University of Novi Sad
INSTITUTE OF FOOD TECHNOLOGY

FINS

foodH congress

28-30. 10. 2014.

Novi Sad, Serbia

XVI International Symposium Feed Technology

ISEKI - Food Association

EFFoST

PROCEEDINGS

ISBN 978-86-7994-044-5

XVI INTERNATIONAL SYMPOSIUM "FEED TECHNOLOGY", NOVI SAD 2014, SERBIA

Publisher

University of Novi Sad
Institute of Food Technology
Bulevar cara Lazara 1.
21000 Novi Sad

Main editor
Dr Jovanka Lević

Editor
Bojana Kokić

Abstract/Paper Review
All abstracts and papers are reviewed by International Scientific Committee and competent researchers

Technical editor
Bojana Kokić

Cover
Boris Bartula, BIS, Novi Sad, Serbia

Printed by
"Futura" – Novi Sad, Serbia

Number of copies
350 copies

Organization of Symposium:

- INSTITUTE OF FOOD TECHNOLOGY, University of Novi Sad, Serbia

Symposium is supported by:

- Ministry of Education, Science and Technological Development of the Republic of Serbia - Belgrade
- Provincial Secretariat for Science and Technological Development - Novi Sad
- Ministry of Agriculture and Environmental Protection of the Republic of Serbia - Belgrade
- Provincial Secretariat of Agriculture, Water Economy and Forestry - Novi Sad
- ISEKI Food association
- EFFoST – The European Federation of Food Science & Technology

INTERNATIONAL SCIENTIFIC COMMITTEE

Dragomir Catalin, National Research Development Institute for Animal Biology and Nutrition, Balotesti, Romania

Jovanka Lević, Institute of Food Technology, University of Novi Sad, Serbia

Luciano Pinnoti, Veterinary Faculty, University Of Milan, Italy

Violeta Jurskiene, Institute of Animal Science, Lihuanian University of Health sciences, Lithuania

Bogdan Yegorov, Odessa National Academy of Food technologies, Ukraine

Mariana Petkova, Institute of Animal Science, Bulgaria

Ilias Giannenas, Aristotle University of Thessaloniki, Greece

Leszek Moscicki, Lublin Univestiy of Life Sciences, Poland

Ionelia Taranu, National Research Development Institute for Animal Biology and Nutrition, Balotesti, Romania

Rodica Diana Criste, National Research Development Institute for Animal Biology and Nutrition, Balotesti, Romania

Maria Chrenkova, Research Institute of Animal Production, Nitra, Slovakia

Jana Hajslova, Institute of Chemical Technology, Prague, Czech Republic

Delia Etleva, Faculty of Agriculture and Environment, Agricultural University of Tirana, Albania

Mia Eeckhout, Faculty of Bio-engineering,Ghent University, Belgum

Arnaud Bouxin, FEFAC, Belgium

Marcela Šperanda, Faculty of Agriculture, University of Osijek, Croatia

Nurgin Memiši, AD Mlekara – Subotica, Serbia

Olivera Đuragić, Institute of Food Technology, University of Novi Sad, Serbia Serbia

Šandor Kormanjoš, Institute of Food Technology, University of Novi Sad, Serbia Serbia

Ljiljana Kostadinović, Institute of Food Technology, University of Novi Sad, Serbia

Radmilo Čolović, Institute of Food Technology, University of Novi Sad, Serbia

Dušica Čolović, Institute of Food Technology, University of Novi Sad, Serbia

Dragan Palić, Institute of Food Technology, University of Novi Sad, Serbia

Miroslav Ćirković, Scientific Veterinary Institute, Novi Sad, Serbia

Rade Jovanović, Institute of Scientific Application in Agriculture, Belgrade, Serbia
Ivan Pavkov, Faculty of Agriculture, University of Novi Sad, Serbia
Sava Pavkov, Institute Gosa, Belgrade, Serbia
Ljiljana Suvajdžić, Faculty of Medicine, University of Novi Sad, Serbia

HONORARY BOARD

Prof. dr Milica Petrović, dean, Faculty of Agriculture Zemun, University of Belgrade, Serbia
Prof. dr Milan Popović, dean, Faculty of Agriculture, University of Novi Sad, Serbia
Prof. dr Zoltan Zavargo, dean, Faculty of Technology, University of Novi Sad, Serbia
Prof. dr Bojana Obradović, dean, Technology and Metallurgy Faculty, University of Novi Sad, Serbia
Prof. dr Miroslav Ćirković, director, Scientific Veterinary Institute Novi Sad, Serbia
Prof. dr. Ćemal Dolićanin, rector, State University of Novi Pazar, Serbia
Dr Petar Kljaić, director, Institute of Pesticides and Environmental Protection, Belgrade, Serbia
Dr Vesna Matekalo-Sverak, Institute of Meat Hygiene and Technology, Novi Sad, Serbia
Dr Jegor Miladinović, Institute of Field and Vegetable Crops, Novi Sad, Serbia
Dr Jelena Begović, director, Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia
Prof. dr Vladeta Stević, dean, Faculty of Agronomy Čačak, University of Kragujevac, Serbia
Prof.dr Neda Mimica Dukić, dean, Faculty of Sciences, University of Novi Sad, Serbia

ORGANIZING BOARD OF SYMPOSIUM

President:

Dr Olivera Đuragić, University of Novi Sad, Institute of Food Technology, Serbia

Members:

Jovanka Lević, Institute of Food Technology, University of Novi Sad, Serbia
Bojana Kokić, Institute of Food Technology, University of Novi Sad, Serbia
Slavica Sredanović, Institute of Food Technology, University of Novi Sad, Serbia
Đuro Vukmirović, Institute of Food Technology, University of Novi Sad, Serbia
Jasna Stevanović, Chember of Comerce, Belgrade, Serbia
Nedeljka Spasevski, Institute of Food Technology, University of Novi Sad, Serbia
Vojislav Banjac, Institute of Food Technology, University of Novi Sad, Serbia
Slađana Rakita, Institute of Food Technology, University of Novi Sad, Serbia
Sanja Teodosin, Institute of Food Technology, University of Novi Sad, Serbia
Zdenka Marković, Institute of Food Technology, University of Novi Sad, Serbia
Albert Kormanjoš, Institute of Food Technology, University of Novi Sad, Serbia
Jovanka Mićić-Veljković, Institute of Food Technology, University of Novi Sad, Serbia

EXECUTIVE COMMITTEE

Dr Jovanka Lević, Institute of Food Technology, University of Novi Sad, Serbia
Prof. Dr Viktor Nedović, Faculty of Agriculture, University of Belgrade, Serbia
Dr Marija Bodroža-Solarov, Institute of Food Technology, University of Novi Sad, Serbia
Dr Milica Pojić, Institute of Food Technology, University of Novi Sad, Serbia
Dr Tea Brlek, Institute of Food Technology, University of Novi Sad, Serbia
MSc Bojana Kokić, Institute of Food Technology, University of Novi Sad, Serbia

CONTENT

PRESENCE OF MYCOBIOTA AND MYCOTOXINS IN SILAGE

Aleksandra Bočarov-Stančić[1]*, Slavica Stanković[2], Jelena Lević[2], Snežana Janković[1], Milan Adamović[3], Željko Novaković[1], Janja Kuzevski[1]

[1]Institute for Science Application in Agriculture, 68b Boulevard despota Stefana, Belgrade, 11000, Serbia
[2]Maize Research Institute Zemun Polje, 1 Slobodana Rajića, Belgrade, 11185, Serbia
[3]Institute for Technology of Nuclear and Other Mineral Raw Materials, 86 Franchet d' ESperey Street, Belgrade, 11000, Serbia

*Corresponding author:
E-mail address: abocarov@ipn.bg.ac.rs

ABSTRACT

The growth of molds and production of mycotoxins in silage depends on proper ensiling, and environmental conditions (oxygen, pH, moisture, etc.). During the initial stages of ensiling, after oxygen depletion, strict aerobes (*Fusarium* species) first disappear followed by other, so-called field mycobiota, *Alternaria* and *Cladosporium* spp., so the dominant mycobiota become fungi tolerant to oxygen deficiency among which the most common are some *Mucorales* and *Penicillium* species, *Aspergillus fumigatus*, *Trichoderma viride*, *Geotrichum candidum, Paecilomyces variotii* and *Monascus ruber*.
Although changes in pH value during ensiling caused by the production of organic acids do not have adverse effect to mycobiota (they can grow between pH 3 and 8), some of these acids (propionic and butyric) have a strong inhibitory effect on most of fungi.
Many mycotoxins (aflatoxin B1, zearalenone, fumonisins, trichothecenes etc.) detected in different types of silage all over the world are in fact produced in the field, considering that in the case of proper ensiling toxigenic mold species are being replaced with characteristic silage mycopopulations. The results of mycological and mycotoxicological investigations of corn and alfalfa silages in Serbia show that the most significant contaminants of these silage types are zearalenone (750-1640 µg/kg) and type A trichothecenes (T - 2 toxin and DAS).
***Keywords**: silage, toxigenic mycobiota, mycotoxins*

INTRODUCTION

The ensiling of animal feed allows preservation of feed quality for a longer period. It takes place due to the presence and the activity of lactic, acetic and butyric acids produced by lactic acid bacteria. In addition to them, yeasts and fungi can also be present in the ensiled material, as well as toxic fungal metabolites - mycotoxins. Silage is a very favorable environment for mycobiota development. Humidity is between 25% and 80%, temperature range is 5-40 °C, while the concentration and availability of nutrients is extremely high (Adamović et al., 2005). It should be emphasized that even in the field potentially toxigenic fungi can infest plants and biosynthesize different mycotoxins, so they can be introduced in the silo during the preparation of silage. Mycobiota mainly develop on the surface of silage or in the parts in which air is not squeezed out enough. Covering silage by various means, including plastic films, is not enough to guarantee that fungi and mycotoxins will not develop, especially in the parts of the silo not sufficiently protected against ingress of air (lateral, frontal and superficial parts of the silo).
Oxygen accessibility plays a major role in the development of characteristic silage mycobiota as well as in their total number. When oxygen is depleted during the initial stage of ensiling, strictly aerobic *Fusarium* species are first to disappear, followed by other so-called field fungi - *Alternaria* and *Cladosporium*. Because of that, the total number of mycobiota is significantly reduced. Fungal species tolerant to a lack of oxygen are some *Mucorales* and *Penicillium* species, *A. fumigatus*, *Trichoderma viride*, *Geotrichum* and *Monascus ruber*. *Byssochlamys nivea*, *Paecilomyces variotii* and *P. roqueforti* are considered to be microaerophilic species or

indifferent to the presence of oxygen (Auerbach, 2003). Another important factor for the growth of silage mycobiota is the natural production of organic acids - lactic, acetic, propionic and butyric. Although changes in pH values do not act detrimental to the growth of fungal species (fungi can grow between pH 3 and pH 8), some of these acids - propionic and butyric - have a strong inhibitory effect on most mycobiota. After opening the silage, the access of oxygen allows the substantial growth of filamentous fungi if other factors (temperature, antimycotic acids, substrate composition, competition etc.) do not restrict their development, and provide that microaerophilic species have an advantage because of earlier proliferation. A number of fungal species occurring in silage can synthesize and excrete toxic secondary metabolites – mycotoxins (*Table 1*).

Table 1. Major mycobiota and their toxic metabolites that can be found in silage

Fungal species	**Mycotoxins**
Pre-harvest infestation	
Alternaria spp.	Alternariol, alternariol monomethyl ether, tenuazonic acid, alternuene
Fusarium culmorum, *F. graminearum*	Deoxynivalenol (DON), zearalenone (ZEA)
F. proliferatum, F. verticillioides	Fumonisins
F. langsethiae, *F. poae*, *F. sporotrichioides*	Diacetoxyscirpenol (DAS), T-2 toxin
Post-harvest infestation	
Aspergillus flavus, *A. parasiticus*	Aflatoxins (AFL)
A. fumigatus	Fumitremorgen, gliotoxin, verruculogen
A. ochraceus, Penicillium aurantiogriseum	Ochratoxins (OTA)
A. versicolor	Cyclopiazonic acid, sterigmatocystin
Byssochlamys nivea	Citrinin, mycophenolic acid,
Monascus ruber	Citrinin, monacolins
Paecilomyces variotii	Patulin
Penicillium citrinum	Citrinin
P. expansum	Patulin, roquefortine A, B, C
P. roqueforti	Mycophenolic acid, roquefortine A, B, C, PR toxin

Sources: Škrinjar et al. (1995), Bočarov-Stančić et al. (2007, 2008); Tangni et al. (2013)

OCCURRENCE OF MYCOBIOTA IN SILAGE

There are relatively little data on the mycobiota infestation of different types of silage in Serbia since some authors present the results obtained for different types of cattle feed collectively, without the allocation of the results obtained for silage (Škrinjar et al., 2011; Krnjaja et al., 2013). In four samples of corn silage contaminated with minimal amounts of aflatoxin B1 - AFB1 (Table 2), only *A. versicolor* was identified (Đorđević et al., 2003). Although Jakić-Dimić et al. (2009) found that during five-year period 18.7% of analyzed corn grain samples were contaminated with *A. flavus* and 18.2% with AFB1, respectively, none of 58 corn silages was contaminated with them. The silage of corn grain and spent mushroom substrate (*Pleurotus ostreatus*) was infested only with genus *Penicillium* – the dominant species was *P. brevicompactum* (Adamović et al., 2007). From six mycobiota identified on alfalfa silage, dominant were *Botryotrichum piluliferum*, *Chaetomium globosum* and *Mucor racemosus*. Potentially toxigenic *Fusarium* species - *F. culmorum, F. semitectum* and *F. sporotrichioides* (Table 1) - were observed only in fresh alfalfa used for ensiling (Đorđević et al., 2003). *F. semitectum* was also identified in one wilted alfalfa sample and it was proved that it can biosynthesize diacetoxyscirpenol – DAS and zearalenone – ZEA (Bočarov-Stančić et al., 2005).

Unlike in Serbia, dominant spoilage fungus in Portuguese corn silages was *A. fumigatus.* Other identified mycobiota belonged to the following genera: *Absidia*, *Aspergillus* spp., *Cladosporium*, *Monascus*, *Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma* etc. (dos Santos et al., 2005). On the other hand, in high moisture corn silages from Slovakia most frequently found molds were *Penicillium* spp. and *Paecillomyces* sp., 56.49% and 32.16%, respectively (Bíro et al., 2009). Mycopopulations detected with the highest frequency of occurrence in corn silages in Lithuania were *Rhyzomucor* (13.7%) and *Aspergillus* (11.7%). Besides other fungal genera, *Fusarium* spp. was found in 6.3% of tested samples (Baliukoniene et al., 2012). Most predominant mycobiota in silage samples in South Africa, mainly in corn silages, was *A. fumigatus* (32%), followed by *A. flavus* (21%) and *A. parasiticus* (20%). Such high frequency of these potentially toxigenic fungal species (Table 1) resulted in the contamination of almost all corn silage samples with aflatoxins (Ndlovu and Dutton, 2013). The differences in the incidence and type of mycobiota in silages in the listed countries and in Serbia are a consequence of diverse agro ecological conditions during the vegetative period of corn and other agricultural crops.

OCCURRENCE OF MYCOTOXINS IN SILAGE

Many mycotoxins (e.g. aflatoxins, DON, ZON, fumonisins, trichothecenes, *Alternaria* toxins) detected in silage all over the world are produced in the field before harvest and ensiling, considering that in appropriate ensiling conditions toxigenic mold species are being replaced by characteristic ensiling mycopopulations.

Table 2. Mycotoxin contamination of silage in Serbia (summarized data)

Type of silage	Mycotoxin	Incidence (%)	Average (µg/kg)	Reference
Pre-harvest mycotoxins				
Corn	ZEA	12.5	140.0	Mašić et al. (2003)
Whole corn plant	ZEA	43.8	1450.0	Adamović et al. (2005)
	DAS	43.8	620.0	
	T-2	25.0	310.0	
Corn	ZEA	63.6	1640.0	
	DAS	18.2	1380.0	
	T-2	27.3	500.0	
Alfalfa	ZEA	100.0	730.0	
	DAS	16.7	250.0	
Alfalfa*	ZEA	100.0	1280.0	Bočarov-Stančić et al. (2005)
	DAS	100.0	250.0	
Corn	ZEA	5.56	500.0	Škrinjar et al. (2011)
Whole corn plant	ZEA	-	1843.0	Krnjaja et al. (2013)
	DON	-	1149.0	
Alfalfa and grass*	ZEA	100.0	2477.5	
	DON	100.0	164.0	
Post-harvest mycotoxins				
Whole corn plant	AFB1	100.0	7.3	Đorđević et al. (2003)
Whole corn plant	AFB1	25.0	3.0	Adamović et al. (2005)
	OTA	37.5	75.0	
Corn	OTA	9.1	130.0	
Alfalfa	AFB1	16.7	3.0	
Corn	OTA	11.1	46.2	Škrinjar et al. (2011)
Whole corn plant	AFB1	-	4.9	Krnjaja et al. (2013)
Alfalfa and grass*	AFB1	100.0	7.3	

** Only one sample was analyzed*

Ten-year investigations of the contamination of different types of silage with mycotoxins (Table 2) have shown that ZEA was the most predominant pre-harvest mycotoxin in Serbia. It was detected in 5.56-63.6% of corn silage samples (grain and whole plant) with the mean concentration from 140 µg/kg to 1843 µg/kg. In the case of alfalfa silage, all examined samples were contaminated with this fusariotoxin; mean concentration of ZEA varied from 730 µg/kg to 2477.5 µg/kg. From other fusariotoxins found less frequently in Serbian silage, the mean concentration of DAS ranged from 250 µg/kg to 1380 µg/kg, DON from 164 µg/kg to 1149 µg/kg and T-2 toxin from 310 to 500 µg/kg (Table 2).

Other authors also reported about frequent contamination of silage with toxins produced by *Fusarium* spp. prior to harvest and ensiling. In corn silage samples submitted for analysis by North Carolina farmers, the dominant contaminant was DON (66%, 1991±2879 µg/kg) although both fumonisins and ZEA were quite often detected (in 37% and 30% of the samples, respectively) (Whitlow et al., 1998). Albeit *Fusarium* species were rarely isolated from corn silage in the Azores, fusariotoxins – fumonisin B1 and DON were found much frequently (in 56% and 40% of the samples, respectively) (dos Santos et al., 2005). In Lithuania, in corn silages from tranches, ZEA was found with the highest mean level of concentration (625 µg/kg), followed by DON (435 µg/kg) (Baliukoniene et al., 2012). Eckard et al. (2011) detected the contamination of 79% of corn silage in Switzerland with ZEA but the mean concentration (181 µg/kg) was much lower than in Serbia (Table 2). Contrary to these investigations, Ndlovu and Dutton (2013) reported that two commonly found *Fusarium* toxins DON and ZEA occurred with low frequency (7% and 10%, respectively) in silage samples in South Africa. According to Signorini et al. (2012) corn silage is a major source of DON and ZEA intake, which often co-occur in this substrate. Up to 56% and 51% of the total DON and ZEA intake from a diet may be provided by concentrated feed and corn silage (Tangni et al. 2013). In high moisture corn silages in Slovakia, fusariotoxin found with the highest concentration (ranging from 179.13 ± 3.04 µg/kg to 249.4 ±24.69 µg/kg) was T-2 toxin (Bíro et al., 2009). Its concentration range was similar with the values reported in Serbia (Table 2). Contrary to that, Eckard et al. (2011) detected T-2 toxin with a much lower mean concentration (36 µg/kg) although found in even 42% of corn silage samples in Switzerland.

A dominant post-harvest mycotoxin in corn and alfalfa silages in Serbia was aflatoxin B1. Its frequency ranged from 16.7% to 37.5% and mean concentrations from 3 µg/kg to 7.3 µg/kg (Table 2). Contrary to Serbia, much higher mean AFB1 concentrations (28±19 µg/kg) were found in maize silage samples in North Carolina (Whitlow et al., 1998). The similar case was in South Africa. According to Ndlovu and Dutton (2013), most commonly occurring mycotoxins in corn silage were aflatoxins (97%) with a concentration range from 0.2 µg/kg to 67 µg/kg, what is much higher than in Serbia (Table 2). The contamination of almost all corn silage samples in South Africa with AFB1 concurred with a high level of *A. flavus* and *A. parasiticus* in the same samples. Another post-harvest mycotoxin – OTA was less frequently found in silage samples in Serbia than AFB1 (9.1-37.5%). Its mean concentration varied from 46.2 µg/kg to 130 µg/kg (Table 2).

In the presented studies, similarities as well as differences in the contamination incidence and level often depended on geographical location and climatic conditions. While presence of AFL is usually associated with tropical and sub-tropical conditions, in a moderate climatic zone in Europe fusariotoxins (DON, ZEA, FUM, DAS, T-2 toxin) and OTA are more frequent contaminants of different types of silage. However, changes that have commenced in climatic conditions may lead to changes in mycobiota and mycotoxins usually present in European samples, as it was the case with the outbreak of *A. flavus* in Serbia in 2012 (Lević et al., 2013). The result of this outbreak was a high contamination frequency of AFB1 (56.4%) in corn harvested in Serbian province of Vojvodina in 2012 (Kiš et al., 2013).

CONCLUSIONS

Ten-year investigations revealed that ZEA was a predominant mycotoxin in corn and alfalfa silages in Serbia, followed by another fusariotoxin – DAS, considering their incidence and detected concentration. Although AFB1 was less often found and with its mean

concentrations within Serbian regulatory levels, climatic changes may lead to the occurrence of unusual mycobiota and mycotoxins in European silage.

ACKNOWLEDGEMENT

The research was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia within the project TR31023.

REFERENCES

Adamović, M. J., Bočarov-Stančić, A. S., Đorđević, N. Ž., Daković, A. S., Adamović, I. D. (2005). Mycotoxins in the silage – causes of creating, aftermath and protection from acting. Proc. Nat. Sci. Matica Srpska Novi Sad, 108, 51-57.

Adamović, M. J, Bočarov-Stančić, A. S., Milenković, I. M., Štrbac, S. S., Adamović, I. D. (2007). The quality of silage of corn grain and spent *P. ostreatus* mushroom substrate. Proc. Nat. Sci., Matica Srpska Novi Sad, 113, 211-218.

Auerbach, H. (2003). Mold growth and mycotoxin contamination of silages: sources, types and solutions, Proceedings of Alltech's Nineteenth Annual Symposium » Nutritional Biotechnology in the Feed and Food Industries« (Ed. T.P. Lyons and K.A. Jacques), Nottingham University Press, Nottingham, pp.247-265.

Baliukoniene, V., Bakutis, B., Vaivadaite, T., Bartkiene, E., Jovašiene, J. (2012). Prevalence of fungi and mycotoxins in silage and milk in Lithuania. Vet. Med. Zoot., 59(81), 3-9.

Bíro, D., Juráček, M., Kačániová, M., Šimko, M., Gálik, B., Michálkova, J., Győngyová, E. (2009). Occurrence of microscopic fungi and mycotoxins in conserved high moisture corn from Slovakia. Ann. Agric. Environ. Med., 16, 227-232.

Bočarov-Stančić, A. S., Adamović, M. J., Đorđević, N. Ž. (2005). Mycopopulations of alfalfa silage with particular review on toxigenic *Fusarium* spp. Proc. Nat. Sci. Matica Srpska Novi Sad, 108, 59-67.

Bočarov-Stančić, A. S., Lević, J. T., Stanković, S. Ž., Krnjaja, V. S., Kovačević, T. M., Tančić, S. L. (2007). The toxigenic potential of *Fusarium poae* originated from wheat. Proc. Nat. Sci. Matica Srpska Novi Sad, 113, 113-123.

Bočarov-Stančić, A., Lević, J., Stanković, S., Tančić, S., Krnjaja, V., Salma, N. (2008). Toxigenic potential of *Fusarium langsethiae* isolates from Serbian wheat kernels. Cereal Research Communications, 36, Suppl. B, 345-346.

dos Santos, V. M., Venâncio, A., Matos, J. E. (2005). Fungi and *Fusarium* mycotoxins in corn silages. (https://repositorium.sdum.uminho.pt/bitstream/1822/3565/1/Trends_in_Mycology.07%5B1%5D.pdf)

Đorđević, N., Adamović, M., Grubić, G., Kotlajić, V., Bočarov-Stančić, A. (2003). Influence of Min-a-Zel Plis on biochemical. microbiological and mycotoxicological parameters on lucerne silage. J. Agric. Sci., 48(2), 171-178.

Đorđević, N., Adamović, M., Grubić, G., Bočarov-Stančić, A. (2004). Uticaj organozeolita i uree na hemijski sastav i kvalitet silaže cele biljke kukuruza. Biotechnol. Anim. Husb., 20(5-6), 187-194.

Eckard, S., Wettstein, F. E., Forrer, H .R., Vogelgsang S (2011). Incidence of Fusarium Species and Mycotoxins in Silage Maize. Toxins., 3(8), 949-967.

Jakić-Dimić, D., Nešić, K., Petrović, M. (2009). Contamination of cereals with aflatoxins, metabolites of fungi A*spergillus flavus*. Biotechnol. Anim. Husb., 25(5-6), 1203-1208.

Kos, J. J., Janić Hajnal, E. P., Mastilović, J. S., Milovanović, I. Lj., Kokić, B. M. (2013). The influence of drought on the occurence of aflatoxins in maize. Jour. Nat. Sci, Matica Srpska Novi Sad, 124, 59—65.

Krnjaja, V., Novaković, Ž., Stojanović, Lj., Ostojić-Andrić, D., Stanišić, N., Nikšić, D., Mandić, V. (2013). Kontaminacija hrane za goveda plesnima i mikotoksinima/Contamination of cattle feed with molds and mycotoxins. Vet. glasnik, 67(1-2), 129-138.

Lević, J., Gošić-Dondo, S., Ivanović, D., Stanković, S., Krnjaja, V., Bočarov-Stančić, A., Stepanić, A. (2013). An outbreak of *Aspergillus* species in response to environmental conditions in Serbia. Pestic. Phytomed., 28(3), 167-179.

Mašić, Z., Bočarov-Stančić, A., Adamović, M., Đilas, S. (2003). Mikotoksini u hrani za životinje u Republici Srbiji. X Simpozijumu „Tehnologija hrane za životinje" (sa međunarodnim učešćem),19.-23. 10.2003.,Vrnjačka Banja. Zbornik radova, 299-305.

Ndlovu, C. S., Dutton, M. F. (2013). A survey of South African silage for fungi and mycotoxins. African Journal of Agricultural Research, 8(32), 4299-4307.

Signorini, M. L., Gaggiotti, M., Molineri, A., Chiericatti, C. A., Zapata de Basílico, M. L., Basílico, J. C., Pisani, M. (2012). Exposure assessment of mycotoxins in cow's milk in Argentina. Food Chem. Toxicol., 50(2), 250-257.

Škrinjar, M., Danev, M., Dimić, G. (1995). Interactive effects of propionic acid and temperature on groth and ochratoxin A production by *Penicillium aurantiogriseum.* Folia Microbiol., 40(3), 253-256.

Škrinjar, M., Jakić-Dimić. D., Blagojev, N., Šošo, V. (2011). Results of one-year investigations of the contamination of dairy cattle feed and raw milk with moulds and mycotoxins. Biotechnol. Anim. Husb., 27 (3), 985-992.

Tangni, E. K., Pussemier, L., Van Hove, F. (2013). Mycotoxin contamining maize and grass silages for dairy cattle feeding: Current state and challanges. J. Anim. Sci. Adv., 3(10), 492-511.
Whitlow, L. W., Hagler, Jr., W. M., Hopkins, B. A. (1998). Mycotoxin occurrence in farmer submitted samples of North Carolina feedstuffs. J. Dairy Sci. 81 (Abstr.):1189, 1989-1997.

SUPPRESSION MEASURES FOR MYCOTOXIN CONTAMINATION OF FOODS AND FEEDS

Aleksandra Bočarov-Stančić[1]*, Marija Bodroža-Solarov[2], Mirjana Stojanović[3], Jovana Vučković-Đisalov[2], Zorica Lopičić[3], Jelena Milojković[3]

[1]Institute for Science Application in Agriculture, 68b Boulevard despota Stefana, Belgrade, 11000, Serbia
[2]Institute of Food Technology, University of Novi Sad, 1 Boulevard cara Lazara, Novi Sad, 21000, Serbia
[3]Institute for Technology of Nuclear and Other Raw Materials, 86 Franchet d' ESperey Street, Belgrade, 11000, Serbia

*Corresponding author:
E-mail address: abocarov@ipn.bg.ac.rs

ABSTRACT

Suppression measures for mycotoxin contamination of foods and feeds include mould growth control and the elimination of biosynthesised mycotoxins.
The control of mycobiota development as a preventive measure starts from sowing (cultivar/variety selection, sowing date, etc.) and continues through the pre-harvest, i.e. vegetative period (selecting appropriate cropping practices, making predictive models, preventing insect attacks, etc.) to the post-harvest period (storage, grain sorting, etc.).
Mycotoxins in foods and feeds can be eliminated by using non-nutritive additives, i.e. different adsorbents (mineral, organic and biological), or by detoxification of mycotoxins by their transformation (using physical or chemical treatments, microorganisms or their enzymes) into non-toxic or less toxic compounds.
A disadvantage of mineral adsorbents is their non-selectivity. Yeast cell wall-derived biological adsorbents were therefore proven to be the best. The transformation of mycotoxins by physical and chemical treatments do not often give satisfactory results because of a change in the nutritional value of the food and feed treated or because of forming toxic compounds. Recently, the transformation of mycotoxins by microorganisms or their enzymes is a procedure that promises to be one of the best methods to increase foods and feeds safety and eliminates all negative effects of their mycotoxin contamination.
Keywords*: suppression measures, mycotoxin contamination, foods, feeds*

INTRODUCTION

Mycotoxins, secondary metabolites biosynthesised by fungi (mycobiota), pose one of the most significant problems that must be taken under control in order to ensure food and feed safety. They represent a serious hazard for animal and human health and have significant economic impact worldwide since 25% of world's agricultural commodities are contaminated with mycotoxins, according to the estimation of the Food and Agriculture Organization (FAO). Approximately, 400 secondary metabolites with toxigenic potential are produced by more than 100 fungal species. These small and stable metabolomes that possess different chemical structure cause diverse biological effects. It is extremely difficult to remove or eliminate these substances when they enter the food/feed chain. Suppression measures for mycotoxin contamination of foods and feeds include preventive measures - mould growth control and the elimination of biosynthesised mycotoxins by adsorption or transformation (by the use of physical or chemical treatments, microorganisms or their enzymes).

CONTROL OF MYCOBIOTA DEVELOPMENT

Sowing. Before sowing, it is necessary to select appropriate crop cultivars/varieties and seeds without insect pests or microbial diseases, in order to obtain healthy, vigorous plants

resistant to mycobiota attack (Clements and White, 2004). Also, hulled species such as spelt wheat proved to be less sensitive on *Alternaria* spp. and their toxic metabolities (Vučković et al., 2012 and 2013) The time of sowing is another important factor. Besides the variety and weather conditions in the vegetative period, the time of sowing has a great influence on the production of spores and plant infection, and partly on the flowering time. If the flowering time coincides with the time of fungal spores release, more frequent and intense attacks of pathogens should be expected (Champeil et al., 2004). In practice, sowing should be carried out in such time to avoid high temperatures and stress caused by drought during seed development and maturation.

Pre-harvest period. Suitable cultivation techniques and removal of agricultural waste of a preceding crop is effective in preventing contamination of the following crop. Crop rotation is very important. For example, when sown after maize, wheat has a 6-fold higher concentration of deoxynivalenol (DON) than wheat whose preceding crop was also wheat, barley or soybean (Krebs et al., 2000). The Codex Alimentarius Commission (2003) gives farmers guidelines for good agricultural practices in the prevention and reduction of the mycotoxin presence in cereals. For example, potatoes and other vegetables, as well as clover and alfalfa (species that are not hosts of *Fusarium* spp.) are proposed to be a preceding crop. There are software applications that help farmers to predict the risk of mycotoxin contamination during the year, depending on weather conditions (Jard et al., 2011). Fungicides should be applied whenever there is a predictable risk. Treatment with insecticides is also recommended since insects attack the outer seed membrane and facilitate seed infection by the producers of mycotoxins. Atoxigenic strains of *A. flavus* and *A. parasiticus* significantly reduce pre-harvest aflatoxin contamination of crops. Soil treatment with these strains also has a positive effect on the reduction of aflatoxin contamination after storage (Dorner and Cole, 2002).

Post-harvest period. Storage is a critical moment in preventing mould growth and mycotoxins biosynthesis in harvested food/feeds. Grain should be stored at a lower temperature and contain less than 15% of moisture in order to eliminate pockets with increased humidity. Low oxygen concentration (<1%) and an increase in the concentration of carbon dioxide are also very effective in preventing the development of fungi. Mixing of grains and prolonged storage should be avoided. Contaminated grain does not have the same colour and density as healthy, so their sorting is required in accordance with these parameters. Although this procedure is not too specific and exhaustive, in the case of heterogeneous mycotoxin contamination, the removal of contaminated grain lots could reduce levels of mycotoxins in the final product. For example, the first step in the production of spaghetti is washing of wheat grain - it removes 23% of deoxynivalenol - DON (Visconti et al., 2004).

ELIMINATION OF MYCOTOXINS FROM FOODS AND FEEDS

Mycotoxin detoxification by adsorption

The process of decontamination od foods and feeds before ingestion should destroy or deactivate mycotoxins, not create new toxic products, maintain nutritional value and do not modify the technological properties of the product. As mycotoxins are very stable, and no physical or chemical procedure can be applied without changing the nutritional value of foods/feedstuffs. At the present time, the most commonly used detoxification method is the utilisation of dietary supplements that can bind mycotoxins and thus reduce the mycotoxins uptake, as well as their distribution in animal blood and target organs. These adsorbents are effective only if they remain stable in the entire gastrointestinal tract so that there is no desorption of bounded mycotoxins during digestion.

Mineral adsorbents. Activated charcoal is effective in the adsorption of aflatoxin B1 (AFB1), DON and zearalenone (ZEA), ineffective in the adsorption of ochratoxin A (OTA) and variably effective in the adsorption of T-2 and HT-2 toxin, depending on the type of animals (mice - yes, chickens - no) (Jard et al., 2011). Among silicate binders, it was shown that hydrated sodium calcium aluminosilicate (HSCAS) can bind 85% AFB1 *in vitro* and prevent aflatoxicosis (Phillips et al., 1988). It is also effective in the adsorption of fumonisin B1 (FUMB1), ineffective in the adsorption of DON, and variably effective in the case of ZEA, T-2 and HT-2 toxin (Jard et al., 2011). Diaz et al. (2002) demonstrated that bentonite agents can bind more than 95% of AFB1. They are less efficient in OTA and FUMB1, and especially T-2 toxin adsorption (Jard et al., 2011). Another type of clay – zeolite is also very effective in AFB1 binding and ineffective in T-2 toxin adsorption, while hydrophobic zeolites (treated with ammonia) are effective in the case of OTA and ZEA binding (Jard et al., 2011). According to the data in the literature (Whitlow, 2006; Manafi et al., 2009), silicate binder - diatemaceous earth has a potential to adsorb *in vitro,* besides AFB1 and OTA and some other mycotoxins (ZEA, T-2 toxin and sterigmatocystin). Our own investigations also demonstrated that bentonite, zeolite and diatomite bound more than 95% of applied AFB1 *in vitro* (Bočarov-Stančić et al., 2011a and 2012c). In the case of OTA, only diatomite adsorbed 66.67% of this toxin. Binding of DON has been observed exclusively at pH 3.0 (25.00 to 50.00%). ZON adsorption index ranged from 12.20 to 37.00% while the amount of adsorbed T-2 toxin ranged from 16.66 to 33.33%. Limitations of these mineral adsorbents are that they are not specific to mycotoxins and can also adsorb other molecules essential for human/animal nutrition.

Organic adsorbents. Humic acid (complex of organic acids) is capable of adsorbing AFB1 and ZEA but not DON (Jard et al., 2011). Beside other mycotoxins, peach and sour cherry pits can also adsorb DON and T-2 toxin (Bočarov-Stančić et al., 2012b; Lopičić et al., 2013a and 2013b) while indigestible fibre from food/feed can bind ZEA and T-2 (Tangni et al., 2006; Carson and Smith, 1983b).

***Biological binders*.** Shetty and Jesperson (2006) have shown that *Saccharomyces cerevisiae* can bind AFB1. Termolysed yeast cell walls were very efficient in adsorption of ZEA, while esterified glucomannan polymer obtained from yeast cell wall, apart from binding AFB1, OTA, also bound T-2 toxin (Jard et al., 2011). Some lactic acid bacteria, propionibacteria and bifidobacteria are capable to adsorb mycotoxins. In the case of AFLB1, ZON, DON, diacetoxyscirpenol (DAS), nivalenol, and other mycotoxins, binding is associated with hydrophobic pockets on the bacterial surface (Haskard et al., 2000; El-Nezami et al., 2004). Our *in vitro* experiments with plant *Myriophyllum spicatum* showed that this aquatic weed adsorbed different quantities of applied mycotoxins: 94.7-96.0% AFB1, 30.0-50.7% OTA, 70.0-75.0 ZEA and 16.7-33.3% T-2 toxin (Bočarov-Stančić et al., 2012b). Due to hydrophobic interactions based on conidial wall characteristics, fungal conidia are efficient in adsorption of ZEA and OTA (Jard et al., 2011).

Mycotoxin detoxification by transformation

Transformation by physical and chemical treatments

Physical treatments. Thermal treatment of mycotoxins leads to little or no reduction under normal cooking conditions, i.e. boiling. Fumonisins are completely destroyed at 220°C. Thermochemical treatment of biomass at 200-320 °C can eliminate between 45 and 83% of AFB1, while pasteurisation is inefficient. DON is stable at 120 °C, moderately stable at 180 °C and partially stable at 210 °C. Heat treatment of food contaminated with zearalenone is not effective (Jard et al., 2011). Mycotoxins are not often affected by irradiation. AFB1 is sensitive to UV, X-rays and γ. The content of the same mycotoxin in peanuts and trichothecenes in corn can be reduced by microwaves (Jard et al., 2011).

Chemical treatments. Oxidising agents (ozone, hydrogen peroxide) react with many functional groups of mycotoxins and are used for the reduction of mycotoxins in contaminated feed. Ozone is effective in the case of AFB1 and OTA, and it changes the biological effects of ZEA and trichothecenes (Jard et al., 2011). Treatment with hydrogen peroxide degrades 83.9% of ZEA (80 °C, 16 h, 10% reagent) (Abd Alla, 1997).
Reducing agents (ascorbic acid, Na-bisulfite etc.) are especially effective in reducing the concentration of AFB1 and DON (Kabak et al., 2006). FUMB1 reaction with reducing sugars prevented induced toxicity of this mycotoxin (65 °C, 48 h). DON is transformed into less toxic derivative by reaction with Na-bisulfite.
Ammoniation is used in the case of corn contaminated with AFB1 (higher temperature and pressure increase efficiency), but the procedure is expensive, does not act on other mycotoxins and affects the quality of the food (Jard et al., 2011).
Acidification - treatment with a strong acid (e.g. HCl) reduces the level of AFB1 by 19.3% for 24 h.
Deamination - nitrite in an aqueous medium reduced the toxicity of FUMB1 (Jard et al., 2011).

Transformation by microorganisms and their enzymes

The use of the aforementioned methods for decontamination of food/feed is limited by a number of factors, such as high price, the loss of food/feed quality, low efficiency, low specificity, etc. On the other hand, there are a large number of microorganisms/enzymes that have the ability to bio transform mycotoxins into less toxic components in the intestinal tract of animals, i.e. before the absorption of mycotoxins happens.

Aflatoxin B1. Besides bacteria *Flavobacterium aurantiacum*, *Stenotrophomonas maltophila* and *Bacillus subtilis,* extracellular enzymes from *Rhodococcus erythropolis* are also able to decontaminate AFB1 (Jard et al., 2011). Macroscopic fungi *Armillariella tabescens and Pleurotus ostreatus*, as well as a number of moulds, can also bio transform this micotoxin. In Serbia, the majority of the analysed atoxigenic representatives of genus *Aspergillus* (*A. clavatus*, *A. flavus*, *A. fumigatus, A. niger*, *A. terreus* etc.) were able to degrade AFB1 *in vitro* but the best biotransformation capability (95%) was shown by one isolate of *Cladosporium* sp. and *Cephalophora tropica* (Bočarov-Stančić et al., 2012a and 2013a).

Ochratoxin A. Many of biodegrading microorganisms and plants are able to transform OTA into less toxic compound OTα (Skrinjar et al., 1996; Jard et al., 2011). For prevention of OTA accumulation in wine *Aureobasidium pullulans* was used as bio control agent (De Fellice et al., 2008). Commercial product based on yeast *Trichosporon mycotoxinivorans* is developed for removing OTA from feed (Molnar et al., 2004). Some macroscopic fungi (*Pleurotus ostreatus*) and moulds like *Aspergillus*, *Penicillium* and *Rhizopus* are also effective in reduction of OTA *in vitro*. According to the results of our own investigations, 57.1% of the tested *A. fumigatus* isolates were able to degrade OTA that was added to an agar cultivation medium and followed by 41.7% of *A. niger* and 15.4% *Rhizopus stolonifer* cultures (Bočarov-Stančić et al., 2011c and 2013a). The most effective fungal isolates in OTA reduction were *Purpureocillium lilacinum* and *Trichoderma* sp. (80.0% and 90.0%, respectively). *P. lilacinum* was even more effective in OTA biodegradation (99.8%) after cultivation on rice grain (Bočarov-Stančić et al., 2011b).

Zearalenone. Soil bacteria are able to reduce ZEA (Jard et al., 2011). *Rhodococcus* and *Nocardia* spp. has been patented for insertion in transgenic plants (Duvick and Rood, 2000; Karlovsky et al., 2003). Beside OTA, yeast *Trichosporon mycotoxinivorans* can also bio transform ZEA (Molnar et al., 2004). Mycoparasitic fungus *Clonostachys rosea,* capable of controlling mycotoxin-producing *F. graminearum* and *F. culmorum,* can detoxify ZEA through the enzyme zearalenone lactonohydrolase (Kosawang et al., 2014).

Trichothecenes. Transformation of DON in less toxic metabolites has been observed by mixed cultures of unidentified microorganisms, *Agrobacterium* and *Rhizobium* strains (Jard et al., 2011). A commercial preparation "Mycofix plus", based on an anaerobic bacterium *Eubacterium* (strain BBSH 797), has been developed for reduction of type A and type B trichothecenes in feed (Schatzmayr et al., 2006). Nakajama et al. (1980) isolated 12 bacterial strains (*Bacillus, Nocardia*-like and some unidentified strains) that were able to use T-2 toxin (type A trichothecene) as a sole source of carbon. Some moulds can bio transform T-2 toxin and DAS. Most of the fungal isolates from feed (*A. niger*, *Mucor racemosus* f. *racemosus* and *Mucor* spp.) were able to degrade this type A trichothecenes added in cultivation media (Bočarov-Stančić et al., 2010). Depending on the duration of fermentation in a liquid culture, an isolate *M. racemosus* f. *racemosus* bio transformed 99.97% DAS and 96.7% T-2 toxin, respectively (Bočarov-Stančić et al., 2011d).

CONCLUSIONS

Efficiency of mycotoxin adsorbents is as follows: AFB1 is particularly good at binding the materials of mineral origin, such as clays and zeolites; biological adsorbents show greater efficiency for OTA, ZEA and FUMB1 binding, while DON, T-2 and HT-2 do not bind easily to any type of adsorbents. Yeast cell wall-derived biological adsorbents were therefore proven to be the best. The transformation of mycotoxins by physical and chemical treatments do not often give satisfactory results due to a change in the nutritional value of the food and feed treated, or due to forming toxic compounds. Recently, the transformation of mycotoxins by microorganisms or their enzymes is a procedure that promises to be one of the best methods to increase foods and feeds safety and eliminates all negative effects of their mycotoxin contamination.

ACKNOWLEDGEMENTS

The research was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia within the projects III46005 and TR31003.

REFERENCES

Abd Alla, E. S. A. M. (1997). Zearalenone: incidence, toxigenic fungi and chemical decontamination in Egyptian cereals. Food Nachrung., 41, 362-365.

Bočarov-Stančić, A., Adamović, M., Salma, N., Bodroža-Solarov, M., Vučković, J., Pantić, V. (2011a). *In vitro* efficacy of mycotoxins adsorption by natural mineral adsorbents. Biotechnology in Animal Husbandry, 27 (3), 1241-125.

Bočarov-Stančić, A., Bodroža-Solarov, M., Bagi, F., Stojšin, V., Vučković, J., Pantić, V. (2012a). Biodegradation of aflatoxin B1 by fungal species. Proceedings of the 15th International Feed Technology Symposium „FEED-TO-FOOD"/COST FEED FOR HEALTH join Workshop, Novi Sad, Serbia, 410-415.

Bočarov-Stančić, A., Lević, J., Bodroža-Solarov, M., Pantić, V., Salma, N., Vučković, J. (2013a). Mycological detoxification of aflatoxin B_1 and ochratoxin A *in vitro.* Proceedings of the 10th International Symposium Modern Trends in Livestick Production, Belgrade, Serbia, 824-834.

Bočarov-Stančić, A. S., Lević, J. T., Salma, N. M., Stanković, S. Ž., Bodroža-Solarov, M. I., Pantić, V. R. (2011b). The role of *Paecilomyces lilacinus* (Thom) Samson and other fungal species in biodegradation of ochratoxin A. Proc. Nat. Sci. Matica Srpska, Novi Sad, 120, 103-109.

Bočarov-Stančić, A., Lopičić, Z., Milojković, J., Adamović, M., Salma, N., Pantić, V. (2012b). The efficiency of *in vitro* adsorption of mycotoxins by adsorbents of plant origin. Proceedings of the 6th Central European Congress on Food, Novi Sad,Serbia, 1131-1136.

Bočarov-Stančić, A., Milojković, J., Lopičić, Z., Stojanović, M., Pantić, V., Stanković, S., Adamović, M. (2012c). Evaluation of natural adsorbents to adsorb fusariotoxins *in vitro*. Proceedings of The First International Symposium on Animal Science, Belgrade, Serbia, 654-664.

Bočarov-Stančić, A., Salma, N., Lević, J., Pantić, V., Stanković, S., Barnić, S. (2010). Microbiological degradation of T-2 toxin and diacetoxyscirpenol under laboratory conditions. Proceedings of the XIV International Symposium FEED TECHNOLOGY, Novi Sad, Serbia, 254-261.

Bočarov-Stančić, A., Stanković, S., Lević, J., Salma, N., Pantić, V. (2011c). *In vitro* biodegradation of ochratoxin A by different fungi. Proceedings of the MICROBIOLOGIA BALCANICA 2011, 7th BALCAN CONGRESS OF MICROBIOLOGY, Belgrade, Serbia (on CD).

Bočarov-Stančić, A. S., Stanković, S. Ž., Lević, J. T., Salma, N. M., Pantić, V. R. Barnić, S. (2011d). *In vitro* degradation of diacetoxyscirpenol and T-2 toxin by use of *Mucor racemosus* Fresen. f. *racemosus* isolate. Proc. Nat. Sci. Matica Srpska, Novi Sad, 121, 51-59.

Carson, M. S., Smith, T. K. (1983). Effect of feeding alfalfa and refined plant fibres on the toxicity and metabolism of T2 toxin in rats. J. Nutr., 113 (2), 304–313.

Champeil, A., Fourbet, J. F., Doré, T., Rossignol, L. (2004). Influence of cropping system on *Fusarium* head blight and mycotoxin levels in winter wheat. Crop Prot., 23 (6), 531–537.

Clements, M. J., White, D. G. (2004). Identifying sources of resistance to aflatoxin and fumonisin contamination in corn grain. J. Toxicol. Toxin Rev., 23 (2–3), 381–396.

Codex Alimentarius (2003). Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and trichothecenes. CAC/RCP 51-2003.

De Felice, D. V., Solfrizzo, M., De Curtis, F., Lima, G., Visconti, A., Castoria, R. (2008). Strains of *Aureobasidium pullulans* can lower ochratoxin A contamination in wine grapes. Phytopathology, 98, 1261–1270.

Diaz, D. E., Hagler, W. M., Jr., Hopkins, B. A., Whitlow, L. W. (2002). Aflatoxin Binders I: *In vitro* binding assay for aflatoxin B1 by several potential sequestering agents. Mycopathologia, 156, 223- 226.

Dorner, J. W., Cole, R. J. (2002). Effect of application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* on subsequent aflatoxin contamination of peanuts in storage. J. Stored Prod. Res., 38, 329–339.

Duvick, J., Rood, T. A. (2000). Zearalenone detoxification compositions and methods. US patent 6074838. Des Moines (IA): Pioneer Hi-Bred International, Inc.

El-Nezami, H., Polychronaki, N., Yuan Kun, L., Haskard, C., Juvonen, R., Salminen, S., Mykkänen, H. (2004). Chemical moieties and interactions involved in the binding of zearalenone to the surface of *Lactobacillus rhamnosus* strains GG. J. Agric. Food Chem., 52 (14), 4577–4581.

Haskard, C., Binnion, C, Ahokas, J. (2000). Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chem-Biol Interact., 128 (1), 39–49.

Jard, G., Liboz, T., Mathieu, F., Guyonvarc, A., Lebrihi, A. (2011). Review of mycotoxin reduction in food and feed: from prevention in the field to detoxification by adsorption or transformation. Food Additives and Contaminants: Part A: Chemistry, Analysis, Control, Exposure and Risk Assessment, 28 (11), 1590-1609.

Kabak, B., Dobson, A. D. W., Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. Crit. Rev. Food Sci. Nutr., 46 (8) 593-619.

Karlovsky, P., Edmund, I. I. I., Crane, H., Gilliam, J. T., Maddox, J. R. (2003). Compositions and methods of zearalenone detoxification. US patent 20030073239. Des Moines (IA): Pioneer Hi-Bred International, Inc.

Kosawang, C., Karlsson, M., Vélëz, Rassmussen, P. H., Collinge, D. B., Jensen, B., Jensen, D. F. (2014). Zearalenone detoxification by zearalenone hydrolase is important for the antagonistic ability of *Clonostachys rosea* against mycotoxigenic *Fusarium graminearum*. Fungal biology, 118 (4), 364-373.

Krebs, H., Streit, B., Forrer, H. R., (2000). Effect of tillage and preceding crops on *Fusarium* infection and deoxynivalenol content of wheat. In T. Alfoldi, W. Lockeretz & U. Niggli (Eds.), The world grows organic. Proceedings of the 13th International IFOAM Scientic Conference. Basel: IOS Press. p. 13.

Lopičić, Z., Bočarov–Stančić, A., Stojanović, M., Milojković, J., Pantić, V., Mihajlović, M., Adamović, M. (2013a). *In vitro* mycotoxins adsorption by sour cherry stones. Proceedings of the 10th International Symposium Modern Trends in Livestick Production, Belgrade, Serbia, 1142-1153.

Lopičić, Z. R., Bočarov–Stančić, A. S., Stojanović, M. D., Milojković, J. V., Pantić, V. R., Adamović, M. J. (2013b). *In vitro* evaluation of the efficacy of peach stones as mycotoxin binders. Proc. Nat. Sci. Matica Srpska, Novi Sad, 124, 289-296.

Manafi, M., Narayanaswamy, H. D., Pirany, N. (2009). *In vitro* binding ability of mycotoxin binder in commercial broiler feed. African J. Agricultural Research, 4 (2), 141-143.

Molnar, O., Schatzmayr, G., Fuchs, E., Prillinger, H. (2004). *Trichosporon mycotoxinivorans* sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. Syst. Appl. Microbiol., 27 (6), 661–671.

Nakayama, K., Kato, A., Ueno, Y., Minoda, Y., Omori, T., Komagata, K. (1980). Studies on metabolism of trichothecene mycotoxins. II Metabolism of T-2 toxin with the soil bacteria. Maikotokishim, 12, 30-32.

Phillips, T. D., Kubena, L. F., Harvey, R. B., Taylor, D. R., Heidelbaugh, N. D. (1988). Hydrated sodium calcium aluminosilicate: a high affinity sorbent for aflatoxin. Poult. Sci., 67 (2), 253–260.

Schatzmayr, G., Zehner, F., Taübel, M., Schatmayr, D., Klimitsch, A., Loibner, A. P., Binder, E.M. (2006). Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res., 50 (6), 543–551.

Shetty, P. H., Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends Food Sci. Technol., 17 (2), 48–55.

Skrinjar, M., Rasic, J. L., Stojicic, V. (1996). Lowering of ochratoxin A level in milk by yoghurt bacteria and bifidobacteria. Folia Microbiol., 41 (1), 26–28.

Tangni, E. K., Simonis, J., Larondelle, Y., De Meeûs D'argenteuil, L. (2006). Biological method for detoxification of a liquid food medium. US patent 2006/0263410 A1. Washington (DC): Clark & Brody.

Visconti, A., Haidukowski, E. M., Pascale, M., Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicol. Lett., 15 (1), 181–189.

Vuckovic, J., Bodroza-Solarov, M., Vujic, Dj., Bocaroc-Stancic, A., Bagi, F. (2013). The protective effect of hulls on *Alternaria* mycotoxins occurence in spelt wheat. J Sci Food Agric., 93, 1996-2001.

Vuckovic, J., Bodroza-Solarov, M., Bagi, F., Bocarov-Stancic, A., Brlek, T., Stojsin, V., Budakov, D. (2012). The effect of hulls on *Alternaria* spp.infection of spelt wheat kernel. Proceedings of the 6th Central European Congress on Food, Novi Sad, Serbia, 527-531.

Whitlow, L. W. (2006): Evaluation of mycotoxin binders. Proceedings of the 4th Mid-Atlantic Nutrition Conference (Zimmerman, N. G. ed.),College Park, University of Meryland, USA, 132-143.

OBTAINING PROTEIN RICH FRACTIONS OF SUNFLOWER MEAL USING AIR CLASSIFICATION

Vojislav Banjac*, Radmilo Čolović, Đuro Vukmirović, Dušica Čolović, Jovanka Lević, Bojana Kokić, Olivera Đuragić

Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: vojislav.banjac@fins.uns.ac.rs

ABSTRACT

Sunflower meal is a by-product of the vegetable oil industry. It remains in large quantities after oil extraction from sunflower seeds and it is mostly used as a feed for all classes of animals. It consists of broken sunflower kernels and fiber rich hulls. Crude fiber is a limiting factor for usage of this feedstuff in diet formulation for monogastric animals. Thus, the removal of sunflower hulls can contribute to an increase of the protein content and therefore increases the nutritional value of it. In this study sunflower meal was first crushed by the use of conical mill and in a second step classified by the use of a cascade air classifier. Starting, unmilled, sunflower meal was also classified by same classifier. The aims were to evaluate the air classification process for obtaining protein enriched fractions, as well as to determine efficiency of conical mill for grinding sunflower meal prior to the air classification. During the classification process air flow and feed rate were varied. Air flow was set at 5, 8.7 and 12.5 m^3/h, respectively, and feed rate was changed by setting rotation speed of dosing element at 30, 60 and 90%. The results showed that an air flow of 5 m^3/h was not efficient for obtaining protein rich fractions of unmilled meal. The lowest values of protein enrichment in coarse fractions of both used meals were obtained at 8.7 m^3/h. The highest used air flow, 12.5 m^3/h, was proved to be the best for obtaining protein rich fractions. Conical mill was efficient tool for preparing sunflower meal prior to the air classification at 12.5 m^3/h. The highest protein enrichment level achieved at this air flow was 12% compared to the protein content of unclassified sunflower meal. Milling also ensured even and undisturbed material flow from feed chute into classifier, thus positively affected the feed rate. Protein content of obtained protein rich fractions was affected by air flow, therefore protein enrichment and yield combination can be adjusted by only modulating this parameter of air classification.
***Keywords**: sunflower meal, air classification, conical mill, protein enrichment*

INTRODUCTION

Sunflower is one of the major oilseed crops in the world, which adapts very well to a wide range of climatic and soil conditions (Laudadio et. al, 2014). Sunflower meal (SFM) remains after solvent extraction of oil from sunflower seeds, and consist of broken sunflower kernels and hulls. It is a by-product produced in large quantities and it is mainly used as relatively cheap protein and energy source in animal feed industry. Thus, the protein content is most important nutritional component of SFM. It ranges from 29 to 48% (Boni et al., 1987; Ramachandran et al., 2007; Geneau-Sbartaï et al., 2008), which depends on factors such as sunflower variety, climate, soil and extraction process (Lomascolo et. al, 2012). Comparing it with other oilseed meals, SFM has high content of raw fiber and contains less antinutritional factors but it has relatively lower protein content (Boni et al., 1987) and low level of lysine (Mérida et. al, 2010).

Sunflower hull, rich in crude fiber, have adverse effect on welfare and performance of monogastric animals. Crude fiber content is a limiting factor for usage of SFM in formulation of diets for this class of animals. The removal of fiber rich hulls from the SFM decreases the fiber content and increases the protein content, as well as the nutritive and economical value. Air classification is a technological process of fractionation that can be applied for certain component enrichment of oilseed meals. Granular materials are separated into fine and coarse fraction by air stream according to size, density and shape of the particles. Air stream rising up inside a classification chamber creates the gravitational-counterflow zone in which

particles experience gravity and drag forces acting in opposite directions. Coarse particles, having terminal settling velocity larger than air flow velocity, move downwards, against the air stream, and fines rise with the stream (Shapiro & Galperin, 2005). Air classification process in combination with sieving was used to fractionate soybean and cottonsead meal into fiber and protein rich fraction (Challa et al., 2010). Wu and Abbott (2003) obtained fine fractions of pin milled, defatted salicornia meal enriched in protein. Beside oilseed meals, air classification is proved to be an useful tool for obtaining protein rich fractions of field pea according to difference in size and density of starch and protein particles (Wu & Nichols, 2005).
In this work unmilled SFM and conical milled SFM were fractionated using cascade zigzag air classifier, device that consists of vertical zigzag channel formed by a several inclined pipes of rectangular cross section. Air flow and feed rate were changed during air classification. The aims were to obtain fractions of SFM enriched in protein and to determine efficiency of conical mill as a tool for grinding SFM prior to the air classification. Particle size reduction in this type of mill is achieved by mechanical impacts between rotary cone and stationary serrated surface. To the best of our knowledge, none of other authors ever used conical mill for grinding sunflower meal. We used it in order to crush large agglomerates consisted of broken sunflower kernels and hulls that exists in SFM, thus tried to improve classification efficiency.

MATERIAL AND METHODS

Sunflower meal was obtained from oil factory "Victoria Oil", Šid. A conical mill (Miag, Braunschweig, Germany) was used to reduce the size of particles and agglomerates of SFM. The adjustable gap between the rotary cone and the stationary serrated surface was set up to its maximum to enable acceptance of large agglomerates (consisting of broken sunflower kernels and hulls) between grinding elements.
Unmilled and conical milled SFM was fractionated by an air classification, using 1-40MZM laboratory zigzag air classifier (Hosokawa Alpine, Augsburg, Germany). Parameters which were varied during air classification of SFM were rotation speed of dosing element and the air flow. Rotation speed of dosing element was set at 30, 60 and 90 %, respectively, and air flow was set at 5, 8.7 and 12.5 m^3/h, respectively.
Feed rate was determined by measuring the time needed for the classification at each combination of air flow and rotation speed of dosing element. It was calculated as:

$$Q = \frac{C + F}{T}$$

where Q represents feed rate, C and F are masses of obtained coarse and fine fractions, respectively, and T represents classification time. Yields of obtained fractions were calculated according to equations:

$$\gamma_C = \frac{C}{C + F} \qquad \gamma_F = \frac{F}{C + F}$$

where γ_C and γ_F represents yield of coarse and fine fractions, respectively, C mass of coarse fraction, and F mass of fine fraction.
Particle size distribution of unmilled and conical milled SFM was determined by standard sieving analysis (ISO Standard 2591-1:1988 (E)) in duplicate, using laboratory sieves ranging from 63 to 2500 µm (Endecotts Ltd., United Kingdom).
Unclassified SFM and air classified SFM fractions were analyzed to moisture content (AOAC Method 934.01) and crude protein content (AOAC 978.04 Method). Additionally, unclassified SFM was analyzed to crude fiber content (AOAC 978.10 Method), crude ash (AOAC Method

942.05) and crude fat content (AOAC 920.39 Method). Chemical analysis of fractions was done in duplicate.
One-way ANOVA and Tukey honestly significant difference test were used to analyze variations of the results. Differences between the means with probability $p<0.05$ were accepted as statistically significant. The level of confidence was set at 95% (STATISTICA 12.0, StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Chemical composition of unclassified SFM is given in Table 1. Figure 1 is showing particle size distribution of unmilled and conical milled SFM. More than 50% of the particles of unmilled SFM and nearly 50% of the particles of conical milled SFM were larger than 1.25 mm. Due to presence of agglomerates, that consisted of broken sunflower kernels and hulls, the highest percent of unmilled SFM particles were larger than 2.5 mm. After milling, quantity of these particles in milled SFM was reduced to half. Conical milled SFM had highest quantity of particles from 1.25 to 2 mm. Therefore, conical mill was proved as an efficient tool for crushing large agglomerates of the starting SFM. More than 99% of particles of bothe meals were larger than 0.125 mm and sharp separation by used zigzag classifier was enabled since this type of air classifiers have cut sizes in wide range, from 0.1 to 10 mm (Shapiro & Galperin, 2005).

Table 1. Chemical composition of unclassified sunflower meal

Chemical composition	**(%)**
Moisture	9.02
Crude protein	35.99*
Crude fiber	19.39*
Crude fat	2.13*
Crude ash	6.75*
Nitrogen free extracts (NFE)	35.73*

** expressed at dry matter basis*

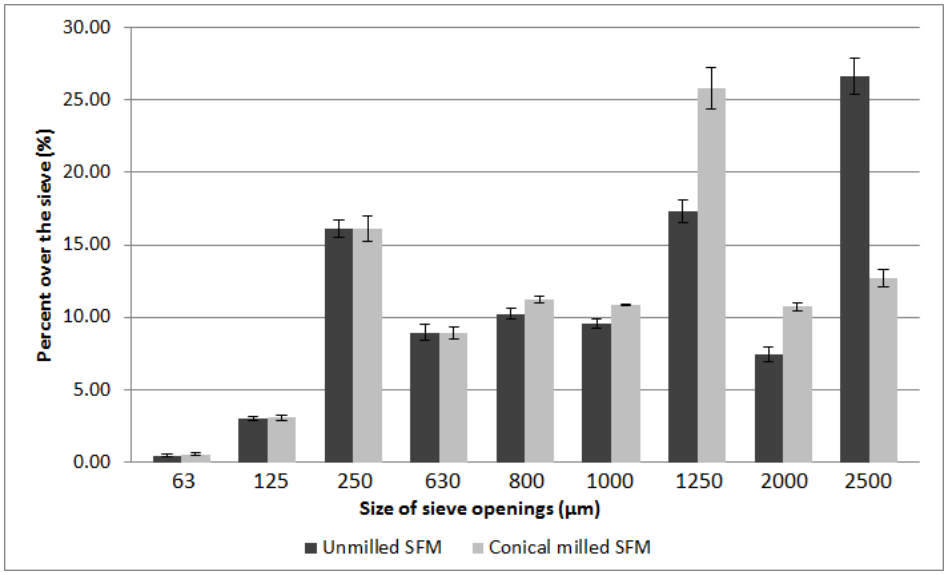

Figure 1. Particle size distribution of unmilled and conical milled sunflower meal (SFM)

The feed rates of classification at each air flow and rotation speed of dosing element, together with the yields of obtained fractions are presented in Table 2.

Table 2. Feed rates and the yields of obtained fractions

Air flow (m^3/h)	Rotation speed of dosing element (%)	Feed rate (kg/h)		Yield (%)			
				Unmilled SFM		Conical milled SFM	
		Unmilled SFM	Conical milled SFM	Coarse fraction*	Fine fraction**	Coarse fraction	Fine fraction
5.0	30	0.26	0.77	92.00	8.00	92.27	7.73
5.0	60	1.84	4.06	92.23	7.77	91.58	8.42
5.0	90	10.03	10.70	94.29	5.71	93.80	6.20
8.7	30	0.53	0.60	78.90	21.10	77.85	22.15
8.7	60	2.71	3.92	77.06	22.94	77.36	22.64
8.7	90	5.02	10.21	81.91	18.09	78.12	21.88
12.5	30	1.02	1.06	56.88	43.12	52.50	47.50
12.5	60	3.72	3.80	58.33	41.67	54.17	45.83
12.5	90	4.70	8.09	56.48	43.52	55.00	45.00

**Fraction of particles that were pulled down by the gravity*
***Fraction of particles that were dragged by the air stream upward*

Higher value of dosing speed had led to higher feed rate and shorter time that was needed for fractionation of the same amount of the material. However, uneven values of feed rate were achieved for unmilled SFM at same rotation speeds of dosing element and at different air flows. Uneven flow of the material from feed chute into classifying chamber was formed due to existence of large agglomerates that was blocking the flow of smaller particles in dosage unit of the classifier. Milling of SFM, along with crushing large agglomerates by conical mill, ensured even flow of the material and therefore stabile feed rates values. Protein contents of obtained SFM fractions are presented in Table 3.

Table 3. Protein content of sunflower meals fractions obtained by air classification

Sunflower meal	Rotation speed of dosing element (%)	Fraction	Air flow (m^3/h)		
			5	8.7	12.5
			Protein content (% d.m.)		
Unmilled	30	Coarse*	$35.68\pm0.06^{de, 1, \dagger}$	$37.92\pm0.03^{g, 2}$	$38.82\pm0.24^{d, 3}$
		Fine**	$36.47\pm0.06^{fg, 3, \dagger}$	$28.13\pm0.12^{a, 1}$	$29.92\pm0.01^{a, 2}$
	60	Coarse	$35.43\pm0.04^{cd, 1, \dagger}$	$37.52\pm0.03^{g, 2}$	$38.56\pm0.11^{de, 3}$
		Fine	$36.20\pm0.02^{ef, 3, \dagger}$	$30.42\pm0.08^{c, 2}$	$29.67\pm0.71^{a, 1}$
	90	Coarse	$35.68\pm0.06^{d,1, \dagger}$	$37.43\pm0.08^{g, 2}$	$38.23\pm0.04^{d, 3}$
		Fine	$36.13\pm0.02^{ef, 3, \dagger}$	$28.94\pm0.03^{b, 1}$	$31.21\pm0.04^{b, 2}$
Conical milled	30	Coarse	$35.00\pm0.02^{c, 1}$	$36.63\pm0.00^{f, 2}$	$40.28\pm0.02^{g, 3}$
		Fine	$37.01\pm0.06^{gh, 2}$	$31.84\pm0.13^{d, 1}$	$31.90\pm0.08^{c, 1}$
	60	Coarse	$34.35\pm0.06^{b,1}$	$36.43\pm0.14^{f, 2, \dagger}$	$39.50\pm0.05^{f, e, 3}$
		Fine	$37.16\pm0.00^{h, 2}$	$32.23\pm0.11^{d, 1}$	$31.83\pm0.05^{c, 1}$
	90	Coarse	$33.02\pm0.06^{a, 1}$	$36.40\pm0.06^{f, 2, \dagger}$	$38.84\pm0.24^{e, 3}$
		Fine	$37.94\pm0.08^{i, 3}$	$33.43\pm0.12^{e, 2}$	$31.94\pm0.08^{c, 1}$

(a,1) Mean value ± standard deviation (n=2); values with different letters in the same column or different number in the same row are significantly different (p<0.05)
† Protein content of fraction is not significantly different (p>0.05) from protein content of unclassified SFM
**Fraction of particles that were pulled down by the gravity*
***Fraction of particles that were dragged by the air stream upward*

Fine fractions of unmilled and conical milled SFM that were obtained at 5 m^3/h had higher protein content than the course fractions and also than the starting SFM. In air classifier, interactions between particles in separation zone are common and air turbulence could influence that fine particles end up in coarse fraction (Johansson & Evertsson, 2012). Therefore, sharpness and efficiency of separation is degraded. Flow rate of 5 m^3/h was probably too low to fiber rich hulls end up as fines, so large hulls and agglomerates in unmilled SFM were pulled down in coarse fraction by gravity. Only very fine particles of hulls and kernels were dragged by the air stream upward and ended up as fines. The air flow of 5 m^3/h was proved to be inefficient for fractionation of unmilled SFM since every obtained fraction had protein content that was not differed significantly ($p>0.05$) from the protein content of starting SFM. This air flow gave fine fractions of conical milled SFM with higher protein content but in small yield (from 6.20 to 8.42 %) and with protein enrichment that was less than 3% compared to the starting material.

Changes in air flow had contributed to the significant changes in protein content ($p<0.05$) of obtained fractions at same level of rotation speed of dosing element. Air flows of 8.7 and 12.5 m^3/h gave coarse fractions that had higher protein content than the fine fraction. These air flows were sufficient enough to drag up significant amount of sunflower hulls into fines. At 8.7 m^3/h any change in dosing speed had not significantly ($p>0.05$) affected changes in protein content of unmilled SFM coarse fractions nor conical milled SFM. Coarse fractions of unmilled SFM had higher protein content than coarse fractions of conical milled SFM obtained at 8.7 m^3/h, thus conical mill was not proved as efficient tool for preparing SFM prior to air classification at this air flow. Protein enrichment of the fraction obtained at 30% rotation speed of dosing element was only 1.78% compared to the starting SFM and protein content of two coarse fractions, the ones obtained at combination of 8.7 m^3/h and at 60% and 90% of maximum rotation speed of dosing element, were not even significantly different comparing it with SFM before classification.

Coarse fractions of both SFM obtained at 12.5 m^3/h had highest protein content of all, thus this air flow was most efficient for obtaining protein rich fractions. Maximum achieved protein enrichment for unmilled SFM compared to the starting material was 7.85%. Increase in rotation speed of dosing element decreased protein content of unmilled SFM coarse fractions at 12.5 m^3/h, but that decrease was not significant ($p>0.05$). Regarding demands for feed rates or yields of unmilled SFM high protein fractions, all three rotation speeds of dosing element can be applied in combination with air flow of 12.5 m^3/h, for achieving the same level of protein enrichment.

Conical milled SFM coarse fractions that were obtained at 12.5 m^3/h were richest in protein content, indicating that conical mill was efficient tool for preparing SFM prior to the air classification at the highest air flow that was used. Highest content of protein, 40.28% d.m., was achieved at 12.5 m^3/h air flow and at 30% rotation speed of dosing element, with protein enrichment of 12% comparing in to the unclassified SFM. As the applied rotation speed of dosing element increased, the protein content of conical milled SFM coarse fractions significantly decreased along with increase of these fractions yields from 52.50% to 55.00%. At the higher dosing rate of the SFM, more material goes to the classifying chamber, so the air stream can drag less grinded hulls into fines. Under influences previously described at the beginning of this section more hulls end up mixed with coarse particles at higher rotation speed of dosing element, which gave fractions that are higher in yield but with lower protein content.

Protein content enrichment of obtained all conical milled SFM fractions was similar to the results of study by Wu and Abbot (2003), in which they obtained protein rich fractions of pinned milled salicornia meal by air classification with protein content higher from 2.9 to 11% than the starting material. Centrifugal separation still proved to be more efficient fractionation technique, since using this method fraction with maximum protein content of 44% d.m. was obtained (Sredanović, 2007).

CONCLUSIONS

Air classification was successfully used for obtaining protein enriched fractions. Protein content and yield of obtained sunflower meal fractions were mainly affected by air flow setting. The increase in air flow resulted in coarse fractions with higher protein content but with lower yield. Lower feed rates led to increase in time for classification of the same amount of the material. Air flow of 5 m^3/h was not efficient for obtaining protein rich fractions of unmilled sunflower meal. Levels of protein enrichment that were achieved at 8.7 m^3/h were the lowest for conical milled meal. Conical mill was efficient tool for preparing sunflower meal prior to the air classification at the highest used air flow. Grinding of sunflower meal gave highest protein enrichment at 12.5 m^3/h and also ensured even and undisturbed material flow in dosing unit of air classifier.

ACKNOWLEDGEMENTS

These results are part of the research within the project III46012, financed by the Ministry of Education, Science and Technological Development, Republic of Serbia. The authors wish to thank Prof. Aleksandar Fišteš on the assistance during performance of experimental procedure, as well as the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina for financial support.

REFERENCES

Association of Analytical Communities (A.O.A.C.) (1998). Official Methods of Analysis (16th ed.). Gaithersburg, MD, USA.

Boni, R., Assogna, A., Grillo, F., Robertiello, A., Petrucci, F., Giacomozzi E., Patricelli, A. (1987). Method for preparing protein hydrolysates soluble in an acid environment, and the hydrolysates obtained. European Patent EP0 271 964 A2.

Challa, R., Srinivasan, R., To, F. (2010). Fractionation of soybean meal, cottonseed meal and wheat middlings using combination of sieving and air classification. Animal Feed Science and Technology, 159, 72-78.

Geneau-Sbartaï, C., Leyris, J., Silvestre, F., Riga, l. L. (2008). Sunflower cake as a natural composite: composition and plastic properties. Journal of Agricultural Food Chemistry, 56, 11198-11208.

ISO 2591-1:1988, "Test sieving-Part 1: Methods using test sieves of woven wire cloth and perforated metal plate."

Johansson, R., Evertsson, M. (2012). CFD simulation of a gravitational air classifier. Minerals Engineering, 33, 20-26.

Laudadio, V., Ceci, E., Nahashon, S.H., Introna, M., Lastela, N.M.B., Tufarellli (2014). Influence of Substituting Dietary Soybean for Air-Classified Sunflower (Helaunthus annuus L.) Meal on Egg Production and Steroid Hormones in Early-Phase Laying Hens. Reproduction in Domestic Animals, 49, 158-163.

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.C., Fine, F. (2012). Rapeseed and sunflower meal: a review on biotechnology status and challenges. Applied microbiology and biotechnology, 95, 1105-1114.

Ramachandran, S., Singh, S.K., Larroche, C, Soccol, CR., Pandey, A. (2007). Oil cakes and their biotechnological applications: a review. Bioresource Technology, 98, 2000–2009.

Shapiro, M. & Galperin, V. (2005). Air classification of solid particles: a review. Chemical Engineering and Processing, 44, 279-285.

Sredanović, S. (2007). Advancement of technological process and quality of sunflower meal, MSc thesis, Faculty of Technology, University of Novi Sad, Serbia.

STATISTICA (Data Analysis Software System), v.12.0 (2013). Stat-Soft, Inc, USA (www. statsoft.com)

Wu, V. & Abbott, T. (2003). Protein enrichment of defatted salicornia meal by air classification. Journal of the American Oil Chemist's Society, 80 (2), 167-169.

Wu, V. & Nichols, N. (2005). Fine grinding and air classification of field pea. Cereal Chemistry, 82(3), 341-344.

THE UNCONTROLLED USE OF ANTIBIOTICS IN PIG PRODUCTION - A THREAT TO PUBLIC HEALTH

Radoslav Došen*, Jasna Prodanov-Radulović, Ivan Pušić, Radomir Ratajac,
Igor Stojanov, Siniša Grubač

Scientific Veterinary Institute "Novi Sad", Rumenački put 20, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: dosen@niv.ns.ac.rs

ABSTRACT

Modern farming is characterized by the tendency of increasing the size and capacity of the farms, which results in increased number of pigs housed at relatively limited space, disturbance of environmental hygiene as well as increased impact of stress factors. Under such conditions, a profitable production implicates successful disease control within the herd. In our country, the majority of farmers is facing financial difficulties and is not able to overcome the drawbacks and problems in the production. Thus, their production strategies often rely on medicamentous therapy. Antibiotics have wide application at pig farms as prophylactic, therapeutic and metaphylactic agents. However, their application leads to the development of resistant microbial strains in both domestic animals and humans. Antimicrobial resistance has emerged as major clinical and public health concern due to its negative effects reflected in prolonged and more complicated therapy course, increased therapy expenses and increased risk of lethal outcomes. A number of consumers and their associations and organizations believe that meat produced without use of antibiotics is safer than that obtained in conventional farming conditions. Our research encompassed industrial farms that have their own veterinary service department or at least one full-time veterinarian, as well as small family-owned farms. The data on prophylactic, therapeutic and metaphylactic measures were obtained from the database and records from the Department of Swine Health protection of the Scientific Veterinary Institute Novi Sad, which were previously obtained from the veterinarians or farm owners. The obtained results indicated a wide range of inadequate procedures in antibiotic application, such as: therapy selection by the owner/farmer himself, free availability of antimicrobial drugs in the market, illegal import, therapy of viral diseases, lack of knowledge on basic principles of pharmacotherapy, lack of effective control in this field.
***Keywords**: pigs, resistance, inadequate use of antibiotics*

INTRODUCTION

The extensive use of antimicrobials in the therapy of human and animal bacterial infections may result in the selection of resistant population of infectious agents that may, in turn, transfer the acquired resistance factors to other bacterial organisms and thereby enable their extensive spread (Levy, 1984; Levy et al., 1988). Numerous authors attempted to understand the relation between the supplementation of antibiotics in animal feed and increased risk of antimicrobial resistance (Stobberingh et al., 1999).

Intensive application of antimicrobials in animal feed industry may lead to severe contamination of animal meet used for human consumption with highly resistant bacterial strains. A number of human infections are caused by such food-born resistant strains and the treatment of human infections is being compromised by increasing resistance of pathogens to antibiotics. Antibiotic-resistant animal pathogens may potentially transfer the resistance towards most important antibiotics used in human medicine (e.g. aminoglycosides, fluoroquinolones, 3rd and 4th generation cephalosphorins), which is the issue of particular concern (Hammerum et al., 2009).

Microbial resistance is a natural biological process and multiplication of resistant strains is influenced by a wide variety of factors. Inadequate (uncontrolled) application of antibiotics in human and veterinary medicine, use of antibiotic for non-therapeutic purposes, environment contamination with antimicrobial drugs, etc. facilitates the multiplication and spread of

resistant organisms. The consequences are highly severe: the population of resistant bacteria is considered responsible for 25,000 deadly outcomes in humans, whilst health care costs reach minimum 1.5 billion Euros. Moreover, it is estimated that some four million patients yearly get infected with resistant microbial strains during hospitalization (WHO, 2014).

In the field of veterinary medicine, it is important to emphasize that a number of pathogens causing digestive and/or respiratory diseases developed resistance to veterinary antimicrobials, thus leading to higher morbidity and mortality rates as well as to increased expenses in swine production (Došen et al., 2011; Prodanov-Radulović et al., 2011). Pursuant to legal directives (EC99/2003), continuous monitoring of antimicrobial resistance of bacteria is performed in EU member countries (Stannarius et al., 2009). Regrettably, an effective system for controlling application of antibiotics and chemotherapeutics in the field of veterinary medicine has not yet been established in the Republic of Serbia (Gavrović et al., 2011).

Antimicrobial resistance of infectious agents results in an increased use of extremely high doses of antibiotics in the prophylaxis, metaphylaxis and therapy (in the feed and drinking water) on pig farms in Serbia. The consequences of such strategy include the emergence, spread and multiplication of highly resistant microbial strains (Došen et al., 2011).

MATERIAL AND METHODS

This research included industrial pig farms that have their own veterinary service department or at least one full-time veterinarian. The material was also collected from small family-owned farms. The data on prophylactic, therapeutic and metaphylactic measures practiced on the investigated farms were obtained from anamnestic and epidemiological databases and records provided by the veterinarians or farm owners. In order to establish the consumption of antimicrobial drugs (antibiotics), our analysis encompassed official records of the Medicines and Medical Devices Agency of Serbia (ALIMS), whereas resistance of human microbial isolates was analysed using the WHO's *2014 report* on global surveillance of *antimicrobial resistance*.

RESULTS AND DISCUSSION

According to the report of the World Health Organization on antimicrobial resistance in 2014, the Republic of Serbia is among the top countries in terms of high resistance of microbial isolates. Resistance to cephalosporins and fluoroquinolones was confirmed in 21.3% and 16.00% *Escherichia coli* isolates, respectively. The resistance rates of *Klebsiella pneumoniae* to cephalosporins and carbapenem are 82.00% and 11.2%, respectively, whereas methicillin (MRSA) resistance was established in 44.5% isolates of *Staphylococcus aureus.* Resistance to penicillin was established in 32.3% isolates of *Streptococcus pneumonia* (WHO, 2014).

The aforementioned results are undoubtedly due to an uncontrolled administration of antibiotics in both human and veterinary medicine. According to the records of Medicines and Medical Devices Agency of Serbia, consumption of veterinary anti-infectious drugs for systemic application reaches the amount of 2,234,264,268.56 Dinars. The half of this amount (1,043,433,007.49 dinars) pertains to pleuromutilins, i.e. tiamulin, which is not surprising considering wide application of these drugs in the prophylaxis and therapy of swine and poultry diseases. Around one fourth of the amount pertains to combinations of antibacterial drugs with other medicinal products (e.g. vitamins) and combinations of several antibacterial drugs.

Massive consumption of antimicrobial drugs in Serbia is closely related to the structure of livestock production, high infectious pressure, poor biosafety measures, and disregarding of principles of good production practices (Došen et al., 2011; Prodanov-Radulović et al., 2013). According to the assessment based on a questionnaire on the level of biosafety, only 0.95% of 111,000 pig-producing farms are classified into the category A of commercial and family-

owned farms, whereas majority of farms is categorized into the category B of family-owned farms (34%) or yards (65%) (Hristov et al., 2012). Furthermore, the swine production plants in Serbia were categorized into three classes according to 15 parameters pertaining to application of health and biosafety measures stipulated in the questionnaire. The majority of production plants were categorized into the class III characterized by the poorest application of the aforementioned measures (total 80.87%), whereas 18.54% of plants were categorized into the class II characterized by somewhat better production conditions. Class I (farms on which all biosafety measures are properly practiced) contained only 0.59% of farms, mainly commercial ones. This is another supporting evidence of unfavourable structure of swine production from the aspect of biosafety (Hristov et al., 2012).

Analysis of four mini swine farms with the capacity 40-350 sows revealed that antimicrobial drugs are administered to all swine categories as well as in all production stages. On the investigated farms, 15 types of antibiotics from almost all antibiotic groups are applied (Table 1). A highly important problem is that farmers (pig producers) administer the drugs to pigs by themselves, and antibiotics in both prophylaxis and therapy are selected based on personal experience. However, only if the expected effects of the administered drug fails and the mortality rate in pigs on the farm increases in spite of self-applied prophylaxis and therapy, the farmers turn to the professionals, i.e. veterinarians.

From the aspect of animal health, the group that poses the highest risk are pig farms that purchase piglets from a number of diverse suppliers. In such production conditions, high infectious pressure often imposes necessity of continuous antimicrobial treatment throughout the entire production process (Došen et al., 2011; Prodanov-Radulović et al., 2013).

Very often, in case of disease outbreak in pigs, the farmers apply therapy based on their own experience or because "the neighbour had successfully cured the disease using particular antibiotic". However, the farmers most commonly use almost every antibiotic that is available on the market (pleuromutilins, tiamulin, quinolone antibacterials, macrolides, lincosamides and streptrogramins, amphenicols, tetracyclines, aminoglycosides and other antibacterial drugs) without establishing the "real" diagnosis or performing the antibiotic susceptibility test (antibiogram) (Došen et al., 2011). In such situation, once the farmer reports to the professional / veterinarian, the problem is already difficult to solve, that is, the veterinarian is faced with a serious issue of selecting the appropriate and effective drug for this disease (Prodanov-Radulović et al., 2011; Prodanov-Radulović et al, 2013).

Table 1. Prophylaxis and therapy in pigs on mini farms (production process from sows to fatteners)

No. of sows	Treatment option	Administration route	Sows	Suckling piglets	Grower pigs	Fattening pigs
40	Prophylaxis	p/o	1,2	4		
	Therapy	p/o		6		
50	Prophylaxis	i/m		14,6	4,6	
	Therapy	p/o		6	3,15,6	
350	Prophylaxis	p/o	1		3,16,	
	Therapy	p/o				5,17, 9
		i/m				5,6, 1+18
180	Prophylaxis	p/o		3,9,19	21	5,11
		i/m	15			
	Therapy	p/o		13		

Table 2. Prophylaxis and therapy on pig fattening farms

No. of fatteners	Treatment option	Administration route	Pre-fatteners	Fatteners
5200	Prophylaxis	p/o	1,2,9,10,6	
350	Prophylaxis	p/o	5,6	
	Therapy	i/m		6,13,8,5
400	Prophylaxis	p/o		1+2, 17
	Therapy	i/m		6,8
600	Prophylaxis	p/o	14+21+22	
	Therapy	p/o		17
		i/m		5
850	Prophylaxis	p/o	1+18,17	
	Therapy	i/m		15,21
220	Prophylaxis	p/o	14+20+21	
	Therapy	p/o		16,18
		i/m		5
300	Therapy	i/m		4,5,6,18

**p/o – peroral administration (in feed or drinking water); i/m – parenteral/intramuscular administration; 1-Lincomycin, 2-Spectinomycin, 3-Amoxicillin, 4-Tulathromycin, 5-Tiamulin, 6-Florfenicol, 7-Cefquinom, 8-Enrofloxacin, 9- Ampicillin, 10-Doxycycline, 11-Penicillin, 12-Streptomycin, 13-Penicillin+Streptomyicin, 14-Oxytetracyclin, 15-Gentamicin, 16-Colistin (polymyxin E),17- Tiamulin + Oxytetracyclin, 18-Tylosin, 19-Tetracyclin,20-Neomycin, 21-Sulfonamid,22-Marbofloxacin, 23 lincomycin+spectinomycin*

This research also encompassed two industrial farms that have their own veterinary service responsible for continuous monitoring of infectious diseases and application of prophylactic measures according to current clinical and pathomorphological condition and laboratory findings including antibiogram results (Tables 3 and 4).

Table 3. Industrial pig farm, category A

No. of sows	Treatment option	Administration route	Sows	Suckling piglets	Grower piglets	Fattening pigs
5000	Prophylaxis	p/o	23	15	3	17
	Therapy	p/o		8,2,20,3,16	6,17,10	6,5,1,3
		i/m	According to the antibiogram and veterinarian's experience			

Table 4. Industrial pig farm, category B

No. of sows	Treatment option	Administration route	Sows	Suckling piglets	Grower pigs	Pre-fatteners	Fatteners
850	Prophylaxis	p/o	5	1,3	1,20	17,1	17
	Therapy	p/o	According to the antibiogram and veterinarian's experience				
		i/m	According to the antibiogram and veterinarian's experience				

Inadequate environment and zoo hygienic conditions that do not comply with the regulations pertaining to certain swine categories represent the most important problem on the majority of pig farms in Serbia. This problem is commonly addressed by inappropriate and unjustified application of antimicrobial drugs (Došen et al., 2011). The situation is further aggravated by

the presence of highly resistant microbial strains, thus prophylactic measures often implicate prolonged administration of therapeutic doses of antibiotics, which is highly improper.

CONCLUSIONS

The Republic of Serbia is among the top European countries in terms of high resistance of microbial isolates. An uncontrolled application of antimicrobial drugs in swine industry is evident on both private farms and mini industrial farms. In that respect, development of a national programme for monitoring the administration of antimicrobial drugs is highly required. Moreover, establishment of continuous control of marketing and application of veterinary drugs as well as education of pig producers and farmers is of crucial importance.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, grants TR 31071.

REFERENCES

Communication from the commission to the European Parliament and the Council (2011). Action plan against the rising threats from Antimicrobial Resistance Brussels, (EC99/2003).

Došen, R., Prodanov-Radulović, J., Pušić, I., Stojanov, I., Stojanović, D., Ratajac, R. (2011): Resistance Escherichia coli isolates to antibiotics from the organ samples originating from swine farms. Biotechnol Anim Husb, 27 (3), 861-866.

Gavrović, M., Ašanin, R., Mišić, D., Jezdimirović, M., Žutić, M. (2011). Investigation of the sensitivity of E.coli strains isolated from domestic animals to antibiotics and hemiotherapeutics in vitro. Acta Veterinaria-Beograd, 61, 1, 21-31.

Hammerum, A.M., Heuer, O.E. (2009). Human health hazards from antimicrobial-resistant Escherichia coli of animal origin. Clin Infect Dis.48 (7),916-21.

Levy, S.B. (1984). Playing antibiotic pool: time to tally the score. New England Journal of Medicine, 311, 663-665.

Levy, S.B., Marshall, B., Schluederberg, S., Rowse, D., Davies, J. (1988). High frequency of antimicrobial resistance in human fecal flora. Antimicrobial Agents and Chemotherapy,32,1801-1806.

Hristov S, Stanković, B., Plavšić, B., Stanojević S. (2012). Biosecurity measures in the prevention and control of classical swine fever. Proceedings of International Conference Prevention of classical swine fever in the border region Croatia – Serbia (STOP – CSF), Novi Sad, Serbia, 290-299.

Prodanov-Radulović, J., Došen, R., Stojanov, I., Pušić, I., Živkov-Baloš, M., Ratajac, R. (2011). Interaction between the mycotoxins and causative agents of swine infective diseases. *Proc. Nat. Sci.*,120, 251-259.

Prodanov-Radulović, J., Došen, R., Stojanov, I., Pušić, I., Ratajac, R. (2013). The necrotic enteritis by Clostridium perfringens in suckling piglets: practical observations, control and diagnostics. The 23rd International Symposium 'New Technologies Incotempotary Animal production', Novi Sad, Serbia,180-182.

Stannarius, C., Bürgi, E., Regula, G., Zychowska, M.A., Zweifel, C., Stephan, R. (2009). Antimicrobial resistance in Escherichia coli strains isolated from Swiss weaned pigs and sows. Schweiz Arch Tierheilkd, 151, 3, 119-125.

Stobberingh E., Van den Bogaard A., London N., Driessen C., Top, J., Willems R. (1999): Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of The Netherlands: evidence for transmission of vancomycin resistance from animals to humans?. Antimicrob. Agents Chemother. 43, 2215–2221.

WORLD HEALTH ORGANISATION (WHO) (2014). Reported or published resistance rates in common bacterial pathogens, by WHO region.

EFFECT OF POPULATION DENSITY ON THE DEVELOPMENT RATE AND THE NUMBER OF RED FLOUR BEETLE *TRIBOLIUM CASTANEUM* (HERBST) OFFSPRING IN COMPLETE ANIMAL FEEDS

Nikola Đukić[1]*, Anđa Vučetić[1], Goran Andrić[2]

[1]Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Zemun, Serbia
[2]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Zemun, Serbia

*Corresponding author:
E-mail address: nikoladjukadj@yahoo.com

Red flour beetle *Tribolium castaneum* is a dangerous pest especially in the ground grain products, which can cause significant damage to the complete animal feed. The aim of this study was to examine still insufficiently known development rate and the number of this pests offspring in complete feeds.
The tests were done under controlled conditions (30±1C^{0}and r.h 55±10%) with four initial population densities of 100, 50, 25 and 10 insects in complete feed for pigs and laying hens and wheat flour (control). Insects were placed in the 50g of above mentioned substrates in four replicates, and extracted after seven days to determine development rate of their offspring and the number and weight of newly emerged adults. Data were statistically analyzed using analysis of variance. We found that the *T. castaneum* development lenght in complete feeds at the highest density was 60 days, while at the lowest density this cycle lasted 20 days. In the control substrate, the length of insects development, at the highest density lasted for 21 days, and 18 days at the lowest. At the highest density, the highest number of offspring (1230) was determined in contol, followed by pig feed (582.8) and significantly lower in the feed for laying hens (260), while at the lowest density there were no statistically significant differences between the substrates. The mass of insects (1.16865mg-1.5653mg) that were developed in feed for laying hens was significantly less than of insects (1.12285mg-1.6686mg) in pig feed, except at the highest density.
The results show that the initial population density significantly influences the development rate, number and weight of *T. castaneum* offspring, which is especially pronounced in the feed for laying hens. Knowing the cycle of reproduction and development of this pest on different substrates may contribute to the timely and economically profitable protection of stored products.
***Keywords**: T. castaneum, population density, development rate, number of offspring, animal feed*

INTRODUCTION

The global feed industry produced 870 million tons of animal feed in 2011., and it is believed that this production will grow by 3% each year (Lević and Sredanović, 2013). As is the case throughout the world and in Serbia, increasing production of feed for domestic animals requires the improvement of existing biotechnological processes, which would also improve its quality and safety. Storage pests, among which are the most numerous stored-product insects, greatly impair the quality and safety of the feed in the facilities of its production and in the storages. Annually, these pests globally destroy 5 - 30% of all stored products (Pugazhvendan et. al., 2009). One of the most serious species of stored-product insects is the red flour beetle, *Tribolium castaneum* Herbst. It belongs to the group of secondary pests, as it feeds on already damaged kernels and various grain products (Campbell, 2010, Mikhaiel, 2011, Pugazhvendan et al., 2012, Romero et al., 2010). The type of stored products which these pests feed on has a major impact on the development rate and the number of their offspring (Lale, et al., 2000, Longstaff, 1995, Sastawa et al., 2009). The nutritional value of the substrate is an important factor for the development and the number of offspring of species *T. castaneum*. Thus, in some of the substrates the number of insects is higher and the length of development is shorter, while in others it is the opposite (Ahamad, 2012, Longstaff, 1995, Soliman and Hardin 1971, Wong and Lee, 2007). In addition to the types of materials and products which the insects feed on, the initial population density of *T. castaneum* can also have a big impact on development, number and weight of its offspring. The lower number and the longer cycle of the offspring development in certain types of

substrates are closely associated with high initial population density of *T. castaneum* (Longstaff, 1995). In addition, the high initial density of the population of *T. castaneum* may affect the lower mass of its offspring (Assie et al., 2008).
Previous studies have mostly been based on an examination of the development rate and number of *T. castaneum* offspring on stored products, which are present in the human diet. There is less available scientific data and information regarding the development and number of offspring of this cosmopolitan pest on stored products, which are a necessary component in the feed industry. The aim of this research is to determine development length, number and weight of the *T. castaneum* offspring with different initial population densities in the complete feed.

MATERIALS AND METHODS

Test insects

Insects from the laboratory population of *T. castaneum* were used as test subjects, and were grown in wheat flour with the addition of brewer's yeast at a constant temperature of 25 ± 10 ^{0}C and a relative humidity of 50 ± 5%.

Substrates

As substrates, we used complete feed for laying hens with 17% protein, and the following composition: corn, soybean and sunflower meal, soybean meal, bread, bran products, industrial alcohol and starch, mineral nutrients, vitamin and mineral mixtures, antioxidants, amino acids, enzymes with vitamin supplements A, D3, E, B2, Mn, Fe, Cu, Zn, S, Co, Se; and complete feed for fattening pigs with 16% protein and the following composition: corn, soybean and sunflower meal, soybean meal, bread, bran products, industrial alcohol and starch, mineral nutrients, vitamin and mineral mixtures, antioxidants, amino acids, enzymes with supplements of vitamins A, D3, Mn, Fe, Cu, Zn, S, Se; and as a control soft wheat flour (type 500) + 5% brewer's yeast was used. All substrates in the experiment were sterilized for 10 h at 600^{0}C before use (Tuncbilek and Kansu, 1996).

Bioassays

Four initial population densities of 100, 50, 25 and 10 test insects were placed in plastic containers with 50 g of the above-mentioned substrate in 4 replicates, and were left in a controlled environment (30 ± 1C^o RH and 55 ± 10%). After one week, the adults were eliminated by sifting from the substrate. This was done for the purpose of monitoring the length of development, determining the number of offspring in substrates, and the mass of the newly emerged adults. The number of offspring was recorded from the moment of emergence of the first adult insect until the appearance of the last one, every 5 days. Once all insects have emerged, a total number and the mass of the insects on different substrates at different population densities were determined.

Statistical analysis of data

Data regarding the number and weight of *T. castaneum* offspring on different substrates and at different population densities were statistically analyzed using oneway ANOVA and a comparison of mean values using Fisher LSD test ($p > 0.05$) (StatSoft, 2005).

RESULTS

Development rate of the *T. castaneum* offspring

We found that the development rate of *T. castaneum* varies considerably depending on the type of substrate and the initial density of the population. In complete feeds at the highest initial population density, the length of development lasted over 60 days, while in the control substrate it lasted three times less (Table 1). At the initial population density of 50 insects, the longest period of development was recorded in the feed for laying hens and it lasted 46 days, while in feed for pigs it was significantly shorter at 27 days. In the control substrate, it

lasted 19 days. At the initial population density of 25 insects the longest period of development was determined in feed for pigs (26 days), slightly shorter (23 days) in feed for laying hens and the shortest in the control substrate (20 days). At the lowest initial density, the differences in the length of development of insects between different substrates were negligible. Thus, the longest cycle of development was in feed for pigs (21 days), one day shorter in feed for laying hens and the shortest in the control substrate (18 days), (Table 1).

Table 1. Development rate and average number of T. castaneum offspring

Development rate and number of *T. castaneum* offspring in different substrates, at initial population densities of 100,50,25 and 10 insects												
Days from first adult emerge*	**Control**				**Feed for pigs**				**Feed for laying hens**			
	100	50	25	10	100	50	25	10	100	50	25	10
0	2.5	2.5	3.5	0.5	0,3	1.0	0.8	0.0	0.0	0.3	0.3	0.0
5	189.3	305.0	245.3	128.3	89.5	141.5	105.3	59.8	1.8	21.0	138.3	154.0
10	442.8	658.8	578.5	228.8	118.3	276.8	301.5	225.3	6.0	77.5	334.5	271.3
15	525.8	180.3	192.8	57.5	64.3	256.8	258.8	125.0	4.0	126.0	283.8	102.5
20	68.3	11.5	16.5	2.8	91.5	234.8	111.5	10.5	8.8	206.3	35.0	5.8
25	1.8	0.0	0.0	0.0	68.3	51.0	12.0	0.0	7.3	173.3	3.3	0.5
30	0.0	0.0	0.0	0.0	36.0	3.3	0.3	0.0	25.3	60.5	0.0	0.0
35	0.0	0.0	0.0	0.0	45.5	0.0	0.0	0.0	36.5	40.5	0.0	0.0
40	0.0	0.0	0.0	0.0	66.8	0.0	0.0	0.0	56.0	25.3	0.0	0.0
45	0.0	0.0	0.0	0.0	80.5	0.0	0.0	0.0	55.0	7.0	0.0	0.0
50	0.0	0.0	0.0	0.0	66.0	0.0	0.0	0.0	32.8	0.5	0.0	0.0
55	0.0	0.0	0.0	0.0	15.5	0.0	0.0	0.0	17.8	0.0	0.0	0.0
60	0.0	0.0	0.0	0.0	3.5	0.0	0.0	0.0	5.5	0.0	0.0	0.0
65	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	3.0	0.0	0.0	0.0
70	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0

**Emerge of the first adult in control 15 days after extracting adults from substrate at all initial population densities, in feed for pigs 17 days after extracting adults from substrate at initial population densities of 100, 50 and 25 insects and 18 days after extracting adults at initial population density of 10 insects and in feed for laying hens 18. days after extracting adults from substrates at initial population densities of 50 and 25 insects and 19 days after extracting adults at initial population density of 100 and 10 insects*

The number of *T. castaneum* offspring

The number of offspring was significantly different depending on the type of substrate and population density (Table 2). At the highest initial population density the lowest number was recorded in the feed for laying hens (260), followed by feed for pigs (582.8), and the maximum number of offspring (1230) was found in the control. At the initial population density of 50 insects the lowest number was again noted in feed for laying hens (738), followed by feed for pigs (965), the highest number was noted in the control substrate (1158). Initial population density of 25 insects produced offspring that were significantly more numerous in the control substrate (1037) in relation to the feed for pigs (790) and the feed for laying hens (795). At the lowest population density there were no statistically significant differences between the tested substrates (Table 2).

Table 2. The average number of T. Castaneum offspring

Substrates	Average number of offspring + SE			
	Densities			
	100	50	25	10
Control	1230.0±50.3[a]	1158.0±69.4[a]	1037.0±40.9[a]	417.8±45.0[a]
Feed for pigs	582.8±34.1[b]	965.0±74.7[ab]	790.0±34.7[b]	4205±63.5[a]
Feed for laying hens	260.0±93.1[c]	738.0±87.3[b]	795.0±50.4[b]	534.0±91.8[a]

Mean within columns followed by the different letters are significantly different at the 0.05 level

The body weight of *T. castaneum* offspring

The results showed that the *T. castaneum* offspring had the lowest average weight at the highest population density in all three tested substrates and that the individuals in the control had significantly more body weight of individuals that have developed in complete feeds (Table 3) .The highest average weight (1.7 and 1.6 mg) of adults was found in the feed for pigs and hens at the lowest population density. The highest average weight (1.8 mg) in the control was found at initial density of 25 insects. At all initial population densities the weight of individuals that have developed in the feed for laying hens was significantly less than that of individuals that have developed in feed for pigs except for the initial popultion density of 100 insects (Table 3).

Table 3. The average weight of T. Castaneum offspring

Substrates	Average weight of one insect (mg) + SE			
	Densities			
	100	50	25	10
Control	1.649±0.015 a[*]	1.768±0.008 a	1.771±0.016 a	1.720±0.018 a
Feed for pigs	1.122±0.009 c	1.535±0.009 b	1.557±0.018 b	1.669±0.018 b
Feed for laying hens	1.169±0.014 b	1.369±0.011 c	1.487±0.013 c	1.565±0.011 c

[*]*Mean within columns followed by the different letters are significantly different at the 0.05 level*

DISCUSSION

The results showed that the type of substrate and initial population density had significantly affected development rate, size and weight of the *T. castaneum* offspring. Insects in complete feeds had a 40 day longer development cycle at the highest compared to the lowest initial population density, whereas in the control, insects had a similar length of the development cycle, at the highest and at the lowest initial population density. Large differences in the insect development rate at high initial population density could be explained by the type of substrate, its composition and nutritional value. Longstaff (1995) found that some substrates such as soft wheat flour with its nutritive values could provide a short cycle of development and a large number of *T. castaneum* offspring. Another important factor is the brewer's yeast presence in the control. Studies that have been carried out earlier (Sokoloff et al., 1966) showed that species *T. castaneum* had a greater need for brewer's yeast than species *Tribolium confusum* Duval, confused flour beetle, and that the addition of brewer's yeast to the substrate caused significantly faster development. Another study conducted by Soliman and Hardin (1972) confirmed that *T. castaneum*, develops much slower in corn flour without additives than in the wheat flour with the addition of yeast.

The number of offspring on different substrates differed significantly at the highest initial population densities. With reducing of the population density, differences in the number of offspring among substrates were gradually reduced too, so at the lowest initial population density these differences weren't statistically significant. The offspring number is related

mainly to the development length, and like development length it is influenced by the composition and nutritional value of the substrate. This fact is confirmed by research conducted by Fardisi et al., (2013) which found that *T. castaneum* could develop in the products of corn distillation which were used as a supplement in animal feed, however, its development in these products lasted much longer, and the number of offspring was significantly lower compared to the flour with the addition of yeast. As in the above mentioned research *T. castaneum* offspring that were developed in complete feeds had a longer cycle of development and were less numerous than the offspring in the flour with brewer's yeast, but only at the higher initial population density. Beside the influence of the composition and the nutritional value of the substrate, another significant limiting factor could affect the number of *T. castaneum* offspring and that is cannibalism. Usually, cannibalism is a consequence of lack of the required amount of food (Li and Arbogast, 1991, Wong and Lee, 2007). In this experiment, during the monitoring of offspring development, at high initial population densities in complete feeds, clear signs of pupae cannibalism by the larvae were observed, i.e. we observed that a large number of pupae were damaged by larvae feeding. These results coincide with research conducted by Longstaff (1995) in which he found pupae cannibalism by larvae at the high initial population densities. As in our research, he eliminated cannibalism by adult insect extraction after a week while leaving the offspring to develop, so that the individuals who had developed faster and were in the pupae stage were eaten by individuals in larvae stage that have been developing slower.
In complete feed for laying hens, offspring from the lowest initial population density had 25% higher mass then the offspring from the highest initial population density, while in the case of complete feed for pigs this difference was as much as 32%. In the control substrate differences between offspring mass at different population densities were insignificant. This data can be explained by the fact that populations with a high density of individuals are often conditioned by the lack of the required amount of nutrients at the time of insect development and that affects the lower mass of insect body. This is confirmed by research conducted by Assie et al. (2008). They also found that females from populations with low initial density are on average 0.22 mg heavier than the females from populations with higher initial density.

CONCLUSION

Based on this study, it may be concluded that the complete feeds could support shorter cycle of development, a higher number and weight of the offspring at the low to moderate initial population density. Higher initial population densities are increasing the length of development and reducing the number of offspring due to lack of food and cannibalism by larvae. These data suggest that at high summer temperatures in the presence of a small number of these insects, even shorter storage of complete feeds could be potentially dangerous. The knowledge of the cycle of reproduction and development of this pest in different substrates may contribute to the timely and economically profitable protection of stored products as well as the determination of the optimal period of their storage.

ACKNOWLEDGEMENTS

Financial support was received from the Ministry of Education, Science, and Technological Development of the Republic of Serbia, Project No. III046012 and Project No. III 46008.

LITERATURE

Ahmad F., Walter, G.H., Raghu, S. (2012). Comparative performance of *Tribolium castaneum* (Herbst) (Coleoptera:Tenebrionidae) across populations, resource types and structural forms of those resources. Journal of Stored Products Research 48, 73-80.

Assie L.K., Brostaux, Y., Haubruge, E. (2008). Density-dependent reproductive success in *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). Journal of Stored Products Research 44, 285-289.

Campbell, J.F., Toews, M.D., Arthur, F.H., Arbogast, R.T. (2010). Long-term monitoring of *Tribolium castaneum* in two flour mills: seasonal patterns and impact of fumigation. Journal of Economic Entomology 103, 991-1001

Fardisi, M., Mason, L. and Ileleji, K. (2013). Development and fecundity rate of *Tribolium castaneum* (Herbst) on Distillers Dried Grains with Solubles. Journal of Stored Products Research 52, 74-77.

Lale, N.E.S., M. Lawan and F.A. Ajayi. (2000). Effects of temperature and yeast supplementation on the development of *Tribolium castaneum* (Herbst) in whole meal and polished flour derived from four cereals in Maiduguri, Nigeria. Journal of Pest Science. 73, 89–92.

Lević J. and Sredanović S. (2012). Feed to food. XV International Feed Technology Symposium, „Feed-to Food"/ COST- „Feed for Health" joint Workshop, Proceedings, 15-16, 03-05 October, 2012, Novi Sad, Serbia.

Li, L. and Arbogast, R. (1991). The effect of grain breakage on fecundity, development, survival, and population increase in maize of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Journal of Stored Products Research 27, 87-94.

Longstaff, B. (1995). An Experimental Study of the Influence of Food Quality and Population Density on the Demographic Performance of *Tribolium castaneum* (Herbst). Journal of Stored Product Research. Vol. 31, No. 2, 123-129.

Mikhaiel, A. (2011). Potential of some volatile oils in protecting packages of irradiated wheat flour against *Ephestia kuehniella* and *Tribolium castaneum*. Journal of Stored Products Research 47, 357-364.

Pugazhvendan, R., Elumalai, K., Ronald Ross, P. and Soundararajan, M., (2009): Repellent Activity of Chosen Plant Species Against *Tribolium castaneum*. World Journal of Zoology 4 (3), 188-190.

Pugazhvendan, R., Ronald Ross, P., Elumalai K. (2012). Insecticidal and repellant activities of plants oil against stored grain pest, *Tribolium castaneum* (Herbst) (Coleoptera:Tenebrionidae). Asian Pacific Journal of Tropical Disease, 412-415.

Romero S., Campbell J., Nechols J. and With K. (2010). Movement Behavior of Red Flour Beetle: Response to Habitat Cues and Patch Boundaries. Environmental Entomology 39, 919-929.

Sastawa, B.M., Turaki, J.M., Kabir B.G. J., Lale, N.E.S.(2009). Effects of resource conditioning, season and intraspecific interaction on progeny development in *Tribolium castaneum*. International Journal of Agriculture and Biology, 11, 158-162.

Soliman, M. and Hardin R. (1972). Variation in Populations of *Tribolium castaneum (*Herbst)(Coleoptera, Tenebrionidae). Journal of Stored Products Research 8, 1-10.

Sokoloff, A., Franklin, I. and Lakhanpal R. (1966). Comparative Studies with *Tribolium* (Coleoptera, Tenebrionidae) - II: Productivity of T. *castaneum* (Herbst) and *T. confusum* Duv. on Natural, Semi-synthetic and Synthetic Diets, Journal of Stored Products Research 1, 313-324.

StatSoft, 2005. STATISTICA (data analysis software system), version 7.1. StatSoft, Tulsa, OK, USA.

Tuncbilek, A. and Kansu, A. (1996). The influence of rearing medium on the irradiation sensitivity of eggs and larvae of the flour beetle *Tribolium confusum* J. du Val. Journal of Stored Products Research 32, 1-6.

Wong, N. and Lee, C. (2011). Realationship between population growth of the red flour beetle *Tribolium castaneum* and protein and carbohydrate content in flour and starch. Journal of Economic Entomology 104, 2087-2093.

AGRI-FOOD CO-PRODUCTS AS ALTERNATIVE DIETARY SUPPLEMENTS AND FARM ANIMAL PRODUCT QUALITY: OPPORTUNITIES, LIMITATIONS AND RESEARCH GAPS

Eleni Kasapidou[1]*, Paraskevi Mitlianga[1], Evangelia Sossidou[2]

[1]Department of Agricultural Technology, Division of Agricultural Products Quality Control, Technological Educational Institution of Western Macedonia, Terma Kontopoulou, 53100 Florina, Greece
[2]Hellenic Agricultural Organization-DEMETER, Veterinary Research Institute of Thessaloniki, P.O. BOX 60272, 57001 Thermi, Thessaloniki, Greece

*Corresponding author:
E-mail address: e.kasapidou@teiwm.gr

ABSTRACT

Agri-food co-products are good sources of valuable compounds such as pigments, polyphenols and flavonoids that possess nutritional and health promoting properties. Supplementation of animal diets with these compounds and subsequent transfer into milk, meat and eggs increase their availability in the human diet. Utilization of agri - food co-products in livestock nutrition helps towards reduction of the environmental burdens arising from food production. Moreover, nowadays consumers demand the production of "clean," "natural" and "green" label food products and are willing to pay significant premiums for such products. At the same time, the global market for feed ingredients is expected to expand due to population growth and increased consumption of animal products in the developing countries. Current research has primarily focused on the utilization of co-products such as grape, tomato, oilseed or citrus pomace that are voluminously produced and have an important environmental impact. Product quality, inconsistency of chemical composition, seasonality, anti-nutritional compounds, logistics, commercial value, and the complicated feed legislation are the main reasons restricting their use. Safety, sustainability, innovation and value addition are key drivers for product differentiation in the competitive animal food product market and thus, the market potential of agri-food co-products in animal nutrition seems very promising. However, focused research on their effect on livestock product quality is still missing.
Keywords*: agri-food co-products, dietary supplements, environment, product quality*

INTRODUCTION

Recently, there has been increased social, political and environmental pressure for the efficient utilization of agricultural production residues (Mirzaei-Aghsaghali and Maheri-Sis, 2008). Utilization of agroindustrial co-products in animal nutrition helps the food industry to comply with the legislation by finding new end-uses of the co-products (Panouillé *et al*., 2007) and by reducing the environmental burden arising from food production (Laufenberg *et al*., 2003). Additionally, inclusion of agri-food co-products in feed rations improves profitability and valorization of the agricultural by-products since feeding food residue to livestock is an efficacious way to upgrade cheap materials into high quality foods (Elferink *et al.,* 2008). Finally, consumers that demand the production of "clean," "natural" and "eco/green" label food products (Grunert, 2005) are also willing to pay significant premiums for such products.
Agri-food co-products are promising sources of valuable substances such as pigments, sugars, organic acids, flavors and functional compounds such as polyphenols and flavonoids that possess favorable technological and nutritional properties (Galanakis, *2012*). The functions of these compounds in the food industry are well documented in review studies (Ayala-Zavala *et al*., 2011; Galanakis, 2012; Laufenberg *et al*., 2003; Oreopoulou and Tzia, 2007). Agri-food residues have been traditionally used in animal nutrition as main feed ingredients (Crawshaw, 2001). However, agri-food co-products as sources of phytochemicals constitute a relatively new class of feed ingredients, and there is limited knowledge on their

aspects of application and functions as well as their bioactivity, bioavailability and interactions with other feed ingredients.
Production of agri-food products yields various amounts of by-/co-products in relation to the type of the raw material and the processing procedure. For most fruits and vegetables, the production of the likely waste is estimated to approximately 30% or more of the processed material (Table 1). The global market for feed ingredients is expected to expand due to population growth and increased consumption of animal food products in the developing countries. Recent reports predict that the global market of all types of feed additives will grow at the compound annual rate of 3.8% in the coming years, and it is projected to reach the worth of approximately $20 million by 2018 (Anonymous, 2014).

Table 1. Percentage of plant origin food wastes and by-products (modified from Fuentes et al., 2004)

Production process	**% of wastes and by-products**
White wine production	20-30
Red wine production	20-30
Fruit and vegetable juice production	30-50
Fruit and vegetable processing and preservation	5-30
Vegetable oil production	40-70
Potato starch production	80
Sugar production from sugar beet	86

The aim of this work is to provide recent knowledge and to outline opportunities, limitations and research gaps on the application of agri-food co-products as alternative dietary supplements in farm animal nutrition.

DEFINITION OF AGRI-FOOD CO-PRODUCTS IN ANIMAL NUTRITION

In this overview, the definition of agri-food co-products covers all products deriving from the various steps of agri-food processing that is suitable for animal consumption, welfare and health as well as human health and safety. According to the European Union Regulation EC 767/2009, feed materials are defined as "products of vegetable or animal origin, whose principal purpose is to meet animals' nutritional needs, in their natural state, fresh or preserved, and products derived from the industrial processing thereof, and organic or inorganic substances, whether or not containing feed additives, which are intended for use in oral animal-feeding either directly as such, or after processing, or in the preparation of compound feed, or as carrier of premixtures". The presented data will be limited to co-products of plant origin (fruits, vegetables and oilseeds) due to the restrictions applying in the use of many animal origin co-products in livestock nutrition in the European Union countries.

MODES OF ACTION OF DIETARY SUPPLEMENTS

Feed additives are considered as substances added in small quantities in the stock feed for nutritional purposes but also for additional functionality on a permanent basis (possibly throughout the entire production period of the respective species) (Windisch *et al.,* 2008). Feed additives are used to improve the health status of the animals since they exhibit anti-bacterial, coccidiostatic, anthelmintic, antiviral or anti-inflammatory activities. They can also promote animal performance by improving feed conversion ratio, increasing nutrient digestibility and helping the animals to reach their genetic potential. Feed additives can also affect other major determinants of food quality for the modern consumer such as nutritional value, sensory characteristics, health enhancement (Bogue *et al*., 2005; Wenk, 2000). Regulation EC 1831/2003, determines the use of additives in animal nutrition and sets out

the rules for their authorization, marketing and labelling. The Commission has also established the European Union Register of Feed Additives (http://ec.europa.eu/food/food/animalnutrition/feedadditives/legisl_en.htm)

SYSTEM DESCRIPTION

Agri-food co-products are collected either from the primary production fields or the processing factories, and they are used either as an unprocessed residue or they are subjected to processing. Processing procedures may involve drying since most of these materials contain high moisture content that leads to product spoilage or subjected to advanced processing/ bio refinery techniques for the collection of specific compounds such as phenols, vitamins, fatty acids, carotenoids, pectin, pigments.
The produced co-products can be incorporated in animal feed formulations either as main feed ingredients to provide crude protein, fat and energy i.e. citrus pulp, as dietary supplements to achieve a particular function i.e. tomato pulp as antioxidants or as dual purpose ingredients that is as main and functional feed ingredients i.e. olive pomace, allowing recycling of the agri-food co-products inside the food chain, that is completed by the production of farm animal products i.e. milk, meat and eggs (Figure 1). Additional information about the application of agri-food co-products in animal diets as main or supplementary feed ingredients can be found in the studies quoted in Table 2.

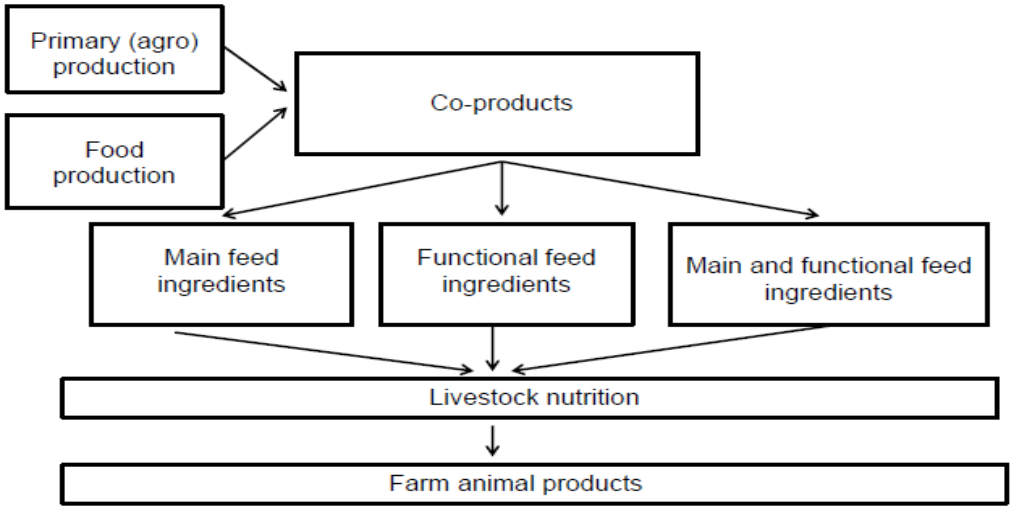

Figure 1. Schematic presentation of the food – feed – food system

EFFECTS OF DIETARY AGRI-FOOD CO-PRODUCTS SUPPLEMENTATION ON FARM ANIMAL PRODUCT QUALITY

Examples of effective dietary supplementation of agri-food co-products on the sensory characteristics and the nutritional value of the produced animal products are presented in Table 1. Research has primarily focused on the utilization of co-products such as grape, tomato, olive or citrus pomace that are voluminously produced and have an important environmental impact. Most of these products have been tested in poultry diets as antioxidants, and the desirable effect was achieved following a dose-response pattern. Another examined quality trait is the lipid profile of small ruminant meat and milk since application of the food co-products resulted in the production of milk and meat a health promoting fatty acid profile. Yolk color enhancement has also been reported in a few studies. In most studies, fresh or minimally processed i.e. dried co-products have been used but in a limited number of trials high value components such as lycopene, phenols, recovered from co-products through the application of various conventional and emerging processing

technologies, have also been used. Utilization of apple, banana, potato, onion, carrot or sugar beet co-products remains underexplored. Additionally, documented research on the antimicrobial function of agri-food co-products is very scarce.
Lack of efficacy of the supplemental feed ingredient in relation to the intended function has also been reported in many cases. However, the potential reasons for the absence of effectiveness have not been thoroughly explored in most studies. Parameters such as supplementation levels, duration of supplementation, product bioavailability and metabolism, product quality and form (fresh, dried, extract) as well as species differences may be related to the unpromising results.

Table 2. Effect of agri-food co-products on farm animal product quality (selection)

Co product	Animal	Effect[1]	Reference
Grape pomace	Broilers	Lipid oxidation	Goñi *et al*., 2007
Dried tomato pomace	Broilers	Cholesterol	Kavitha *et al*., 2007
Hesperidin	Broilers	Lipid oxidation	Simitzis *et al*., 2011
Grape seed extract	Broilers	Lipid oxidation	Smet *et al*., 2008
Citrus pulp	Broilers	Fatty acid composition	Mourão *et al*., 2008
Tomato pomace	Quails	Lipid oxidation	Sahin *et al*., 2006
Tomato powder	Quails	Color (yolk)	Karadas *et al*., 2006
Hesperidin	Layers	Lipid oxidation	Goliomytis *et al*., 2014
Grape pomace	Ewes	Fatty acid composition (milk)	Tsiplakou and Zervas, 2007
Olive by products	Ewes & Goats	Fatty acid composition (milk)	Molina Alcaide & Yáñez-Ruiz, 2008
Olive cake	Ewes	Fatty acid composition	Vargas-Bello-Pérez *et al*., 2013
Olive cake	Lambs	Lipid oxidation	Luciano *et al*., 2013
Dried citrus pulp	Lambs	Lipid oxidation	Inserra *et al*., 2014
Olive pomace	Rabbits	Lipid oxidation	Dal Bosco *et al.*, 2012
Tomato pomace	Rabbits	Fatty acid composition	Peiretti *et al.*, 2012
Olive pomace	Pigs	Lipid oxidation Fatty acid composition	Doyle *et al*., 2006
Fermented grape pomace	Pigs	Fatty acid composition (subcutaneous fat)	Yan and Kim, 2011

[1] *effect on meat unless otherwise stated*

FACTORS AFFECTING THE COMMERCIAL APPLICATION OF AGRI-FOOD CO-PRODUCTS IN ANIMAL NUTRITION

The main three parameters affecting application of alternative feed ingredients in animal nutrition are related to species and anti-nutritional factors, production logistics and the profit from the product extending from the primary producer to both the feed industry and the livestock producer. The presence of anti-nutritional factors can adversely affect production yields prohibiting the utilization of agri-food products in livestock nutrition. Anti-nutritional factors can be species dependent and related to differences in their bio-efficacy between monogastrics and ruminants, though. With regard to production logistics, there should be adequate product quantity to support a consistent supply chain and to enable incorporation in various types of feed formulations. Finally, the value and the profit of the co-product should be sufficient to attract the product producer, feed and livestock producers, whereas the relative economic value of the co-products should remain low to gain an advantage in the competitive feed market. The complicated feed legislation and the hidden/unknown costs

related to storage and handling may also discourage animal nutritionists from using this type of feed ingredients (Williams, 2014).
The application of co-products in animal nutrition is also limited by parameters such as product inconsistency dependent upon plant material botanical origin and the method of initial processing; product quality, composition, stability, functionality and safety; palatability of the produced ration; the lack of thorough documentation on the positive and adverse effects of these ingredients in animal performance, and end-product quality in commercial trials.

CONCLUSIONS

Available information shows that agri-food co-products can be used in livestock nutrition as novel feed additives for the production of clean and green label farm animal products with improved sensory characteristics and nutritional value, in compliance with consumer's wishes. Recent advances in the recovery of valuable compounds from agri-food processing co-products would allow the cost effective utilization of these products in animal nutrition. Commercial application of agri-food co-products as dietary supplements poses an interesting challenge for field scientists aiming to overcome the problems of environmental burden and production costs whilst maintaining animal health and production yields.

FUTURE RESEARCH

Future research should focus on:

- The effect of processing on product composition as well the type and the concentration of the active/ target feed ingredients.
- The development of processing or biorefinery techniques that eliminate or negate the anti-nutritional factors of the co-products.
- The interactions and the synergistic functions of the dietary (functional) feed ingredients with the main feed ingredients in various types of feed formulations.
- The determination of optimum supplementation levels and the bioavailability of the feed ingredients in relation to species and required function.
- The production of affordable products with a standard composition that can be easily stored (extracts, powders) for a reasonable shelf life.
- The evaluation of the functionality of this type of feed ingredients on large scale (commercial) feeding trials.

REFERENCES

Anonymous, (2014). Animal feed additives market worth $20,233.2 Million by 2018 http://www.marketsandmarkets.com/PressReleases/animal-feed.asp (accessed 15 June, 2014)

Ayala-Zavala, J.F., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J.A., Siddiqui, M.W., Dávila-Aviña, J.E. & González-Aguilar, G.A. (2011). Agro-industrial potential of exotic fruit byproducts as a source of food additives. Food Research International, 44 (7), 1866-1874.

Bogue, J., Coleman, T. & Sorenson, D. (2005). Determinants of consumers' dietary behaviour for health-enhancing foods. British Food Journal, 107(1), 4-16.

Brenes, A., Viveros, A., Goñi, I., Centeno, C., Sáyago-Ayerdy, S.G., Arija, I., Saura-Calixto, F. (2008). Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry Science, 87 (2), 307-316.

Crawshaw, R. (2001). Co-product feeds: animal feeds from the food and drinks industries. Nottingham University Press, UK.

Dal Bosco, A., Mourvaki, E., Cardinali, R., Servili, M., Sebastiani, B., Ruggeri, S., Mattioli, S., Taticchi, A., Esposto, S., Castellini, C. (2012). Effect of dietary supplementation with olive pomaces on the performance and meat quality of growing rabbits. Meat Science, 92 (4), 783-788.

Doyle, S.P., Harrison, K.R., Daley, C.A., Hamilton, P.C., Sinnott, D.K. (2006). Effects of feeding olive pomace on the fatty acid profile of pork. Proceedings, Western Section, American Society of Animal Science, 57, 216-218.

Elferink, E.V., Nonhebel, S. & Moll, H.C. (2008). Feeding livestock food residue and the consequences for the environmental impact of meat. Journal of Cleaner Production, 16 (12), 1227-1233.

European Union (2003). Regulation No 1831/2003 of the European parliament and of the council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union, L268, 29-43.

European Union (2009). Regulation No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed. Official Journal of the European Union, L 229, 1-28.

Fuentes, L. de las., Sanders, B., Lorenzo, A. & Alber, S. (2004). AWARENET: Agro-Food Wastes Minimisation and Reduction Network. Workshop Report IN: K.W. Waldron, C.B. Faulds and A.C. Smith (eds.) "Total Food 2004, exploiting co-products – minimizing waste" Proceedings Volume, Institute of Food Research, pp. 233-244.

Galanakis, C.M. (2012). Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends in Food Science and Technology, 26 (2), 68-87.

Goliomytis, M., Orfanou, H., Petrou. E., Charismiadou. M.A., Simitzis, P.E., Deligeorgis, S.G. (2014). Effect of hesperidin dietary supplementation on hen performance, egg quality and yolk oxidative stability. British Poultry Science, 55 (1), 98-104.

Goñi, I., Brenes, A., Centeno, C., Viveros, A., Saura-Calixto, F., Rebolé, A., Arija, I., Estevez, R. (2007). Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility, and susceptibility to meat lipid oxidation in chickens. Poultry Science, 86 (3), 508-516.

Grunent, K.G. (2005). Food quality and safety: consumer perception and demand. European Review of Agricultural Economics, 32 (3), 369-391.

Inserra, L., Priolo, A., Biondi, L., Lanza, M., Bognanno, M., Gravador, R., Luciano, G. (2014). Dietary citrus pulp reduces lipid oxidation in lamb meat. Meat science, 96 (4) 1489-1493.

Karadas, F., Grammenidis, E., Surai, P.F., Acamovic, T., Sparks, N.H.C. (2006). Effects of carotenoids from lucerne, marigold and tomato on egg yolk pigmentation and carotenoid composition. British poultry science, 47 (5), 561-566.

Kavitha, P., Ramana, J.V., Prasad, J.R., Reddy, P.V.V.S. & Reddy, P.S. (2007). Effect of dried tomato (Lucopersicon esculentuni) pomace inclusion is broiler diets on serum and muscle cholesterol content. Indian Journal of Animal Sciences, 77 (4), 338-343.

Lau, D.W. & King, A.J. (2003.) Pre- and post-mortem use of grape seed extract in dark poultry meat to inhibit development of thiobarbituric acid reactive substances. Journal of Agricultural and Food Chemistry, 51 (6), 1602-1607.

Laufenberg, G., Kunz, B., Nystroem, M. (2003). Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. Bioresource Technology, 87 (2), 167-198.

Luciano, G, Pauselli, M., Servili, M., Mourvaki, E., Serra, A., Monahan, F.J., Lanza, M., Priolo, A., Zinnai, A., Mele, M. (2013). Dietary olive cake reduces the oxidation of lipids, including cholesterol, in lamb meat enriched in polyunsaturated fatty acids. Meat Science, 93 (3), 703–714.

Mirzaei-Aghsaghali, A., Maheri-Sis, N. (2008). Nutritive value of some agroindustrial by-products for ruminants - A Review. World Journal of Zoology, 3 (2), 40-46.

Molina-Alcaide, E., Yáñez-Ruiz, D. R. (2008). Potential use of olive by-products in ruminant feeding: A review. Animal Feed Science and Technology, 147(1-3), 247-264.

Mourão, J.L., Pinheiro, V.M., Prates, J.A.M., Bessa, R.J.B., Ferreira, L.M.A., Fontes, C.M.G.A., Ponte, P.I.P. (2008). Effect of dietary dehydrated pasture and citrus pulp on the performance and meat quality of broiler chickens. Poultry Science, 87 (4), 733-743.

Oreopoulou, V. & Tzia, C. (2007). Utilization of plant by-products for the recovery of proteins, dietary fibers, antioxidants, and colorants. In V. Oreopoulou, R. Winfried (Eds), Utilization of by-products and treatment of waste in the food industry (pp. 209-232). Springer, New York.

Panouillé, M., Ralet, M.C., Bonnin, E., Thibault, J.F. (2007). Recovery and reuse of trimmings and pulps from fruit and vegetable processing. In K. Waldron (Ed.), Handbook of waste management and co-product recovery in food processing, Vol. 1 (pp. 417-447). Cambridge: Woodhead Publishing Limited.

Peiretti, P.G., Gai, F., Rotolo, L., Brugiapaglia, A., Gasco, L. (2013). Effects of tomato pomace supplementation on carcass characteristics and meat quality of fattening rabbits. Meat science, 95 (2), 345-351.

Sahin, N., Orhan, C., Tuzcu, M., Sahin, K., Kucuk, O. (2008). The effects of tomato powder supplementation on performance and lipid peroxidation in quail. Poultry Science, 87 (2), 276-283.

Simitzis, P.E., Symeon, G.K., Charismiadou, M.A., Ayoutanti, A.G., Deligeorgis, S.G. (2011). The effects of dietary hesperidin supplementation on broiler performance and chicken meat characteristics. Canadian Journal of Animal Science, 91 (2), 275-282

Smet, K., Raes, K., Huyghebaert, G., Haak, L., Arnouts, S., De Smet, S. (2008). Lipid and protein oxidation of broiler meat as influenced by dietary natural antioxidant supplementation. Poultry Science, 87 (8), 1682-1688.

Tsiplakou, E. & Zervas, G. (2008). The effect of dietary inclusion of olive tree leaves and grape marc on the content of conjugated linoleic acid and vaccenic acid in the milk of dairy sheep and goats. Journal of Dairy Research, 75 (3), 270-278.

Vargas-Bello-Pérez, E., Vera, R.R., Aguilar, C., Lira, R., Peña, I., Fernández, J. (2013). Feeding olive cake to ewes improves fatty acid profile of milk and cheese. Animal Feed Science and Technology, 184 (1), 94-99.

Wenk, C. (2000). Recent advances in animal feed additives such as metabolic modifiers, antimicrobial agents, probiotics, enzymes and highly available minerals – Review. Asian-Australasian Journal of Animal Sciences, 13 (1), 86-95.

Williams, P.E.V. (2014). Alternative feed ingredients – Overview from an end-users perspective with specific reference to bioethanol co-products. Advances in Animal Biosciences, 5(1), 1.

Windisch, W., Schedle, K., Plitzner, C., Kroismayr, A. (2007). Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science, 86 (E Suppl.), E140-E148.

Yan, L., Kim, I.H. (2011). Effect of dietary grape pomace fermented by Saccharomyces boulardii on the growth performance, nutrient digestibility and meat quality in finishing pigs. Asian-Australian Journal of Animal Science, 24(12), 1763-1770.

FATTY ACID COMPOSITION AND MEAT QUALITY TRAITS OF BROILER CHICKENS FED A DIET FORMULATED WITH FLAXSEED CO-EXTRUDATES

Predrag Ikonić[1]*, Dušica Čolović[1], Tatjana Tasić[1], Đorđe Okanović[1], Natalija Džinić[2], Jasmina Gubić[1], Jovanka Lević[1]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: predrag.ikonic@fins.uns.ac.rs

ABSTRACT

The aim of this work was to investigate the influence of diet supplemented with flaxseed co-extrudates on quality and fatty acid composition of chicken breast meat. Broiler chickens were fed a mash diet, a starter until 21 days and finisher diet, until slaughter at 35 days of age. Two diets, control (C) and flaxseed (F), were assessed with the aim of increasing the content of n-3 polyunsaturated fatty acids and evaluating their influence on proximate composition, technological and sensory properties of breast meat. The F diet was formulated with two types of co-extrudates (5%), flaxseed-soybean meal (starter diet) and flaxseed-sunflower meal (finisher diet). 120 broiler chickens were assigned to each diet. The use of F diet did not influence significantly (*P<0.05*) the technological parameters of breast meat. On the other hand, it enhanced the α-linolenic (ALA) (7.76% vs. 3.54%), EPA and DPA (EPA+DHA, 0.45% vs. 0.29%) fatty acids content in meat and drastically reduced the n-6/n-3 ratio (9-4). Sensory attributes of roasted breast meat samples were negatively affected by supplementation with flaxseed co-extrudates.
***Keywords**: breast meat, flaxseed, co-extrudates, fatty acids*

INTRODUCTION

Several studies suggest that omega-3 fatty acids have the beneficial influence on human health, including anti-atherogenic, anti-thrombotic and anti-inflammatory effects, as well as reduction of risk of coronary heart disease (CHD) (Ruxton et al., 2004; Givens and Gibbs, 2006).

Fat is one of the essential substances in human and animal nutrition, as it is an important source of energy, essential fatty acids and liposoluble vitamins (Krejčí-Treu et al., 2010). A number of experimental studies show that animal fat-rich diets are directly related to the risk of cardiovascular diseases and colon cancer (Jiménez-Colmenero et al., 2001; Roynette et al., 2004). However, meat is a primary source of dietary fat, especially saturated one. Hence, the increasing interest in meat fatty acid composition stems mainly from the need to find ways to produce healthier meat, i.e. with a higher ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA) and a more favorable balance between n-6 and n-3 PUFA (Wood et al., 2003).

Poultry production was the fastest growing livestock industry in recent years. In the same time, the consumption of poultry meat increased markedly throughout the world. Thus, the quality of chicken meat and fat is of great importance both for the producers and the consumers (Kavouridou et al., 2008; Magdelaine et al., 2008).

It has been observed that the content of fatty acids in meat of food animals (including broiler chickens) depends largely on the content and composition of these acids in the diet (Schiavone et al. 2004; Krejčí-Treu et al., 2010). Flaxseed, due to its high α-linolenic acid (ALA), has gained attention as a dietary ingredient for animals (Nam et al., 1997; Wood et al., 2003; Juárez et al., 2011). However, its usage in animal nutrition is limited, due to the presence of antinutritive components - cyanogenic glycosides. One of the ways for detoxification of flaxseed is an extrusion process (Wu et al., 2008). Additionally, high oil content in flaxseed may cause extrusion difficulties. In order to overcome the aforementioned

problem, flaxseed is usually co-extruded with protein rich materials, which show great ability of oil adsorption (Thacker et al., 2004; Lević and Sredanović, 2012).
The objective of this study was to determine the influence of the consumption of diet formulated with flaxseed co-extrudates on quality and fatty acid composition of chicken breast meat.

MATERIAL AND METHODS

Experimental design and diets

Broiler chickens were fed with a mash diet. A starter diet was given until 21 days, followed by a finisher diet, until slaughter at 35 days of age. Two different diets were assessed: control (C) and flaxseed diet (F). For flaxseed diet, two types of co-extrudates were prepared. Co-extrudate flaxseed-soybean meal (5%) was used for preparation of starter mash, since soybean meal has less content of cellulose than sunflower meal, which is limitation factor in breeding of young broiler chickens. Finisher mash was prepared with 5% of co-extrudate flaxseed-sunflower meal.
120 broiler chickens were assigned to each diet. Chickens were divided in four boxes for every diet (30 birds in one box), in order to eliminate the influence of breeding environmental conditions on broiler performances.
The animals were slaughtered in a commercial abattoir, according to the routine procedure. After chilling (24h *post mortem*), the carcasses of twelve birds per treatment (three per box) were transferred under refrigerated conditions (0-4ºC) to the laboratory of Institute of Food Technology (FINS) in Novi Sad. The birds were chosen as a representatives of their experimental group and box, taking into account average mass of 30 birds from each box. After cutting and deboning, the breast meat samples were subjected to physicochemical and sensorial analysis, while the rest of the samples were homogenized and stored at -20°C, pending fatty acid analysis.

Physicochemical and sensory analysis

The pH of breast meat samples was determined using the portable pH meter Testo 205 (Testo AG, USA) equipped with a combined penetration tip with temperature probe. Color was measured on the fresh cross-section using Minolta Chroma Meter CR-400, and the color characteristics were presented in CIE L*a*b* system (CIE, 1976).
Moisture, fat, protein (Kjeldahl N x 6.25) and ash contents in breast meat samples were quantified using the ISO recommended standards 1442:1997 (ISO, 1997), 1443:1973 (ISO, 1973), 937:1978 (ISO, 1978) and 936:1998 (ISO, 1998), respectively.
In order to analyze sensory characteristics of breast meat, the samples were roasted in a convection air oven at 175^{o}C for 45 min. and cooled to room temperature for 1 h. Six trained panelists, experienced in the sensory evaluation of various meats were engaged. Sensory evaluation (appearance-color, smell, taste, tenderness, juiciness, chewingness and overall impression) was carried out according to the point system of analytical descriptive test using a scale from 1 to 5 (1-unacceptable, 5-excellent).

Fatty acid analysis

Supercritical fluid extraction with CO_2 was used for preparation of fat extracts. Extractions were performed on a LECO TFA2000 fat analyzer (LECO Corporation, MI, USA). Temperature, pressure and extraction flow rates were adopted from existing procedures for meat samples (LECO Corporation, 2003).
Fatty acid methyl esters were prepared from the extracted lipids by transmethylation method that uses 14% wt. boron trifluoride/methanol solution (Sigma Aldrich, MO, USA), as recommended method for this type of samples (Karlović and Andrić, 1996). Nitrogen gas (99.99%, Messer, Germany) was used for removing boron trifluoride/methanol solution and

n-heptane (99.99%, J.T. Baker, NJ, USA) from fatty acid methyl esters. Obtained samples were analyzed by a gas chromatograph Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detector (GC-FID), auto-injection module for liquid, equipped with fused silica capillary column (DB-WAX 30 m, 0.25 mm, 0.50 um). The oven temperature was programmed at 90ºC for 4 min, increased from 70 to 150ºC at rate of 15ºC/ min and then held at 190 ºC for 5 min. Carrier gas was helium (purity > 99.9997 vol %, flow rate = 1.26 ml/min, produced by Messer, Germany). The fatty acids peaks were identified by comparison of retention times with retention times of standards from Supelco 37 component fatty acid methyl ester mix and with data from internal data library, based on previous experiments and fatty acid methyl ester determination on GC-MS. Results were expressed as percentages of total fatty acids.

Statistical analysis

One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 12.0 for Windows (Stat Soft, Tulsa, Oklahoma, USA). Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

The obtained results of pH and color characteristics (L*a*b*) of breast meat showed not significant influence ($P>0.05$) of diet formulated with flaxseed co-extrudates (F) on these technological parameters (Table 1). Based on the pH value and the lightness (L*), as a quality parameters, the breast muscle samples of both C and F group of chickens were classified as "normal" (Wilkins et al., 2000; Barbut et al., 2005).

Table 1. Breast meat pH and color characteristics of control (C) and flaxseed (F) diet fed chickens

Group	pH	L*	a*	b*
C	6.01±0.14 [a]	48.43±3.79[a]	2.39±0.65[a]	1.71±1.88[b]
F	5.92±0.20[a]	51.27±4.98[a,b]	1.94±0.98[a]	2.45±2.20[a,b]

[a , b] *Means within the same column with different superscript letters are different (P<0.05)*

Regarding the proximate chemical composition (Table 2), it can be seen that F diet had a significant effect ($P<0.05$) on fat and protein content in breast meat. Namely, fat content in breast muscles of F group chickens was higher comparing to C group, being 0.34% and 0.31%, respectively. On the contrary, protein content was 0.4% lower in breast meat of chickens fed a diet formulated with flaxseed co-extrudates (F).

Table 2. Proximate breast meat chemical composition of control (C) and flaxseed (F) diet fed chickens

Group	Moisture (%)	Fat (%)	Protein (%)	Ash (%)
C	75.0±0.33[a]	0.31±0.01[a]	21.4±0.11[b]	1.19±0.14[a]
F	75.3±0.78[a]	0.34±0.02[b]	21.0±0.27[a]	1.11±0.04[a]

[a , b] *Means within the same column with different superscript letters are different (P<0.05)*

Fatty acids profile of breast muscles from broiler chickens fed a control and flaxseed diet is presented in Table 3. The use of experimental F diet produced a notable effect on the composition of fatty acids in breast muscles in chickens. In general, dietary flaxseed led to decreases in the levels of saturated fatty acids (SFA) (16:0, 18:0), polyunsaturated fatty acids (PUFA) (18:2n-6, 18:3n-6, 20:2, 20:4n-6), total n-6 and the n-6/n-3 ratio, and increases in the levels of monounsaturated fatty acids (MUFA) (16:1, 18:1n-9), ALA (18:3n-3), sum of docosahexaenoic and eicosapentaenoic acid (DHA+EPA), PUFA/SFA ratio and total n-3 fatty acids. A number of studies have reported similar increases in the n-3 fatty acids in breast muscle when feeding increasing levels of flaxseed to broiler chickens (Woods and Fearon,

2009; Krejčí-Treu et al., 2010). Thus, flaxseed co-extrudates added into F diet affected positively the content of ALA (7.76%) compared to C group (3.54%), and consequently the total content of n-3 fatty acids (8.33%) and n-6/n-3 ratio (3.69). This finding is very important in terms of human nutrition as decreased n-6/n-3 ratio indicates an increased dietetic value of fat and meets the recommendations (1-4:1) (Simopoulos, 1999).

Table 3. Breast meat fatty acid composition of control (C) and flaxseed (F) diet fed chickens

Fatty acids (%)	C	F
C14:0	0.46	0.47
C16:0	20.37	19.59
C18:0	7.99	7.03
C16:1	2.49	3.14
C18:1 n-9	28.70	30.12
C18:2 n-6	33.86	29.80
C18:3 n-6	0.25	0.20
C18:3 n-3	3.54	7.76
C 20:0	0.11	0.12
C20:1	0.19	0.20
C20:2	0.38	0.27
C20:4 n6	1.29	0.73
C20:3 n3+n6	0.08	0.12
DHA+EPA	0.29	0.45
SFA	28.93	27.21
MUFA	31.36	33.46
PUFA	39.69	39.32
PUFA/SFA	1.37	1.45
n-3	3.91	8.33
n-6	35.40	30.72
n-6/ n-3	9.05	3.69

SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, EPA - eicosapentaenoic acid, DPA – docosahexaenoic acid

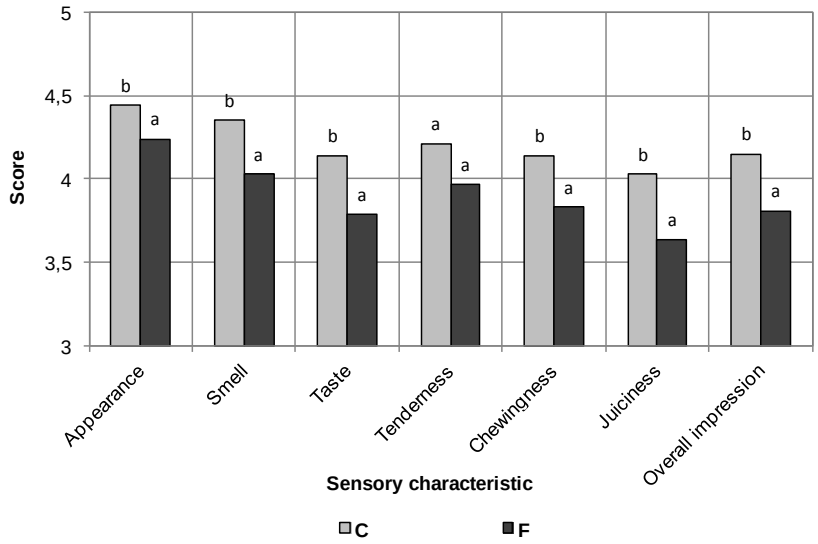

Figure 1. Breast meat sensory characteristics of control (C) and flaxseed (F) diet fed chickens

The results of the sensory evaluation of roasted breast meat samples of C and F diet fed chickens show a significant effect of flaxseed (Fig. 1). All sensory attribute scores were lower

(*P<0.05*) for a breast meat obtained from F diet fed animals. The differences were particularly pronounced for smell, taste and juiciness, as these sensory characteristics were graded 0.32, 0.35 and 0.39 units lower, respectively, for F group samples. This finding is in concordance with results previously reported by several authors (Wood et al., 2003; Azcona et al., 2008; Woods et al., 2009; Juárez et al., 2011), who observed the negative effect of dietary flaxseed on sensory attributes of meat.

CONCLUSIONS

The use of flaxseed co-extrudates diet did not influence the technological parameters of breast meat. On the other hand, it increased the content of α-linolenic (ALA), EPA and DPA fatty acids in meat and drastically reduced the n-6/n-3 ratio (9 - 4). Considering the sensory quality, the supplementation with flaxseed negatively affected all sensory attributes of roasted breast meat samples.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. III46012. The authors are thankful to Provincial Secretariat for Science and Technological Development of Autonomous Province of Vojvodina for financial support.

REFERENCES

Azcona, J.O., Garcia, P.T., Cossu, M.E., Iglesias, B.F., Picallo, A., Perez, C., Gallinger, C.I., Schang, M.J., , Canet, Z.E. (2008). Meat quality of Argentinean ''Camperos'' chicken enhanced in omega-3 and omega-9 fatty acids. Meat Science, 79, 437–443.

Barbut, S., Zhang, L., Marcone M. (2005). Effects of Pale, Normal and Dark Chickens Poultry Breast Meat on Microstructure Extracable Proteins and Cooking of Marinated Fillets. Poultry Science, 84, 797-802.

CIE (1976). International Commission on Illumination, Colorimetry: Official Recommendation of the International Commission on Illumination. Publication CIE No. (E-1.31) Bureau Central de la CIE, Paris, France.

Givens, D.I., Gibbs, R.A. (2006). Very long chain n-3 polyunsaturated fatty acids in the food chain in the UK and the potential of animal-derived foods to increase intake. British Nutrition Foundation, Nutrition Bulletin, 31, 104–110.

International Organization for Standardization (ISO) (1973). International standards meat and meat products, Determination of total fat content (1443:1973). Genève, Switzerland.

International Organization for Standardization (ISO) (1978). International standards meat and meat products, Determination of nitrogen content (937:1978). Genève, Switzerland.

International Organization for Standardization (ISO) (1997). International standards meat and meat products, Determination of moisture content (1442:1997). Genève, Switzerland.

International Organization for Standardization (ISO) (1998). International standards meat and meat products, Determination of ash content (936:1998). Genève, Switzerland.

Jiménez-Colmenero, F., Carballo, J., Cofrades, S. (2001). Healthier meat and meat products: Their role as functional foods. Meat Science, 59(1), 5–13.

Karlović, Đ., Andrić, N. (1996). Kontrola kvaliteta semena uljarica. Tehnološki fakultet Novi Sad, Savezni zavod za standardizaciju, Beograd, Srbija.

Kavouridou, K., Barroeta, A.C., Villaverde, C., Manzanilla, E.G., Baucells, M.D. (2008). Fatty acid, protein and energy gain of broilers fed different dietary vegetable oils. Spanish Journal of Agricultural Research, 6, 210-218.

Krejcí-Treu, T., Straková, E., Suchý, P., Herzig. I. (2010). Effect of vegetable oil fortified feeds on the content of fatty acids in breast and thigh muscles in broiler chickens. Acta Veterinaria Brno, 79, S21-S28.

Lević, J., Sredanović, S. (2012). Suncokretova sačma. Institut za prehrambene tehnologije, Novi Sad.

Magdelaine, P., Spiess, M.P., Valceschini, E. (2008). Poultry meat consumption trends in Europe. World Poultry Science Journal, 64, 53–64.

Nam, K., Lee, H., Min, B., Kang, C. (1997). Influence of dietary supplementation with linseed and vitamin E on fatty acids, α-tocopherol and lipid peroxidation in muscles of broiler chicks. Anim. Feed Sci. Technol. 66:149–158.

Roynette, C.E., Calder, P.C., Dupertuis, Y.M., Pichard, C. (2004). n-3 Polyunsaturated fatty acids and colon cancer prevention. Clinical Nutrition, 23, 139–151.

Ruxton, C.H.S., Reed, S.C., Simpson, M.J.A., Millington, K.J. (2004). The health benefits of omega-3 polyunsaturated fatty acids: A review of the evidence. Journal of Human Nutrition and Dietetics, 17(5), 449–459.

Schiavone, A., Ramboli, I., Chiarini, R., Marzoni, M. (2004). Influence of dietary lipid source and strain on fatty acid composition of Muscovy duck meat. Journal of Animal Physiology and Animal Nutrition, 88, 88-93.

Simopoulos, A.P. (1999). Essential fatty acids in health and chronic disease. The American Journal of Clinical Nutrition, 70, 560-569.

Thacker, P.A., Racz, V.J., Soita, H.W. (2004). Performance and carcass characteristics of growing-finishing pigs fed barley-based diets supplemented with Linpro (extruded whole flaxseed and peas) or soybean meal. Canadian Journal of Animal Science, 84 (4), 681-688.

Wilkins, L.J., Brown, S.N., Phillips, A.J., Warriss, P.D. (2000). Variation in the color of broiler breast fillets in the UK. British Poultry Science, 41, 308–312.

Wood, J.D., Richardson, R.I., Nute, G.R., Fisher, A.V., Campo, M.M., Kasapidou, E., Sheard P.R., Enser M. (2003). Effects of fatty acids on meat quality: A review. Meat Science, 66(1), 21–32.

Woods, V. B., Fearon, A. M. (2009). Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: a review. Livestock Science, 126, 1-20.

Wu, M., Li, D., Zhou, Y-G., Brooks, M. S-L., Chen, X.D., Mao, Z.H. (2008). Extrusion detoxification technique on flaxseed by uniform design optimization. Separation and Purification Technology, 61, 51-59.

THE QUALITY OF CORN STILLAGE OF BIOETHANOL PRODUCTION

Šandor Kormanjoš[1]*, Slavko Filipović[1], Ljiljana Kostadinović[1], Olivera Đuragić[1], Sanja Teodosin[1], Vera Radović[2]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, 32000 Čačak, Serbia

*Corresponding author:
E-mail address: sandor.kormanjos@fins.uns.ac.rs

ABSTRACT

High consumption and depletion of fossil fuels require finding the alternative solution. Among the alternative energy resources an important place takes the biofuels, in particular bioethanol as a renewable and environmental friendly fuel.
The highest percentage of bioethanol in the industry is produced from corn grain. During the bioethanol production process the high amount of by-products are formed. Since the by-products of bioethanol industry are excellent sources of proteins and energy for animals, these are commonly used as components for the animal feed preparation. Valorization of by-products provides greater productivity and environmental protection.
As a part of this study, it was examined the overall quality of whole stillage that remains after the distillation of ethanol, wet cake and liquid phase obtained after separation of whole stillage.
The samples were analyzed to crude protein content by the Kjeldahl method and aflatoxin content by the Elisa test. Furthermore, content of heavy metals was analyzed by the atomic absorption spectrophotometer.
The contents of crude protein in the dry matter of the whole stillage, wet cake and liquid phase were 21.50%, 34.27% and 8.31%, respectively. Aflatoxin content in all the samples was <0.005 mg/kg. Moreover, the content of heavy metals (As, Pb, Cd and Hg) meets the requirements of the current national regulative - Pravilnik o kvalitetu hrane za životinje.
The obtained results showed that by-products of bioethanol industry can be used as components for animal feeds.
***Keywords**: corn, bioethanol, stillage, feed*

INTRODUCTION

Bioethanol is a biofuel which is worldwide used as a substitute for fossil fuels. Use of a mixture of bioethanol and gasoline can significantly reduce the need for petroleum as well as greenhouse gas emissions and environmental pollution (Semenčenko *et al.*, 2011).
The high consumption and depletion of fossil fuels in the last few years, which occurs due to the development of industry and technology, requires finding of an alternative solution. Among the alternative energy resources biofuels play a major role today, but bioethanol has a special place as a renewable and environmentally suitable fuel. In the production of bioethanol it is important that it is obtained by fermentation from renewable raw materials that contain carbohydrates (corn, sugar cane, potatoes, Jerusalem artichokes etc.). Sugar fermentation of biomass is done by microorganisms, traditionally using yeasts, and in newer technologies using certain types of bacteria.
The production of bioethanol generates significant amounts of by-products (from 1 t of corn 315-330 kg of by-products is obtained) which combustion or destruction in any other way can cause a great environmental damage (Mézes, 2013). Utilization of by-products of bioethanol production from corn significantly improves its profitability.
The composition of corn stillage contains all of the components present in raw materials other than carbohydrates and yeast, and the newly generated intermediate phases which yeast cannot metabolize to ethanol (Mojović *et al.*, 2007).

MATERIAL AND METHODS

As a part of the research, it was investigated the quality of total whole stillage that remains after the distillation of ethanol, wet cake and liquid phase obtained after separation of whole stillage.
For these study the minimum of three replicate were performed for each sample.
Moisture, crude protein, crude fat, crude fiber, pH, BEM and mineral matter content of corn stillage were according to the standard methods described by regulation - Pravilnik o metodama uzimanja uzoraka i metodama vršenja fizičkih, hemijskih i mikrobioloških analiza stočne hrane (1987).
Total sugar content was determined in accordance to the regulation - Pravilnik o metodama fizičkih i hemijskih analiza za kontrolu kvaliteta žita, mlinskih i pekarskih proizvoda, testanina i brzo smrznutih testa (1988).
The total number of bacteria was determined by the method EN ISO 4833:2008, *Coagulase positive staphylococci* by method EN ISO 6881-1:2008, *Clostridium perfringens* by method EN ISO 7937:2008, *Salmonella* spp by method EN ISO 6579:2008. The total number of yeasts and molds was determined using EN ISO 21527 -2:2008, the total number of *Escherichia coli* using EN ISO 16649-2:2008, and *Sulphite-reducing clostridia by* method EN ISO 15213:2003. The content of aflatoxins (B1+B2+G1+G2) was determined by ELISA test using a kit aflatoxin Veratox 8031 High Sensitivity Test.
For the determination of As, Pb and Cd AAS method - FINSLab-5.4-3M-004/13 was used.
Determination of total mercury-Hg content was performed at using an automatic mercury analyzer AMA 254 according to AAS method - FINSLab-5.4-3M-005.
The results given in tables are reported as the mean ± standard deviations (SD) of a number (n) of independent determinations. The one way ANOVA analysis was performed to assess data differences between various groups using Statistics software version 12 (StatSoft inc. 2013). Significant differences among treatment means were analyzed by Duncan's multiple range tests. The data means were considered different at $P < 0.05$.

RESULTS AND DISCUSSION

Water and solid (insoluble) substances which remain after the distillation of ethanol are called the whole stillage, and are composed mainly of water, fiber, protein and fat. In addition to unchanged starting material originating from the used raw material, stillage contains yeast cells and metabolic products of yeast fermentation process such as B-group vitamins and some growth factors (Mojović *et al.*, 2010). After separation of the whole stillage, liquid phase and wet cake are obtained. During the production of bioethanol, starch grains are consumed and converted into alcohol and carbon dioxide. As a consequence of the starch spending, the concentration of the remaining nutrients is increased approximately three times (Spiehs *et al*., 2002). Chemical indicators of the quality of the surveyed by-products from the production of bioethanol are shown in Table 1. According to obtained results, protein and cellulose fractions are mostly present in the dry matter of wet cake, 34.27% and 6.76%, respectively. Total sugars were most abundant in the dry matter of the liquid phase, 29.18%.
By-products of bioethanol industry are excellent sources of protein and energy for animals and therefore often used as a component in feed for domestic animals. Nevertheless, there is often a problem of storage and removal of unused stillage (Mojović *et al.*, 2009; Rakin *et al.*, 2009; Pejin *et al.*, 2009). The chemical composition of stillage depends on the composition of starting corn. According to research of Belyea *et al.* (2004) average chemical composition of dry corn stillage is as follows (g/100 g of dry matter): fat 11.9; protein 31.3; crude fiber 10.2; fibers soluble in acid 17.2; ashes 4.6 and starch 5.1.

Table 1. Chemical parameters of the by-products of bioethanol production

Parameters	Whole stillage	In dry matter	Liquid phase	In dry matter	Wet cake	In dry matter
Moisture content (%)	87.91±1.2^{b}	-	90.37±1.0^{b}	-	63.15±1.4^{a}	-
Crude protein content (%)	2.60±0.09^{b}	21.50±0.5^{e}	0.80±0.05^{a}	8.31±0.2^{c}	12.63±0.3^{d}	34.27±0.9^{f}
Ash content (%)	0.01±0.001^{a}	0.08±0.002^{b}	0.02±0.001^{a}	0.21±0.008^{c}	1.40±0.01^{d}	3.81±0.02^{e}
Crude celullose content (%)	0.25±0.002^{a}	2.07±0.03^{c}	0.50±0.004^{b}	5.19±0.1^{d}	2.49±0.09^{c}	6.76±0.2^{e}
Crude fat (%)	1.14±0.05^{b}	9.42±0.09^{e}	0.73±0.003^{a}	7.58±0.03^{d}	2.89±0.01^{c}	7.84±0.02^{d}
Total sugar content (%)	0.89±0.001^{a}	7.36±0.02^{e}	2.81±0.01^{c}	29.18±0.15^{f}	1.57±0.02^{b}	4.26±0.025^{d}
BEM (%)	8.09±0.2^{a}	66.91±1.0^{e}	7.58±0.1^{b}	78.71±0.15^{f}	17.44±0.2^{c}	47.33±0.45^{d}
pH	3.48±0.01^{a}	-	3.48±0.015^{a}	-	3.75±0.01^{b}	-

Mean±SD (n=3)
$^{a-f}$ Means within a row with no common superscript differ significantly at P < 0.05;

Studies have shown that there is no significant correlation between the content of some nutrients in the dry stillage. Thus, for example, the protein content is very variable, as influenced by the relationship between the amount and composition of wet stillage, which vary from batch to batch, and generally is not sufficiently standardized and controlled. Almost all proteins of corn are lagging in dry meal, since the yeasts used for fermentation do not contain proteolytic enzymes that could degrade those (Balyea *et al.*, 2004).
In the market you can find fresh and dry stillage. Fresh stillage can be used for food on farms near the ethanol-producing facilities (liquid diet, preparation of stillage) because it is perishable, while dried stillage can be used throughout the year. The content of heavy metals in by-products of bioethanol production is shown in Table 2.

Table 2. The content of heavy metals in by-products from the production of bioethanol

Parameters	Whole stillage	Liquid phase	Wet cake
As, mg/kg	<0.5	<0.5	<0.5
Pb, mg/kg	0.86	<0.25	<0.25
Cd, mg/kg	<0.05	<0.05	<0.05
Hg, mg/kg	0.00174	0.00563	0.00361

The results showed that the content of arsenic, lead, cadmium and mercury are in the range allowed by the requirements of the current national regulation - Pravilnika o kvalitetu hrane za životinje (2010), which means that, in terms of heavy metals, by-products of bioethanol production are safe for application in domestic animal nutrition.
The content of mycotoxins in corn processing by-product deserves a special attention (Mézes, 2013). In relation to the content of mycotoxins in the starting raw material, by-product contains three times higher amount of mycotoxins. Based on the EU directive from 2012, the maximum value of aflatoxin B1 in all feedstuffs is 20 mg/kg calculated on the 88% dry matter.

Table 3. Microbiological content and content of aflatoxin in the by-products of bioethanol production

Microorganism	Whole stillage	Liquid phase	Wet cake
Salmonella spp	not detected in 50 g	not detected in 50 g	not detected in 50 g
Coagulase-positive staphylococci *(*cfu/g)	<100	<100	<100
*C.perfringens (*cfu/g)	<10	<10	<10
*SRC (*cfu/g)	<10	<10	<10
*E. coli (*cfu/g)	<10	<10	<10
TVCYM *(*cfu/g)	2500	not detected	not detected
TVAC (cfu/g)	4100	5300	3500
Mycotoxins			
Aflatoxins B1+B2+G1+G2 (mg/kg)	<0.005	<0.005	<0.005

Cfu- colony forming units
SRC- *Sulphite-reducing clostridia*
TVCYM - Total viable count yeasts and moulds
TVAC- total viable aerobic count

Based on the content of microorganisms and aflatoxins in the by-products of bioethanol production, the tested samples meet the requirements of the regulation - Pravilnika o kvalitetu hrane za životinje (2010). Aflatoxin content complies with the recommendations of the EU and is safe for animal feed (Table 3).
According to Mézes (2013) corn stillage is a suitable feed for cattle, pigs and chickens diet. The author recommends the application of 25-30% in feed for dairy cows. Since the digestion in the stomach is great, during preparation of recipes it should be taken care about the optimal ratio of protein bypass. Digestibility of cellulose is good, and the content of milk fat can be increased by 0.3-0.5% (Mézes, 2013). The phosphorus content is high (1%), and therefore it is necessary to increase calcium supplement (Mézes, 2013). Since pigs are sensitive to the taste of food, for piglets and fattening pigs can be admixed 5-10% of stillage and 15-20% of corn stillage in sow's diet (Mézes, 2013). In the food for laying hens may interfere maximum of 10% of corn stillage.

CONCLUSIONS

The production of bioethanol generates significant amounts of by-products (from 1 t of corn 315-330 kg of by-products is obtained). Based on the chemical composition of the corn stillage obtained as a byproduct in the production of bioethanol, it s concluded that stillage represents an excellent source of protein and energy for animals and can therefore be used as a component of feed for domestic animals or used for consumption in fresh condition. Moreover, in terms of heavy metals content, the analyzed by-products from the production of bioethanol are safe feedstuffs. Based on the content of microorganisms and aflatoxins in the by-products from the bioethanol industry, it can be concluded that they are suitable for animal feed.

ACKNOWLEDGEMENTS

The paper is a part of the research work on the project III-46012 financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia.
Many thanks to Provincial secretariat of science and technological development for financial support.

REFERENCES

Belyea, R.L., Rausch, K.D., Tumbleson, M.E. (2004). Composition of corn and distillers dried grains with solubles from dry grind ethanol processing. Bioresource technology, 94, 293-298.

EN ISO 15213 (2003). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions.

EN ISO 21527 (2008). Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds - Part 2: Colony count technique in products with water activity less than or equal to 0,95.

EN ISO 6888-1 (2008). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coagulase positive staphylococci (*Staphylococcus aureus* and other species).Part -1 Tehnique using Baird Parker agar medium.

Mézes, M. (2013). A bioüzemanyag-gyártás melléktermékeinek felhasználása a takarmányozásban. Agro Napló Országos mezőgazdasági szakfolyóirat, 17, 95-96.

Mojović, D., Pejin, M., Rakin, M., Vukašinović, J., Pejin, O., Grujić, S., Nikolić, M., Radosavljević, M. (2010). Investigations of the possibilities of stillage utilization from the bioethanol production on corn. Journal on processing and energy in agriculture, 14 (1), 54–57.

Mojović, Lj., Pejin, D., Lazić, M. (2007). Bioetanol kao gorivo – stanje i perspektive. Tehnološki fakultet, Leskovac, Srbija..

Mojović, L., Pejin, D., Grujić, O., Markov, S., Pejin, J., Rakin, M., Vukašinović, M., Nikolić, S., Savić, D. (2009). Progress in the production of bioethanol on starch-based feedstocks. Chemical Industry & Chemical Engineering Quarterly, 15 (4), 221–226.

Pejin, D., Mojović, Lj., Grujić, O., Pejin, J., Rakin, M. (2009). The bioethanol production with the thin stillage recirculation. Chemical Industry & Chemical Engineering Quarterly, 15 (1), 49–52.

Pravilnik o kvalitetu hrane za životinje, Službeni glasnik RS, broj 4/2010.

Pravilnik o metodama fizičkih i hemijskih analiza za kontrolu kvaliteta žita, mlinskih i pekarskih proizvoda, testanina i brzo smrznutih testa, *Službeni list SFRJ*, broj 74/1988.

Pravilnik o metodama uzimanja uzoraka i metodama vršenja fizičkih, hemijskih i mikrobioloških analiza stočne hrane, Sl. list SFRJ, 15, 421/456, 1987.

Rakin, M., Mojović, L., Nikolić, S., Vukašinović-Sekulić, M., Pejin, D. (2009). Poboljšanje kvaliteta džibre kao stočne hrane nakon proizvodnje bioetanola. Ecologica, 16 (54), 151–154.

Semenčenko, V., Mojović, Lj., Petrović, S., Ocić, O. (2011). Novi trendovi u proizvodnji bioetanola. Hemijska Industrija, 65 (2), 103-114.

Spiehs, M.J., Whitney, M.H., Shurson, G.C. (2002). Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota. Journal of Animal Science, 80 (10), 2639-2645.

SRPS EN ISO 16649-2 (2008). Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli*. Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

SRPS EN ISO 4833 (2008). Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms. Colony-count technique at 30 degrees C.

SRPS EN ISO 6579 (2008). Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp.

SRPS EN ISO 7937 (2008). Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of *Clostridium perfringens* — Colony-count technique.

STATISTICA (Data Analysis Software System) v.12.0 (2013). Stat-Soft, Inc, USA (www. statsoft.com).

IMPACT OF TECHNOLOGICAL PROCESSES OF ANIMAL FEED PRODUCTION ON VITAMIN A STABILITY

Ljiljana Kostadinović*, Sanja Teodosin, Jovanka Lević, Nedeljka Spasevski, Radmilo Čolović, Vojislav Banjac, Đuro Vukmirović

Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: ljiljana.kostadinovic@fins.uns.ac.rs

ABSTRACT

This aim of this study was to determine if technological processes of expanding and pelleting affect the stability of formulated commercial form of retinol-acetate (vitamin A) in feeds for broilers and piglets. The stability of vitamin A in pelleted feed for broilers and expanded feed for piglets was monitored during the storage under storehouse conditions (at temperature 22ºC and relative humidity 54%) during the period of three months. The vitamin A content was determined by the High Performance Liquid Chromatography (HPLC) at 326 nm.
At the beginning of the study, the concentration of vitamin A in pelleted feed for broilers decreased by 3%, while its average concentration in the sample of expanded feed for piglets decreased by 1%. The concentrations of vitamin A in untreated samples of feed for broilers and piglets decreased during the three month storage period by 28% and 20%, respectively. As well, in the samples of pelleted feed for broilers and expanded feed for piglets, concentrations of vitamin A decreased by 47% and 36%, respectively. Obtained results between pelleted and expanded feed samples show the pelleting process has a greater impact on the degradation of vitamin A than expanding process.
After first month, the difference in vitamin A stability in untreated feed samples was higher than in treated feed samples, but after three months of storage this distinction was significant ($P< 0.05$). The difference in decrease of vitamin A content between pelleted and expanded feed samples after a defined period of storage was around 11%, which is statistically significant distinction.
Vitamin A showed the appropriate stability within the three month long period of storage. Furthermore, decrease of vitamin A content, between pelleted and expanded feed samples, was statistically significant and shows that the pelleting process has a higher influence on the degradation of vitamin A than expanding process.
***Keywords**: pelleting, expanding, stability, vitamin A, HPLC*

INTRODUCTION

Adequate nutrition is a very important for ensuring good health and maximum production of domestic animals. The main aim of proper nutrition of animals is to provide the proper amount and ratio of nutrients and energy and thus enable it to achieve optimum production performances and maintain health (Smith and Somade, 1994).
From this reason, from year to year, the increasing use of various additives in domestic animals feeding is noted. While the needs of the domestic animals in the vitamins is extremely small, as compared to the energy and proteins, the absence of any vitamin portions causes a deficit of specific marks, causing a reduction in production (McDowell, 2000). Also, sub-acute cases deficits may exist when deficiency symptoms are not visible to the naked eye. These borderline deficits in vitamins are economically costly, difficult to repair, since flow unnoticed and regularly without intervention.
Vitamins are more likely to be damaged by the feed manufacturing process, because vitamins are sensitive organic compounds and that can be denaturated by water, oxygen, trace minerals, heat and other factors (Rickman *et al.,* 2007).
In last few decades thermal processing, such as pelleting, expansion and extrusion, of animal feeds are widely used as a way to improve efficiency of gain, nutrient digestibility and to improve feed handling (Fairfield, 2003). However, all types of thermal processing have been shown to reduce effectiveness of functional ingredients such as enzymes and less

stable nutrients such as vitamins (Dozier, 2002). In pelleting, the most important factors are friction, pressure, heat, humidity and conditioning time. Friction and pressure expose vitamin molecules to chemical destruction. Heat and humidity accelerate most chemical reactions, while conditioning time prolongs redox and other chemical reactions (Coelho, 2002).
According to all above mentioned, the study was carried out in order to determine if technological processes of expanding and pelleting affect the stability of formulated commercial form of retinol-acetate (vitamin A) in feeds for broilers and piglets.

MATERIAL AND METHODS

Materials

The vitamin A standard (retinol-acetate, analytical standard min.99.9%, Lot. No.: LB87725V) were purchased from Supelco analytical, USA. Methanol (HPLC grade), methanol, ethanol (extra pure grade) were purchased from Sigma-Aldrich.
Stock solution of retinol-acetate (1 mg/mL) was prepared in 2-propanol. This stock solution was used to prepare a series of standard solutions for calibration curve.
The working standard solutions chosen for the calibration curve were prepared daily by dissolving the stock standard solution into methanol, in the appropriate proportions, in order to obtain the following concentrations: 2, 6, 10, 14 and 18 µg/mL. All samples were filtered through a 0.45 µm membrane filter (Millipore). Dark volumetric flasks were tightly capped until analysis to protect the samples from light.

Storage conditions

Samples were stored in glass bottles (370 mL volume) under storehouse conditions (in the dark, at the temperature of 22ºC and at the relative humidity of 54%) during the period of three months. The samples were analyzed at the beginning of the storage period and after each two weeks, a total of three months.

Samples

As samples were used four different animal feeds:
1. Untreated complete mixture for broilers - UTB
2. Pelleted feed for broilers: - PEB
3. Untreated complete mixture for piglets - UTP
4. Expanded feed for piglets: - EXP

One kilogram of untreated feed for broilers contained minimum 3.6 mg/kg of vitamin A and untreated feed for piglets contained approximately 4.5 mg/kg of vitamin A. Vitamin A contained in the samples was in the form of coated retinol-acetate.
Conditioning of both of complete mixtures was performed in double-shaft steam conditioner Muyang SLHSJ0.2A (China), until materials reached temperature of 80°C. After the end of conditioning process, material moisture content in feed for broilers amounted 15.50%, while in feed for piglets amounted 25.5%.
Complete mixture for broilers was pelleted on a flat die pellet press 14-175, AMANDUS KAHL GmbH & Co. KG (Germany). A die with 6 mm diameter of the openings and with press way of 36 mm was used. The pellets were collected at pelleting temperature of 60°C.
A single screw annular gap expander (OEE 8, AMANDUS KAHL GmbH & Co. KG, Germany) with a length-to-diameter ratio of 8.5:1.0 and capacity of 100 kg/h was used for obtaining expanded mixture for piglets at 130 ± 1°C.
After pelleting and expanding, the products were stored for 24 hours under room conditions in order to reach stabile temperature and subsequently milled by hummer mill with sieve opening of 4 mm.

Retinol-acetate extraction from animal feed

The retinol-acetate extraction and cleanup procedure for the determination of vitamin A was performed according to the official methods of the Association of Official Analytical Chemists (*AOAC, 1995*).

A minimum of five replicate extractions were performed for each sample and each sample was analyzed at least five times by HPLC analysis.

HPLC analysis of retinol-acetate

Chromatographic analysis was performed on the High-Performance Liquid Chromatography instrument (Agilent 1200 system) equipped with Chemstation Software (Agilent Technologies). For the separation was used a 2.1x100 mm Zorbax eclipse plus C_{18} (Agilent, USA), 1.8µm HPLC column. The mobile phase consisted of methanol at a flow-rate of 0.3 ml/min and a pressure of 230 bars. The Agilent USA photodiode array UV-visible detector was made at wavelength of 326 nm for the detection. An aliquot of 5 µ solution was injected for HPLC analysis.

All quantification was determined by comparing the retention times and spectra of retinol-acetate from samples and standards and by peak areas using an Agilent integrator.

Statistical Analyses

To compare means, the paired-samples *t*-test of the STATISTICA Version 12 (2013) statistical software package was used.

RESULTS AND DISCUSSION

During storage for three months in storehouse it is noticed a slight decrease in vitamin A content in untreated feed samples over time, which is shown in Figure 1. At the beginning of the study, the vitamin A contents in the initial samples of complete mixture for broilers (UTB) and complete mixture for piglets (UTP) were in the amount specified by the manufacturer (minimum 3.6 mg/kg of vitamin A in feed for broilers and minimum 4.5 mg/kg of vitamin A in feed for piglets).

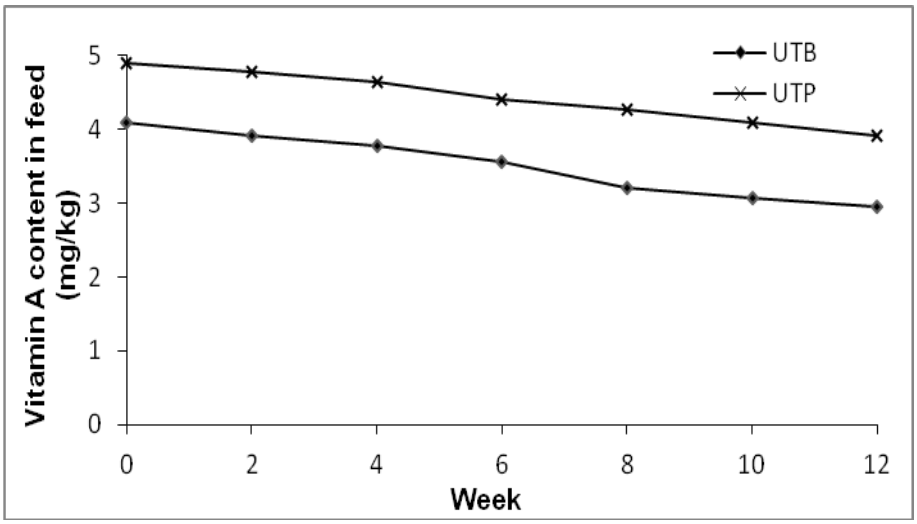

Figure 1. The average concentrations of vitamin A (mg/kg) in samples of untreated mixture for broilers (BUT) and untreated mixture for piglets (PUT) and the fraction of their initial concentrations at the beginning of the study and after each two weeks, total of three months

Even after two weeks of storage was observed slightly reduced content of vitamin A in these samples, but the decrease was not statistically significant comparing to initial amounts. However, after four weeks of storage in storehouse, the vitamin A content decreases by 7.4% in untreated feed for broilers and by 5.3% in untreated feed for piglets. These values,

after 12 weeks of storage, ranged 28% and 20%, respectively, which is statistically significant distinction ($P < 0.05$).
On the other side, at the beginning of the study the concentration of vitamin A in pelleted feed for broilers (PEB) decreased by 3%, while its average concentration in the sample of expanded feed for piglets (EXP) decreased by 1% (Figure 2). These results showed that technological processes of pelleting and expanding have the impact on vitamin A degradation, which is in accordance to results obtained by Kostadinović et al. (2013).

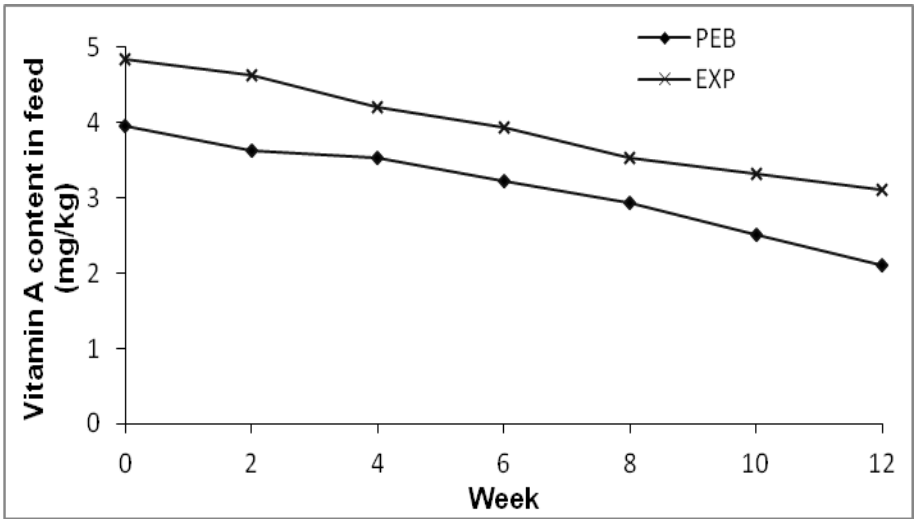

Figure 2. The average concentrations of vitamin A (mg/kg) in samples of pelleted feed for broilers (PEB) and expanded feed for piglets (EXP) and the fraction of their initial concentrations at the beginning of the study and after each two weeks, total of three months

After six weeks in storehouse, the stability of vitamin A in the treated feed samples (PEB) and EXP) was lower and significantly different than in the untreated feed samples (UTB and UTP). The vitamin A content in pelleted feed for broilers and expanded feed for piglets were lower by approximately 18.4% and 18.6% in compare to initial vitamin A content, while these values reached 13.0% and 9.9% in untreated feed for broilers and untreated feed for piglets, respectively. At the end of the experiment, the concentration of vitamin A in the pelleted feed for broilers decreased by 47%, whereas in the expanded feed for pigets this amount was by 36% lower than at the beginning of the experiment.
Storage losses will depend on the moisture content of the product, the environmental temperature and humidity, and the packaging of the diet. Storage locations should ideally be environmentally control, but this is often not practical. Manufacturers usually suggest that the diet is stored in a cool, dry location but in practice many users must accept ambient conditions.
The results obtained in this study can be explained taking into account the formulated commercial form of retinol-acetate (vitamin A) obtained by spray congealing, where emulsion, containing gelatin and sugars, is sprayed in a tower and slowly dried with cold air, starch and silica. In this way it has been achieved more resistant coating that can sustain higher pressure, friction, temperature and humidity (Coelho, 2002). However, the five double bonds in retinol-acetate still make the compound sensitive to oxidations, which is the reason for the vitamin A degradation during storage.
Furthermore, Kostadinović et al. (2014) reported pelleting process has a greater impact on the degradation of vitamin A than expanding process, which fully corresponds to the results obtained during this experiment. The losses of vitamin A content were statistically significant in both cases, but pelleting process caused higher degradation of examined vitamin.

CONCLUSIONS

Regarding to obtain results it can be said examined vitamin A showed the appropriate stability within the three month long period of storage in all examined samples. However, vitamin A was more stable in the untreated feed samples for broilers and piglets compared to treated feed samples. Furthermore, decrease in vitamin A content was statistically significant between pelleted and expanded feed samples and shows that the pelleting process has a higher impact on the degradation of vitamin A than expanding process.
A possible solution to this problem may be the addition of vitamin A in higher concentrations, in which case the contents of vitamin A will decrease to the desired value after technological processing of animal feed. The option most commonly proposed is vitamin application after pelleting and expanding. Furthermore, should try to reduce temperature and conditioning time during technological processes and reduce feed storage, in order to achieve desired vitamin A content in animal feeds.

ACKNOWLEDGEMENTS

The paper is a part of the research work on the project III-46012 financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia. The authors wish to thank the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina for financial support.

REFERENCES

Association of Official Analytical Chemists (AOAC) (1995). Official Methods of Analysis of the Association of Official Analytical Chemists. Determination of Vitamin A in Complete Feeds, Premixes and Vitamin Concentrates with HPLC. 16th ed., Washington.

Coelho, M. (2002). Vitamin Stability in Premixes and Feeds: A Practical Approach in Ruminant Diets, Proceedings 13th Annual Florida Ruminant Nutrition Symposium, Best Western, Gainesville, Florida, 127-145.

Dozier, W.A. (2002). Mash conditioning and vitamin stability. Feed International, 23, 20.

Fairfield, D. (2003). Pelleting for profit—Part 1. Feed and Feeding Digest, 54, 1 – 5.

Kostadinović, Lj., Teodosin, S., Spasevski, N., Đuragić, O., Banjac, V., Vukmirović, Đ., Sredanović, S. (2013). Effect of pelleting and expanding processes on stability of vitamin E in animal feeds. Food & Feed Research, 40 (2), 109-114.

Kostadinović, Lj., Teodosin, S., Lević, J., Čolović, R., Banjac, V., Vukmirović, Đ., Sredanović, S. (2014). Effect of pelleting and expanding processes on vitamin A stability in animal feeds. Journal on processing and energy in agriculture (PTEP), 18 (1), 44-46.

Rickman, J.C.; Bruhn, C.M.; Barrett, D.M. (2007). Nutritional composition of fresh, frozen, and canned fruits and vegetables II. Vitamin A and carotenoids, vitamin E, minerals and fiber. J. Sci. Food Agric., 87, 1185–1196.

McDowell, L.R. (2000). Vitamins in Animal and Human Nutrition, Iowa State University Press, Ames, Iowa.

Smith, O.B., Somade, B. (1994). Interactions between nutrition and reproduction in farm animals, International Foundation seminar on Animal Reproduction, Niamey, Niger.

STATISTICA (Data Analysis Software System) (2013). v.12.0., Stat-Soft, Inc., USA (www. statsoft.com).

THE IMPACT OF FEED PROCESSING ON THE ESSENTIAL OIL OF *ORIGANUM VULGARE*

Ljiljana Kostadinović[1]*, Sanja Teodosin[1], Sava Pavkov[2], Jovanka Lević[1]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]European University, Faculty of Pharmacy, Trg Mladenaca 5, 21000 Novi Sad, Republic of Serbia

*Corresponding author:
E-mail address: ljiljana.kostadinovic@fins.uns.ac.rs

ABSTRACT

From antiquity, essential oils of medical plants and their derivatives have being used for flavoring foods and beverages and for medication. These additives have been usefully used in animal nutrition for improvement of health and animal wellbeing, since they have high antimicrobial and antioxidant activities. In the present study, the effect of pelleting process on essential oil composition and stability was investigated, since they are very unstable during thermal processing which is widely used these days in feedstuff production.
The composition of essential oil obtained by hydro distillation from the plant *Origanum vulgare*, which was added into feed for broilers in concentration of 2 g/kg, was analyzed by GC/FID before and after pelleting process. After pelleting of feed, the essential oil was also isolated from animal diet by hydro distillation. Analysis of essential oils obtained before and after pelleting process showed some quantitative differences. Origano essential oil was characterized by the presence of thymol (19.9%) and carvacrol (61.8%) at the beginning of the experiment. After pelleting process the concentration of thymol and carvacrol amounted to 15.3% and 50.4%, respectively.
It was concluded that pelleting process had significant effect on thymol and carvacrol stability in animal feed, i.e. on reducing their initial contents in animal feed.
***Keywords**: feed, pelleting, essential oil, stability*

INTRODUCTION

Parts of plants, or the products thereof have been used since ancient times in the diet of humans and animals in order to improve the flavor of food, as well as in medical purposes, for the prevention or treatment of various diseases (Negi, 2012; Burt, 2004). In recent years, numerous studies were dealing with the isolation of bioactive compounds from plants, which are known to have antioxidant, antimicrobial, or some other beneficial effects (Burt, 2004). Whether it comes to herbal extracts, essential oils, or organic acids, these natural products are a good alternative to artificial preparations, finding more and more use in the production of animal feed, primarily in order to increase food safety.
Carvacrol (5-isopropyl-2-methylphenol) is one of the main components of oregano oil with proven antioxidant, antifungal and insecticidal effect (Burt, 2004; Chami *et al*., 2005; Panella *et al*., 2005; Tampieri *et al*., 2005; Liolios *et al.*, 2009). In addition to antioxidant, carvacrol has well known antimicrobial activity. The exact mechanism of action of this compound on microorganisms is not yet known, but the majority of scientific studies indicate that its activity is based on the destruction of the cell membranes of microorganisms (Helander *et al.,* 1998; Inamuco, 2012).
Thymol (5-methyl-2-isopropylphenol) is part of a naturally occurring class of compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides such as carvacrol. Numerous studies have demonstrated the antimicrobial effects of thymol, ranging from inducing antibiotic susceptibility in drug-resistant pathogens to powerful antioxidant properties (Zarrini *et al*., 2010). Compounds in the essential oils of one type of oregano have demonstrated antimutagenic effects, and in particular carvacrol and thymol demonstrated a strong antimutagenic effect (Mezzoug *et al*., 2007).
In the past decade, the use of aromatic herbs, essential oils and plant extracts became a special interest in animal nutrition, initially because it was established that these drugs can

effectively replace antibiotics and growth promoters, and for their the other positive effects (Vieira *et al*., 2008).
On the other side, the processing of feedstuffs before ingestion, to improve digestibility, has become a major focus in nutrition researches. Animal feed manufacturing involves the use of a variety of feed technological processes like pelleting, expanding and extrusion to produce compound feeds (Riaz, 2000). Nearly every one of these operations can have positive influence on essential ingredients in feed, which is their primary aim, but can also have adverse effects. Hereby, process should be optimized so that products meet the required nutritional quality of animal feed.
Essential oils are sensitive materials which can easily suffer degradation under the action of oxygen, light and moderate temperatures. Therefore, an adequate application of the essential oil which takes into account these aspects is required for commercial use.
Because of all above mentioned, this study was carried out to determine if pelleting process affect the stability of thymol and carvacrol present in essential oil isolated from *Origanum vulgare*, which was added into the feed for broilers.

MATERIAL AND METHODS

Oil isolation

The essential oil was isolated from dried plant material by hydro distillation, according to the standard procedure reported in the Sixth European Pharmacopoeia (2008). Along with Clevenger type apparatus, duration of distillation was 2 hours. Oil samples were dried with anhydrous sodium sulphate, dissolved in ethanol and analyzed by GC/FID.

Essential oil analysis

The essential oil samples were analyzed by a gas chromatograph Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detector (GC-FID), auto-injection module for liquid, equipped with fused silica capillary column (DB–WAX 30 m, 0.25 mm, 0.50 µm). Carrier gas was helium (purity>99.9997 vol%, produced by Messer, Germany), at a flow rate of 1.26 mL/min. The composition of essential oil was determined by comparing the retention times of oil components (thymol or carvacrol) with those of standards from data library. Results were expressed as mass of thymol or carvacrol (g) per 100 g of essential oil.

Feed

Animal feed used in this study was starter diet mixture for broilers, whose composition is shown in Table 1.
In starter diet mixture was added 2 g/kg of essential oil obtained from the plant *Origanum vulgare*.

Pelleting conditions

The starter diet mixture for broilers was conditioned in double-shaft steam conditioner Muyang SLHSJ0.2A (China), until material reached temperature of 80°C. Water was directly added into feed mash during conditioning. After conditioning process, moisture content of feed for broilers was 15.8%.
Complete mixture for broilers was pelleted on a flat die pellet press 14-175, AMANDUS KAHL GmbH & Co. KG (Germany). A die with 6 mm diameter of the openings and with press way of 36 mm was used (diameter to length ratio 1:6). The pellets were collected at pelleting temperature of 60°C. The speed of passage of material was 18.6 kg/h.
After pelleting, the product was stored for 24 hours under room conditions in order to achieve stabile temperature and subsequently milled by hummer mill with sieve opening of 4 mm.

Data Analysis
Results are presented in the tables as the mean ± standard deviations (SD) of a number (n) of independent determinations. The one way ANOVA analysis (STATISTICA 12.0) was performed to assess data differences among various groups.

Table 1. Composition of starter diet mixture for broilers

Ingredients	(%)
Corn flour	41.8
Soybean meal	37.2
Full fat soy bean	12.5
Soybean oil	4.0
Monocalcium phosphate	1.4
Dl Methionine	0.3
Limestone	1.6
Lysine	0.2
Premix*	1.0

**Vitamins and minerals provided per kilogram of diet: Vitamin A, 3600000 IU; Vitamin B_1, 720 mg; Vitamin B_2, 2640 mg; Pantothenic acid, 4000 mg; Nicotinic acid, 12000 mg; Vitamin B_6, 1200 mg; Folic acid, 400 mg; Vitamin B_{12}, 6 mg; Vitamin D_3, 800000; Vitamin E, 7200 IU; Vitamin K_3, 800 mg; Biotin, 40 mg; Antioxidant, 100000 mg; Choline chloride, 5000 mg; Manganese, 40000 mg; Zinc, 33880 mg; Iron, 20000 mg; Copper, 4000 mg; Iodine, 400 mg; Selenium, 80 mg*

RESULTS AND DISCUSSION

The composition of essential oil from *Origanum vulgare* was characterized by the dominant presence of two substances, thymol and carvacrol. The essential oil composition before pelleting process and after thermal processing is shown in Table 2.

Table 2. The essential oil composition before and after pelleting process

Treatment	Essential oil compositions			
	γ-terpinene	p-cymene	carvacrol	thymol
Before pelleting	3.1 ± 0.2^{a}	11.6 ± 0.4^{a}	61.8 ± 1.1^{b}	19.9 ± 0.7^{b}
After pelleting	2.9 ± 0.3^{a}	10.7 ± 0.2^{b}	50.4 ± 0.9^{a}	15.3 ± 0.2^{a}

Values are presented as means ±SD of six replicates ;
$^{a-b}$Means within a column with no common superscript differ significantlly at $P < 0.05$.

Most of processes used in feed manufacturing are designed to increase the value of feed ingredients, to improve growth rate, efficiency of gain, nutrient digestibility, and to ameliorate feed manipulation (Fairfield, 2003). On the other hand, these processes are harmful to labile nutrients that can be easily damaged (Dozier, 2002), which was proved by results obtained in this study. In order to establish a more suitable and payable way of essential oil application, it was necessary to investigate to what extent this thermal process affect the essential oil composition.
At the beginning of the study, the initial concentration of thymol in complete mixture for broilers was around 19.9 %. In contrast to this, the concentration of thymol in pelleted feed for broilers decreased for 76.9% of its initial value.
The initial concentration of carvacrol in essential oil obtained from *Origanum vulgare* plant was 61.8%. Following the process of pelleting a statistically significant ($P < 0.05$) reduction of carvacrol content in the feed of broilers was observed. The concentration of carvacrol after thermal processing amounted to 50.4%, which shows that reduction of carvacrol concentration after pelleting was nearly 18.5% of its initial value.

Comparing percentage of reduction in the content of thymol and carvacrol, it has been found that thymol is slightly more sensitive than carvacrol, in terms of high temperature, humidity and pressure. These results show that pelleting process has greater impact on the degradation of thymol compared to carvacrol.
Nowadays, there is a wide range of macro and micro components in liquid form and increasing trend in feed industry to use them. In order to achieve a high quality of feed, and in the same time of animal products, a development of suitable technical equipment for their application is absolutely necessary. New technologies are developed to achieve: better pellet quality, higher liquid contents in pellets, better protection of heat sensitive micro ingredients. Vacuum-coating is possible technological tool for post-pelleting application of liquids in animal feed. On the other side, common goals in the development of essential oil formulations are to protect the essential oil from degradation or from losses by evaporation, to achieve a controlled release, and to facilitate handling. This problem can be overcome by producing a dry formulation by microencapsulation. This is a relatively new technology intended for protection, stabilization, and slow dismissal of food ingredients. The encapsulating is used generally for starch, proteins, gums, lipids, essential oils or any combination of them. Methods of encapsulation of food ingredients include spray-drying, freeze-drying, co-crystallization and molecular inclusion.
Therefore, all above mentioned types of liquid applications may be a possible solution for essential oil application in animal feed, in order to achieve high-quality feed, which has a positive effect on the animal health, and hence on the people health.

CONCLUSIONS

Based on the obtained results it can be concluded that pelleting process had an impact on the degradation on examined components of essential oil, thymol and carvacrol, and that losses of mentioned components in treated feeds were generally high.
Average reduction of thymol and carvacrol was about 23.1% and around 18.5%, respectively. This is important difference in terms of nutritional, but economic aspects also.
A possible solution to this problem may be the addition of essential oil in higher concentrations, in which case the contents of examined components would decrease to the desired value after technological processing of animal feed. Furthermore, future studies should consider some other possible ways of essential oil application into animal feed, which will not have negative impact on thymol and carvacrol stability.

ACKNOWLEDGEMENTS

The paper is a part of the research work on the project III-46012 financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia and project 114-451-4685/2013-03 financed by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina.

REFERENCES

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. International Journal of Food Microbiology, 94 (3), 223-53.
Chami, N., Bennis, S., Chami, F., Aboussekhra, A., Remmal, A. (2005). A study of anticandidal activity of carvacrol and eugenol in vitro and in vivo. Oral Microbioliology Immunology, 20 (2), 106-111.
Dozier, W.A. (2002). Mash conditioning and vitamin stability. Feed International, 23, 20 – 24.
European pharmacopoeia (2008). 6th ed., Council of Europe, Strasbourg, France.
Fairfield, D. (2003). Pelleting for profit—Part 1. Feed and Feeding Digest, 54 (6), 1 – 5.
Helander, I.M., Alakomı, H.L., Latva-Kala, K., Mattıla-Sandholm, T., Pol, I., Smıd, E.J., Gorrıs, L.G.M., Von Wrıght, A. (1998). Characterization of the action of selected essential oil components on gram-negative bacteria. Journal of Agricultural and Food Chemistry, 46 (9), 3590–3595.
Inamuco, J.J., Veenendaal, A., Burt, S., Post, J., Tjeerdsma-van Bokhoven, J., Haagsman, H., Veldhuizen, E. (2012). Sub-lethal levels of carvacrol reduce Salmonella Typhimurium motility and invasion of porcine epithelial cells. Veterinary Microbiology, 157 (1-2), 200-207.

Liolios, C.C., Gortzi, O., Lalas, S., Tsaknis, J., Chinou, I. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. Food Chemistry, 112 (1), 77–83.

Martín, A., Varona, S., Navarrete, A., José Cocero, M. (2010). Encapsulation and co-precipitation processes with supercritical fluids: applications with essential oils. The Open Chemical Engineering Journal, 4, 31-41.

Mezzoug, N., Elhadri, A., Dallouh, A., Amkiss, S., Skali, N.S., Abrini, J., Zhiri, A., Baudoux, D., Diallo, B., El Jaziri, M., Idaomar, M. (2007). Investigation of the mutagenic and antimutagenic effects of *Origanum compactum* essential oil and some of its constituents. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 629 (2), 100.

Negi, P.S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology, 156 (1), 7-17.

Panella, N.A., Dolan, M.C., Karchesy, J.J., Xiong, Y., Peralta-Cruz, J., Khasawneh, M., Montenieri, J.A., Maupin, G.O. (2005). Use of novel compounds for pest control: Insecticidal and acaricidal activity of essential oil components from heartwood of Alaska Yellow Cedar. Journal of Medical Entomology, 42 (3), 352-358.

Riaz, M.N. (2000). Extruders in Food Applications, CRC Press, Taylor & Francis Group, Boca Raton, Filadelfia.

STATISTICA (Data Analysis Software System) (2013). v.12.0., Stat-Soft, Inc., USA (www. statsoft.com).

Tampieri, M., Galuppi, R., Macchioni, F., Carelle, M.S., Falcioni, L., Cioni, P.L., Morelli, I. (2005). The inhibitin of *Candida albicans* by selected essential oils and their major components. Mycopathologia, 159 (3), 339-345.

Vieira, S.L., Oyarzabal, O.A., Freitas, D.M., Berres, J., Peña, J.E.M., Torres, C.A., Coneglian, J.L.B. (2008). Performance of Broilers Fed Diets Supplemented with Sanguinarine-Like Alkaloids and Organic Acids. The Journal of Applied Poultry Research, 17 (1), 128–133.

Zarrini, G., Bahari-Delgosha, Z., Mollazadeh-Moghaddam, K., Shahverdi, A.R. (2010). Post-antibacterial effect of thymol. Pharmaceutical Biology, 48 (6), 633–636.

MEAT QUALITY OF RABBITS AFTER ADMINISTRATION OF LANTIBIOTIC GALLIDERMIN

Ľubica Chrastinová[1*], Andrea Lauková[2], Mária Chrenková[1], Zuzana Formelová[1], Mária Poláčiková[1], Anna Kandričáková[2], Klaudia Čobanová[2], Monika Pogány Simonová[2], Viola Strompfová[2], Ľubomír Ondruška[1], Ondrej Bučko[4], Zuzana Mlyneková[1], Anna Kalafová[3], Monika Schneidgenova[3]

[1]National Agricultural and Food Centre, Research Institute of Animal Production, Nitra - Lužianky, Hlohovecká 2, 951 41, Slovakia
[2]Institute of Animal Physiology Slovak Academy of Sciences, Šoltésovej 4-6, 04001 Košice, Slovakia
[3]Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, [4]Department of Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia

*Corresponding author:
E-mail address: chrastinova@vuzv.sk

ABSTRACT

The aim of our work was to test *in vivo* application of lantibiotic gallidermin in the model experiment using rabbits animal. The effect of gallidermin on selected parameters of meat quality was the priority of the study. A total of 48 weaned rabbits (35th day of age, both sexes) were divided into 2 groups (experimental- EG / gallidermin and control- C) with 24 animal in each group. Maternal albinotic line (crossbreed New Zealand White, Buskat Rabbit, French Silver) and paternal acromalictic line (crossbreed Nitra's Rabbit, Californian Rabbit, Big Light Silver) were used. The rabbits in group EG were additionally administered by 5µl of gallidermin per animal/day from 1st day to day 21st applied into the drinking water. In the morning on day 21 and 48, four animals from each group were slaughtered and sampled for testing. Meat quality was analysed from a sample of MLD (50 g) for parameters characterizing the content of nutrients (content of water, proteins, fat, content of amino acids and fatty acids composition) and processing technology parameters (electric conductivity, pH, colour). Of the amino acids and fat acids content investigated in this study statistically insignificant changes ($P > 0.05$) were observed. By this study, lean rabbit meat could be a high quality protein source due to its well-balanced essential amino acid composition. A positive influence of gallidermin on animal health was noted.
Keywords: *rabbits, gallidermin, meat quality*

INTRODUCTION

Gallidermin is an antimicrobial proteinaceus substance produced by the strain *Staphylococcus gallinarum* TU 3928 (Kellner *et al*., 1988; Jack *et al*., 1998). It belongs to a group of polycyclic bacteriocins called lantibiotics (Jack *et al.,* 1998). Lantibiotics contain the rare thioether amino acids lanthionine and/or methyllanthionine. Antimicrobial activity of gallidermin is directed mainly against Gram-positive bacteria, especially against the representants of the species *Propionibacterium acne* or against multi-resistant strains of *Staphylococcus aureus* (Kempf *et al.*, 1999). The main studies conducted with gallidermin have included in vitro tests of its inhibitory activity associated with skin diseases agents. However, never its *in vivo* antibacterial activity and/or its influence concerning the food animals was tested yet as well as nor its influence on the biochemical parameters and meat quality of food animals. In this study, the effect of gallidermin added to drinking water was tested on weight gain, selected parameters as well as the nutritional quality of rabbit's meat.

MATERIAL AND METHODS

A total of 48 weaned rabbits (35th day of age, both sexes) were divided into 2 groups (experimental - EG /gallidermin and control - C) with 24 animal in each group. The rabbits of meat line M91, maternal albinotic line (crossbreed New Zealand white, Buskat rabbit, French

silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in this experiment. The experiment lasted 48 days. Rabbits were kept in the standard cages (0.61 m x 0.34 m x 0.33 m), 2 animals per cage. A cycle of 16 h of light and 8 h of dark was used throughout the experiment. Temperature and humidity in the building were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed the building air temperature to be maintained within 22±4˚C throughout the experiment. Relative humidity was about 70±5%. The rabbits were fed with a commercial diet (pellets of 3.5 mm in diameter). The ingredients and chemical composition of this diet is presented in Table 1. In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by ethical committee. The ME content was calculated by the equation of Wiseman *et al.* (1992). Chemical analyses were conducted according to AOAC (1995) with the considerations mentioned by Egran (2001) for dry mater (DM), crude protein (CP), crude fibre (CF), crude fat, nitrogen free extract, ash and organic matter. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analyzed sequentially (Van Soest *et al*., 1991) with a thermo stable amylase pre-treatment and starch according to the alpha-amyloglucosidase method.
The animals in EG and C were fed by the diet for growing rabbits (Table 1) during the experiment with access to water *ad libitum*.

Table 1. Composition and nutrient content of granulated diet for growing rabbits

Ingredients	**Content (%)**	**Chemical analysis**	**Original matter ($g.kg^{-1}$)**
Lucerne meal	36	Crude protein (N*6,25)	177.99
Extracted sunflower meal	5.5	Crude fibre	146.97
Extracted rapeseed meal	5.5	Fat	36.08
Wheat bran	9	Ash	97.32
Oats	13	Starch	129.05
Malt sprouts	15	Organic matter	847.49
DDGS	5	Acidodetergent fibre (ADF)	185.13
Sodium chloride	0.3	Neutraldetergent fibre (ADF)	315.49
Mineral and vitamin mixture*	1.7	Calcium	9.73
Barley grains	8	Phosphorus	6.94
Limestone	1	ME ($MJ.kg^{-1}$)	11.35

**Premix contains per kg: calcium, 6.73 g; phosphorous, 4.13 g; magnesium, 1.90 g; sodium, 1.36 g; potassium, 11.21 g; iron, 0.36 g; zinc, 0.13 g; copper, 0.03 g; selenium, 0.2 mg.*
Vitamin mixture provided per kg of diet: vitamin A 1500000 IU; vitamin D3 125000 IU; vitamin E, 5000 mg; vitamin B1, 100 mg; vitamin B2, 500 mg; vitamin B6, 200 mg; vitamin B12, 0.01 mg; vitamin K3, 0.5 mg; biotin,10 mg; folic acid, 25 mg; nicotinic acid, 4000 mg, choline chloride, 100000 mg. DDGS: dried distillers grains with solubles

The rabbits in group EG were additionally administered by 5µl of gallidermin (Enzo Life Sciences corp.) per animal/day from the 1st day to day 21 was applied into the drinking water. Gallidermin for use was chilled at +4°C. The dosage of gallidermin was resulted in accordance with our previous *in vitro* studies in laboratory Institute of Animal Physiology Slovak Academy Sciences in Košice. Body weight and feed consumption were registered weekly. Rabbits were fed *ad libitum* (Table 2) and they had free access to drinking water from nipple drinkers during the experiment. In the morning on day 21 and 48, four animals

from each group were slaughtered and samples were taken. After electro-stunning (90 V for 5 sec), rabbits were slaughtered in an experimental slaughterhouse by cutting the carotid and jugular veins, the bleeding out, the MLD samples were separated by removing the skin and connective tissue chilled and stored for 24 h at +4°C until physico chemical analysis started. The ultimate pH was determined after 24 h (*post mortem*) using a Radelkis OP-109 (Jenway, England) with a combined electrode penetrating 3 mm into samples. The electrical conductivity ($\mu S.cm^{-1}$) defined as locations of muscles were evaluated using PMV 51 (Tecpro GmbH, Germany), colour characteristic were expressed by CIE L*a*b system (lightness-L*, 0: black and 100: white), (redness and greenness-a*; yellowness and blueness-b*) using a Lab. Miniscan. Lightness measurements at room temperature were also measured. The content of water, protein and fat were estimated using an INFRATEC 1265 (Germany) spectroscope and expressed in g/100g; from these values, the energy value was calculated: EV (kJ/100g) = 16.75 x protein content + 37.65 x fat content.
The water holding capacity was determined by the compress method at constant pressure (Hašek and Palanská, 1976). The fatty acid (FA) composition of samples MLD were determined (Ouhayoun, 1992) out by gas chromatography of fatty acid methyl ester (FAME) on GC 6890N (Agilent Technologies, Switzerland). Results were expressed as percentages of total fatty acids. Fatty acid composition varies a lot and is expressed as share of SFA (saturated fatty acid) MUFA (monounsaturated fatty acid), PUFA (polyunsaturated fatty acids), P/S and n6/n3 index. The amino acids composition of diet was analyzed by ion-exchange chromatography on AAA (Ingos Prague, Czech Republic) after acid hydrolysis 6M HCl and methionine and cystine after oxidation hydrolysis. Weight feed mixture was checked daily, and average daily weight gain and feed conversion were calculated mathematically as well as mortality at the end of the experiment.
The results were quoted as mean ± standard deviation (SD); statistical evaluation of the results were performed by the one-way ANOVA and Tukey test multiple comparison tests with the level of significance set at $P \leq 0.05$ ± standard deviation (SD).

RESULTS AND DISCUSSION

The study was carried in the National Agricultural and Food Centre, Research Institute for Animal Production Nitra. Among the experimental groups were not found significant differences in feed intake, feed conversion ratio and carcass value in the fattening experiment. Results regarding the zootechnical parameters are shown in Table 2.

Table 2. Effect of treatment on performance of rabbits

Characteristic (n=24)	**Age at slaughter (56 days)**		**Age at slaughter (83 days)**	
	1EC-with gallidermin	**Control-C**	**1EC- not gallidermin**	**Control-C**
Growing period	35-56 days		56-83 days	
Initial leve weight (g)	974±103	957±59	1828±156	1817±14
Final weight (g)	2060±119	1963±33	2713±183	2563±93
Daily weight gain (g/day)	40.905	40.933	34.627	29.362
Feed conversion ratio (g/g)	2.269	2.229	3.544	3.376
Carcass yield (%)	53.45	53.10	57.10	56.39
Mortality (n)	0	0	1	3

Average daily gain from weaning (35d) slaughtered at 56 d of age was reached 40.9 g/d; was not influenced with gallidermin supplement in the rabbits of group EC compared with C. Increase in average body weight gain (about 5.26 g) was noted in the rabbits of group E

compared with C. A positive influence of gallidermin was found on animal health, particularly to favourable effects in gastro-intestinal tract. Results of selected meat quality parameters (content of water, content of proteins, fat, content of amino acids, fatty acids, content of electric conductivity, pH, colour) are presented in Table 3. The fatty acids composition in MLD muscles is shown in Table 3. The rabbit muscles are a low-fat meat. The intramuscular lipid was characterized by the highest percentage of monounsaturated fatty acids (MUFA) (53.19-56.30%). In this study the intramuscular lipids in the MLD muscles were also characterized by a higher percentage of saturated (SFA) (39.18-41.03%) and lower percentage of polyunsaturated fatty acids (PUFA) (7.66-9.58%).

Table 3. Effect of gallidermin on selected processing technology parameters and physico-chemical characteristics of MLD muscles 24h post mortem ($\bar{x}$ ± SD)

Characteristic (n=4)	Age at slaughter (56 days)		Age at slaughter (83 days)	
	1EC- with Gallidermin	Control-C	1EC- not Gallidermin	Control-C
Water g/100g	74.03±0.19	74.11±0.12	74.19±0.15	73.97±0.37
Protein g/100g	23.03±0.26	23.31±0.20	23.83±0.14	23.75±0.17
Fat g/100g	0.86±0.19	0.67±0.12	1.21±0.20	0.75±0.87
Color L	53.54±1.94	55.79±0.70	50.08±1.18	51.03±3.46
Electric conductivity	1.09±0.19	0.84±0.23	0.92±0.48	1.60±0.42
Cholesterol g/100g	0.24±0.06	0.22±0.05	0.23±0.07	0.25±0.06
pH_{24}	5.72±0.04	5.73±0.02	5.47±0.15	5.47±0.15
Water holding capacity	36.09±3.50	36.09±3.50	31.66±1.87	31.66±1.87
EV(kJ.100g^{-1})	419.13±6.54	415.64±4.83	444.57±7.55*	430.41±7.21
Fatty acids (% of total FA)				
Lauric a. (C12:0)	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
Myristic a. (C14:0)	1.22±0.04	1.21±0.03	1.25±0.03	1.25±0.02
Palmitic a. (C16:0)	24.51±0.24*	24.36±0.30	24.39±0.17	24.46±0.24
Margaric a. (C17:0)	0.34±0.05	0.33±0.05	0.33±0.01	0.35±0.01
Stearic a. (C18:0)	11.30±0.18	11.44±0.18	11.45±0.25	11.53±0.08
Vaccenic a. (C18:1n9t)	4.28±0.10	4.39±0.06*	4.43±0.12	4.27±0.08
Oleic a. (C18:1n9c)	42.32±1.30	40.04±1.97	44.46±1.18	43.34±2.27
Linolic a. (C18:2n6c)	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00
Linolenic a. (C18:3n3)	0.22±0.02	0.24±0.02	0.23±0.01	0.22±0.02
Eicosenoic a. (C20:1n-11)	0.47±0.02*	0.42±0.01	0.54±0.01*	0.48±0.04
Eicosapentaenoic a.(C20:5n-3)	0.09±0.01	0.10±0.01	0.10±0.01	0.10±0.01
Arachidonic a. (C20:4n-6)	1.39±0.24	1.70±0.23*	1.56±0.13	1.60±0.33
Docosapentaenic a. (C22:5n-6)	0.14±0.01	0.14±0.01	0.14±0.01	0.14±0.01
Docosahexaenic a. (C22:6n-3)	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
SFA	40.02±1.12	40.67±0.87	39.18±1.46	41.03±0.48*
MUFA	53.59±0.72	53.19±2.16	56.30±1.17*	54.18±1.13
PUFA	7.66±0.92	9.58±0.78	7.80±0.43	8.03±2.52
PUFA/ SFA	0.20±0.02	0.24±0.02	0.20±0.01	0.20±0.06
ω3	0.52±0.07	0.54±0.06	0.45±0.05	0.51±0.07
ω6	6.89±1.07	8.74±0.88	6.90±0.43	6.84±2.75
CLA	0.13±0.01	0.14±0.01	0.14±0.01	0.14±0.01

**P≤0.05*

However, margaric (heptadecanoic) (C17:0) fatty acid in lower proportions was found also in the muscle and subcutaneous tissue of pigs and their wild boar hybrids (Razmaitë and Švirmickas, 2010). But proportions of such polyunsaturated fatty acids as eicosatetraenoic (C20:4n-6) were higher in intramuscular lipids in the lipid tissue of the control. As a consequence, the ratio of C18:2n-6/C18:3n-3 and the ratio of n-6/n-3, including all n-6 and n-3 polyunsaturated fatty acids, were similar. The recommended ratio of polyunsaturated fatty acids to saturated fatty acids (P/S) in the human diets should be increased to above 0.4.

Since some meats (muscle and adipose tissue) naturally have a P/S ratio of around 0.1 (Wood *et al*., 2003). More recently, nutritionists have focused on the type of PUFA and the balance in the diet between n-6 PUFA and n-3 PUFA (Givens *et al*., 2006; Wood *et al.,* 2008). The recommendation for this ratio is of less than 4. However, domestic animals produce an undesirably high n-6/n-3 ratio in meat (Wood *et al*., 2003). Moreover, it can be observed that n-6/n-3 ratio was more favourable than that of hooded and harp seals (Brunborg *et al*., 2006) and of equal value with some species of fish and crabs (Jankowska *et al*., 2010; Chen *et al*., 2007). Although the rabbits are monogastric animals, Martysiak-Żurowska *et al*. (2009) reported the presence of trans fatty acids with 4.3-4.5% of the main vaccenic acid (trans-11-18:1) in the rabbit fat depot. In our study trans fatty acids were not detected. There are recommendations that the value of 2% of the total energy intake as trans fatty acids consumption level should not be exceeded (Léger *et al*., 2007) but on the other hand, the evidence presented in the studies of human diet history shows that for a very long period and until very recently the humans had been regularly consuming ruminant fats contributing up to 5% trans fatty acids in the fats eaten by hunters and later in foods for most agriculturists (Ackman, 1997). Moreover, natural trans vaccenic acid could be metabolized into conjugated linoleic acid and, besides, vaccenic acid did not inhibit the metabolic conversion of linoleic to arachidonic acid (Kummerow, 2009). Food and Drug Administration (FDA) assumed that some of trans acids might be from the natural vaccenic acid that had no harmful effects and suggested that approximately 2.6% of the total daily fat intake were from trans fat and 50% of the trans fatty acids might be from vaccenic acid. Therefore, the presence of vaccenic acid (trans-11-18:1) in the rabbit fat, should not be a limiting factor for the use of meat. Little quantity CLA in meat MLD rabbits is consequences fermentation in caecum and gains caecotrophy (Marounek *et al.,* 2006).

Table 4. The essential amino acid composition of MLD muscles (g/100g)

Characteristic (n=4)	**Age at slaughter (56 days)**		**Age at slaughter (83 days)**	
	1EC- with Gallidermin	**Control-C**	**1EC- not Gallidermin**	**Control-C**
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Threonine	1.08±0.04	1.12±0.04	1.07±0.01	1.07±0.04
Valine	1.03±0.03	1.07±0.03	1.00±0.03	1.01±0.04
Methionine	0.74±0.02	0.80±0.02	0.72±0.01	0.74±0.02
Cystine	0.34±0.01	0.35±0.01	0.34±0.01	0.34±0.01
Isoleucine	0.93±0.03	0.99±0.05	0.89±0.01	0.91±0.07
Leucine	1.99±0.05	2.08±0.07	1.91±0.01	1.95±0.09
Phenylalanine	1.03±0.03	1.07±0.03	0.98±0.01	1.04±0.01
Histidine	1.18±0.05	1.23±0.05	1.10±0.03	1.13±0.08
Lysine	2.09±0.07	2.20±0.08	1.99±0.01	2.17±0.05
Arginine	1.56±0.05	1.64±0.06	1.49±0.01	1.53±0.08
Σ EAA	11.97±0.04	12.55±0.05	11.49±0.02	11.89±0.05

The amino acid composition of MLD muscles is shown in Table 4. Rabbit protein contained a high amount of lysine, leucine, arginine, isoleucine, leucine, histidine, valine, threonine, phenylalanine, methionine and cystine in decreasing amounts. Moreover, the sequence of other amino acids is similar to the sequence of amino acids in other meats. The essential amino acid composition is one of the most important nutritional qualities of protein. Nowadays histidine is considered to be an essential amino acid because of the detrimental effects on haemoglobin concentrations (Report of a Joint WHO/ FAO/ UNU Expert Consultation, 2007). Although tryptophan were not detected, yet according to all of the detected amino acid scores, the protein in MLD muscle was well-balanced in essential amino acid composition and is of high quality. By this study, lean rabbit meat could be a high quality

protein source due to its well-balanced essential amino acid composition. The higher percentage of MUFA in the intramuscular fat was determined in fine trial.

ACKNOWLEDGEMENTS

The authors would like also to thank Dr. R. Jurčik and Dr. V. Parkanyi for help with the rabbits treatment and for their excellent technical assistance in the experiments.

REFERENCES

Ackman, R. G. (1997). Has evolution long-term coexistence adapted us to cope with *trans* fatty acids. Journal of Food Lipids, 4, p. 295-318.

AOAC (1995). Official Methods of Analysis. Washington, DC: *Association of Official Analytical Chemists,* 1018 p.

E.G.R.A.N. (2001). Technical note: Attempts to harmonize chemical analyses of feeds and faeces for rabbit feed evaluation. *World Rabbit Sci*., 2001, vol. 9, p.57- 64.

Brunborg, L. A., Julshamn, K., Nortvedt, R., Frøyland, L. (2006). Nutritional composition of blubber and meat of hooded seal (*Cystophora cristata*) and harp seal (*Phagophilus groenlandicus*) from Greenland. Food Chemistry, 96, p. 524-531.

Givens, D. I., Kliem, K. E., Gibbs, R. A. (2006). The role of meat as a source of n-3 polyunsaturated fatty acids in the human diet. Meat Science, 74, p. 209-218

Hašek, A., Palanská, O. (1976). Determination of water holding in the meat by apparatus with constant pressure. Poultry Industry, vol. 18, p. 228-233.

Chen, D. W., Zhang, M., Shrestha, S. (2007). Compositional characteristics and nutritional quality of Chinese mitten crab (*Eriocheir sinensis*). Food Chemistry, 103, 1343-1349.

Jack, R.W., Bierbaum, G., Sahl, H.G. (1998). Lantibiotics and related peptides. Berlin:Springer Verlag.

Jankowska, B., Zakêœ, Z., Źmijewski, T., Szczepkowski, M. (2010). Fatty acid profile of muscles, liver and mesenteric fat in wild and reared perch (*Perca fluviatilis* L.). Food Chemistry, 118, p.764-768.

Kellner, G., Jung, T., Horner, H., Zahner, N., Schnell, K., Entian, D., Gotz, F. (1988). Gallidermin: a new lanthionine-containing polypeptide antibiotic. Eur. J. Biochem. 177, p. 53-59.

Kempf, M., Theobald, U., Fiedler, H. P. (1999). Correlation between the consumption of amino acids and the production of the antibiotic gallidermin by *Staphylococcus gallinarum*. Biotechnology letters, 21, p. 959-963.

Kummerow, F. A. (2009). The negative effects of hydrogenated trans fats and what to do about them. Atherosclerosis, 205, p. 458 - 465.

Léger, C. L., Razanamahefa, L., Margaritis, I. (2007). Health risks and benefits of trans fatty acids including conjugated fatty acids in food-Synopsis of the AFSSA report and recommendations. European Journal of Lipid Science and Technology, 109, p. 887-890.

Marounek, M., Dokoupilová, A., Skřivanová, V., Berladyn, A. (2006). Conjugated linoleic acid (CLA) and selenium (Se) content of meat of rabbits fed diets enriched with CLA and Se. Annals 3rd Rabbit Congress of the Americas (CD ROM). Maringa City, Brazil, 21.–23. 8. 2006.

Martysiak-Żurowska, D., Zalewski, K., Kamieniarz, R. (2009). Unusual odd-chain and trans-octadecenoic fatty acids in tissues of feral European beaver (Castor fiber), Eurasian badger (Meles meles) and racoon dog (Nyctereutes procyonoides). Comparative Biochemistry and Physiology*,* 153B, p. 145-148.

Ouhayoun, J. (1992). Rabbit meat characteristics and qualitative variability. Cuni-Sci., 7, 1-15.

Pogány Simonová, M., Chrastinová, Ľ., Mojto, J., Lauková, A., Szabóová, R., Rafay, J. (2010). Quality of rabbit meat and phyto-additives. Czech Journal of Food Sciences, 28, p. 161-167.

Razmaitë, V. Švirmickas, G. J. (2010). Effect of introgression of wild boar into Lithuanian indigenous wattle pigs on fat composition in pork under conventional rearing. Veterinarija ir Zootechnika*,* 49, 71, p. 66-72.

Van Soest, J.P., Robertson, J.B., Lewis, B.A. (1991). Methods for dietary fibre, neutral detergent fibre and non starch polysaccharides in relation to animal nutrition. J. Dairy Sci., vol. 74, p.3583–3594.

Wood, J.D., Richardson, R. I., Nute, G.R., Fisher, A.V., Campo, M.M., Kasapidou, E., Sheard, P.R., Enser, M. (2003). Effects of fatty acids on meat quality: a review. Meat Science*,* 66, p**.** 21-32.

WHO/FAO/UNU (2007). Protein and amino acid requirements in human nutrition. Report of a Joint WHO/FAO/UNU Expert Consultation, World Health Organization technical report series 935, WHO, Geneva

Wood, J. D., Enser, M., Fisher, A V., Nute, G. R., Sheard, P. R., Richardson, R. I., Hughes, S. I., Whittington, F. M. (2008). Fat deposition, fatty acid composition and meat quality: A review. Meat Science*,* 78, p. 343-358.

Wiseman, J., Villamide, M. J., De Blas, C., Carabano, M. J., Carabano, R. M. (1992). Prediction of the digestible energy and digestibility of gross energy of feed for rabbits. 1. Individual classes of feeds. Anim. Feeds. Sci. Technol*.,* 39, p. 27-38.

BACTERIAL BIOFILM: AN ANCIENT SURVIVAL STRATEGY OF BACTERIA IN THE BASIS OF THE NEW APPROACH TO UNDERSTANDING THE PATHOGENESIS OF SOME INFECTIONS IN VETERINARY MEDICINE

Dubravka Milanov*, Maja Velhner, Bojana Prunić, Marko Pajić,Jelena Petrović

Scientific Veterinary Institute „Novi Sad", Rumenački put 20, 21 000 Novi Sad, Serbia

*Corresponding author:
E-mail address: dubravka@niv.ns.ac.rs

ABSTRACT

The ability of biofilm formation is an integral feature of prokaryotes and, from the context of evolution it presents the strategy for survival and the maintenance of homeostasis within the unfavourable environmental conditions. In a hostile environment, the bacteria in the time that is measured in minutes from "swimmers" turn into "stickers", maintaining a specific dormancy state and waiting for more favourable conditions for life. It is now known that more than 60% of human infections in developed countries are caused by biofilm, and they become a new category of infectious diseases that are radically different from acute epidemic infections that were dominant until the mid-twentieth century. Biofilm infections cannot be cured using the conventional antibiotics, although some improvements can be achieved during the acute phase of disease. In many cases, standard laboratory techniques did not enable the isolation of the causative agent of such infection thus it was concluded that inflammatory processes are sterile. In veterinary medicine, biofilm infections are investigated to a lesser extent, and still most of the information is derived from an analogy with infections in human medicine. In this paper, we present the relevant facts about a new approach to understanding the pathogenesis of certain infections of importance in veterinary medicine from the aspect of bacterial biofilms. Biofilm infections are persistent, recurrent and failure of therapy by using antibiotics poses a need to search new prophylactic, therapeutic and control methods and strategies.
Keywords: *biofilm, infection, veterinary medicine*

INTRODUCTION

Bacterial biofilm

In view of their total biomass and prevalence, bacteria are the most successful form of life on the Earth. They inhabit the environments and tolerate conditions that would kill other species (way below Earth's surface, depths of the oceans, thermal springs, upper layers of the atmosphere...). Their ability to adapt to diverse, often extremely unfavourable environmental conditions is attributed to the range of survival mechanisms, which have been evolving throughout millions of years and are responsible for the formation of bacterial structures known as a *biofilm*.

Biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002).

Biofilm is a strategy of bacterial survival in all ecosystems including the body of humans and animals. All microscopic "cracks" on the body of a human and animals are deep, dark and humid places suitable for bacterial growth and survival. Only in the space between human tooth enamel and gum tissue, more than 500 diverse bacterial species can be found (dental plaque is most probably the best well-known example of bacterial biofilm). The prevalence of the bacteria in our body is best illustrated by the fact that out of some 90 trillion of body cells, only 10 percent of these are human (http://www.breathdoc.com /articles/biofilm.html). Isolation of bacteria in the laboratory from diverse biomaterials and their cultivation on nutritive media practically offers an insight into the biology of these organisms that is characteristic for planktonic population (planktonic phenotype). However, when attached to the living (or

abiotic) surfaces, the bacteria trigger a whole range or processes resulting in radical declining from planktonic phenotype.

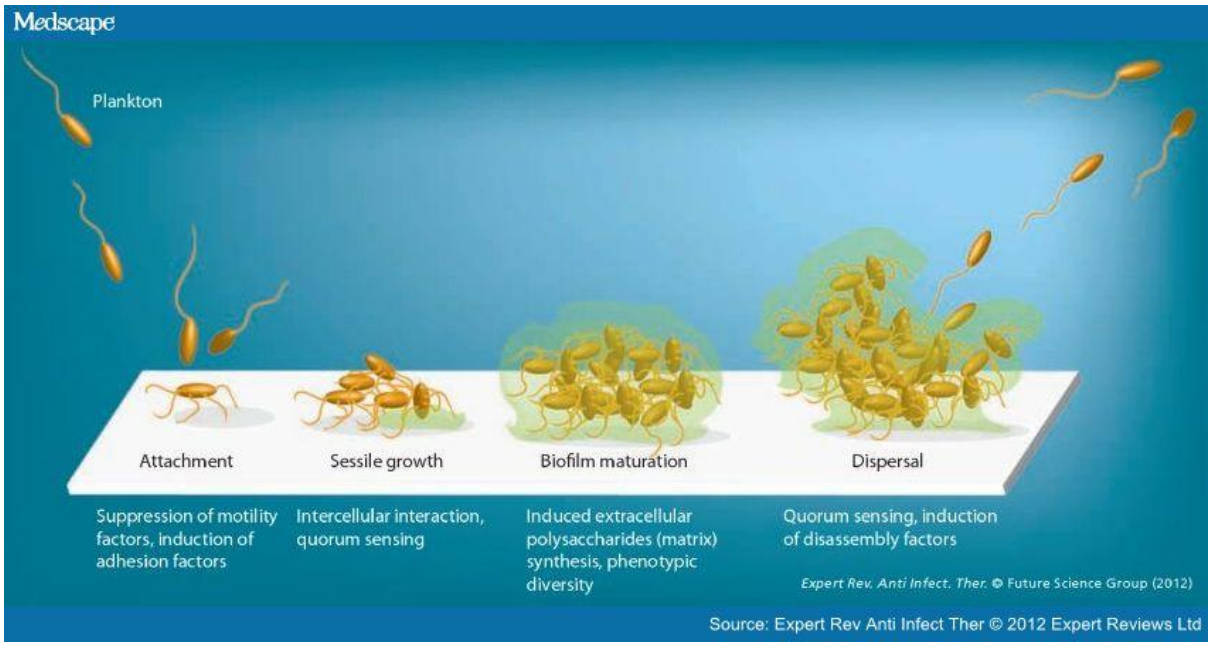

Figure 1: Schematic model representing distinct developmental stages of microbial biofilms. Each stage is associated with specific sets of phenotypic switches, facilitated by tightly regulated changes in gene expression patterns (Source: http://www.medscape.com/viewarticle/774267_4)

In a living being, the bacteria adhere to specific cell-surface receptors (tropism) via specialized adhesins, which are generally regarded as virulence factors. In the pathogenesis of some infections, the process culminates with bacterial internalization into the cell. Rapid bacterial multiplication results in the formation of micro colonies, on the surface or inside the epithelial cells (intracellular bacterial communities – IBCs). Maturation of such micro colonies and their transformation into biofilm structures is regulated by intercellular signalling mediated by specific bacterial products known as signal molecules, i.e. autoinducers (Figure 1). Once the critical concentration threshold of signal molecules („*quorum sensing*") in particular environment is achieved, the activation of genes other than planktonic population is triggered leading to the synthesis of new structural proteins and enzymes. In dense micro colonies of a biofilm, bacteria behave as multicellular communities, thus the term „*sociomicrobiology*" was introduced into microbiology (Parsek and Greenberg, 2005). Biofilm maturation is coupled with the production of extracellular polymeric substance (matrix) that contains water, polysaccharides, proteins, nucleic acids, lipids/phospholipids, absorbed nutrients and metabolic products. The biofilm matrix provides effective protection of bacteria against immune system effectors and, to a lesser extent, against antibiotics. Contrary to planktonic population, bacteria in a biofilm sustain in a slow-growth or dormant state almost without any cell division. Such status undoubtedly contributes to their increased resistance to antibiotics, which may be even 1000 times higher as compared with the planktonic population of the same species (Mah and O'Toole, 2001; Gilbert *et al*., 2007). Sometimes, the bacteria may detach from the biofilm, while either preserving the biofilm-phenotype (detachment due to erosion and sloughing) or recovering the planktonic phenotype (if the detachment is due to the growth and multiplication). There are some opinions that, in nature, the planktonic phenotype plays a role only as a dissemination-mechanism until targeting new colonization sites for biofilm formation.

The twentieth century is characterized by intensive research activities on planktonic bacterial phenotypes and the results of the these researches provided enormous achievements in the field of health protection of humans and animals and control of acute infectious diseases. At

the beginning of the 21st century, the new category of emerging infectious diseases is defined – *biofilm infections*.

Biofilm infections

Biofilm infections emerged as the new category of infectious diseases, which are considered to make over 60% of all bacterial infections in developed countries or, according to some data, even over 80%. *Parsek and Singh* (2003) proposed four basic criteria to define biofilm-associated infections:

- "the pathogenic bacteria are surface associated;
- direct examination reveals bacteria in clusters, encased in a matrix;
- infection is localized;
- infection is resistant to antibiotic therapy despite the antibiotic sensitivity of the constituent planktonic organisms."

Distinctive feature of biofilm infections is their localization in particular tissues/organs. The most common examples of biofilm infections in human medicine include chronic cystitis (caused by uropathogenic strains of *Escherichia coli*, UPEC), pulmonary infections in patients with cystic fibrosis (*Pseudomonas aeruginosa and Burkholderia cepacia*), chronic pyelonephritis (*Proteus vulgaris*), chronic prostatitis (*Escherichia coli* and other Gram-negative bacteria), otitis media (*Haemophilus influenzae*, *Streptococcus pneumoniae*). These infections commonly take chronic course with intermittent periods without overt symptoms and periodical exacerbations. The relation between protective power of the body and invasive capacity of bacteria in biofilm, may be considered a pat-position. The bacteria in biofilm enable host's living and thus, indirectly, provide their own habitat by "turning down" the virulent potential and down regulate their growth and metabolism. Bacteria release antigens that are recognized by host's immune system; however, there are no effective mechanisms able to adequately destroy bacterial biofilm population. The bacteria are protected by a matrix, which presents an effective physical barrier to phagocyte cells, lymphocytes and antibodies. The immune complexes and enzymes produced by phagocytes can even cause tissue damage thus providing the substratum for further development of the biofilm.

Since the bacteria are attached to the surface, encased in a matrix and in a dormant state, their isolation from swabs or body fluids on nutritive media is mostly impossible. Biofilm bacteria are viable yet often not cultivable in common conditions of microbiological laboratories. Isolation failures often compromised the reputation of microbiological laboratories by identifying agents of chronic prostatitis, otitis media in children of other biofilm infections that were therefore considered "sterile" inflammatory processes. Nowadays, it is well established that bacterial isolation on nutritive media in laboratory conditions can be successful only in case of planktonic populations that make only small percentage of bacterial population within the body and that is only periodically identified in biofilm-forming bacteria (during process acutization and in recurrent clinical symptoms).

Direct microscopic examination of infected tissues (bioptate or post-mortem examination) reveals bacteria embedded in a matrix, whereas molecular methods provide the evidence on their viability. In that respect, molecular methods are considered most reliable in the diagnostics of biofilm infections. Moreover, in some recent researches, these methods proved capable of detecting and identifying yet unknown bacteria that would never grow on agar plates. From the diagnostic point of view, the specificity of biofilm infections is reflected in the need of abandoning the generally accepted approach in microbiological laboratories, i.e. looking for one single infectious agent. So far, we have been trained to "choose" one bacterial species and consider it responsible for the infection (which is usually the case in acute planktonic infections). However, in chronic biofilm infections, one single bacterial species is rarely involved in the etiology of infection even though it may play a crucial role. Biofilms represent the environment with distinctive parts that offer optimal conditions for growth and multiplication of particular bacterial species while at the same time biofilm as a

whole contributes to the stability of biofilm structure. Single-species biofilm (so far, most widely investigated in laboratory conditions) are extremely rare in natural environments, including human and animal body. Conventional antibiotic therapy is commonly ineffective in biofilm infections. Antibiotics destroy only bacteria in superficial biofilm layers, while a number of persister-cells are left behind in the biofilm, which are naturally resistant to antibiotics. The ineffectiveness of antibiotics can also be attributed to particular physico-chemical factors in deeper biofilm layers such as pH as well as the concentrations of carbon dioxide, oxygen, divalent cations, pyrimidine and water. The dose of antibiotics required to destroy the bacteria in biofilm would kill the patient. In biofilm infections, surgical treatment is indicated whenever possible. Otherwise, life-long administration of antibiotics is inevitable. The attempts to control biofilm by breaking intercellular bacterial communication are to date far from success, as the cell communication in human and animal body is mediated by similar genetic code as that of the bacteria. Biofilm infections require novel approaches to understanding of the pathogenesis and discovering new methods in both prophylaxis and therapy.

Biofilm infections in veterinary medicine

Many of bacterial species that are well-established agents of human biofilm infections are important animal pathogens, thus likely involved in analogous biofilm infections in animals. Evidently, there is not as much direct body of evidence on biofilm infections in veterinary medicine but a number of *in vitro* researches were performed. To date, there have been a number of reports confirming the involvement of bacterial biofilms in the etiology of pneumonia, liver abscesses, enteritis, wound infections and mastitis (Francey *et al.*, 2000; Olson *et al*., 2002; Clutterbuck *et al*., 2007; Melchior *et al*., 2006; Oliveira *et al*., 2006).

Table 1: Biofilm infections in veterinary medicine

Infections	**Bacterial species in biofilm**
Wound infections (horses, dogs, cats) and joint infections (horses)	*Acinetobacter baumannii*
Wound infections, enteritis (horses)	*Aeromonas hydrophila*
Peritonitis (horses)	*Actinobacillus equuili*
Mastitis (cows)	*Staphylococcus aureus, S. epidermidis, Streptococcus uberis*
Pneumoniae (horses, cows, pigs)	*Aeromonas hydrophila*
Respiratory tract infections (pigs)	*Haemophilus parasuis* *Streptococcus suis* type 2
Pyoderma (dogs)	*Staphylococcus pseudintermedius*
Pleuropneumonia in cattle	*Mycoplasma mycoides* subsp. *mycoides*
Skeletal muscle infections (horses)	*Klebsiella pneumoniae*
Bovine Respiratory Disease Complex (BRDC)	*Histophilus somni*

In the majority of cases, the causative agents of biofilm infections are bacteria that are part of normal microbial flora of the skin and mucosa and any disruption of tissue integrity can trigger the infection. Thus, normal skin microbiota such as *Acinetobacter baumannii,* are most commonly responsible for wound infections and nosocomial infection outbreaks in dogs and cats (Francey *et al.*, 2000). *A.baumannii* easily colonizes inert surfaces such as intravenous jugular catheter, thus representing a causative agent of nosocomial infections in horses (Vaneechoutte *et al*., 2003). *A.baumannii* isolates originating from purulent wounds of horses manifest resistance to penicillin and gentamycin, which is characteristic for biofilm bacteria (Clutterbuck *et al*., 2007). Postoperative infections in horses are often caused by *Actinobacillus equuli,* a commensal inhabiting the upper respiratory tract. Chronic pyoderma

in canines may be the result of underlying bacterial biofilm on the skin formed by *S. pseudintermedius* (Romero *et al*., 2010). The majority of *S. pseudintermedius* isolates obtained from dogs were able to produce biofilm in vitro on 316L stainless-steel orthopaedic bone screw, and this may be an important virulence factor in the rapid emergence of this bacterium in veterinary hospitals worldwide (Singh *et al*., 2013).
Persistent respiratory infections of swine caused by *Haemophilus parasuis* are associated with the ability of particular isolates and serotypes of this organism to form biofilm in vivo (Jin *et al*., 2006). Furthermore, *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm* SC) was investigated in a biofilm model that might be of paramount importance in the pathogenesis of contagious bovine pleuropneumonia (CBPP) (McAuliffe *et al*., 2008). Biofilm-behaviour patterns in vitro were also defined for some other organisms such as *Histophilus somni* (Sandal *et al.*, 2009), *Pasteurella multocida* and *Mannheimia haemolytica* (Romero *et al*., 2010). Bacterial species of importance for veterinary medicine, such as *Corynebacterium pseudotuberculosis, Pseudomonas aeruginosa, S. aureus, Staphylococcus hyicus* and *E. coli* successfully form biofilm in vitro, which is confirmed using Calgary Biofilm Device-CBD (Olson *et al*., 2002). Most likely, the same organisms are able to form biofilm in vivo, which may play a crucial role in the development of chronic infections.
In cattle, the Bovine Respiratory Disease Complex (BRDC) is highly challenging disease causing huge health and economic losses. The researchers from the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech, have been investigating the role of the biofilm in the development of this syndrome, with particular emphasis of *Histophilus somni* biofilm. Biofilm protects the organism against antibiotic therapy and enables its dissemination outside the respiratory tract to the myocardium or nervous tissue, which finally may result in lethal outcome (http://www.eurekalert.org/pubreleases/2008-04/vt-vcr040308.php). The goal of these investigations is the understanding of molecular basis of biofilm formation that would be the starting point in identifying the strategy for prevention of biofilm development and discovering an effective therapy against existing bacterial biofilms.
Mastitis of dairy cattle caused by *S. aureus* is the most well known and commonly investigated representative of biofilm infections in veterinary medicine (Vasudevan *et al*., 2003; Cucarella *et al*., 2004; Fox *et al*., 2005; Oliveira *et al*., 2006). In spite of some improvement that can be accomplished by administration of antibiotics, the majority of infections remain persistent and are thus considered untreatable disease. Physiological changes in the udder are most likely responsible for changes in the expression of virulence-genes of S*. aureus*, which than turns from the *'defensive'* biofilm growth into a phase of *'offensive'* growth resulting in an inflammatory process and clinical disease manifestation (Melchior, 2006). From the clinical point of view, this is the shift from subclinical to clinical mastitis and vice versa. Microscopic examination of mammary tissue in acute and chronic infections revealed bacteria located in clusters within the alveoli and lactiferous ducts (Mellchior *et al.*, 2006). *S. aureus* biofilm formation is considered a virulence factor in the development of mastitis in dairy cattle. *Ica* (intracellular adhesion) gene clusters, particularly *ica*A and *ica*D, are of crucial importance in *S. aureus* biofilm formation and are most commonly identified in bovine mastitis isolates (Vasudevan *et al*., 2003; Cucarela *et al*., 2004). However, not all isolates that possess these genes produce biofilm *in vitro*, suggesting that combination of phenotypic and genotypic methods is most desirable when examining clinical isolates (Vasudevan *et al*., 2003). In our epizootiological area, *ica*A and *ica*D genes were identified in bovine mastitis isolates, in both strong biofilm producers and isolates that did not produce biofilm *in vitro* (unpublished results) (Figure 2).

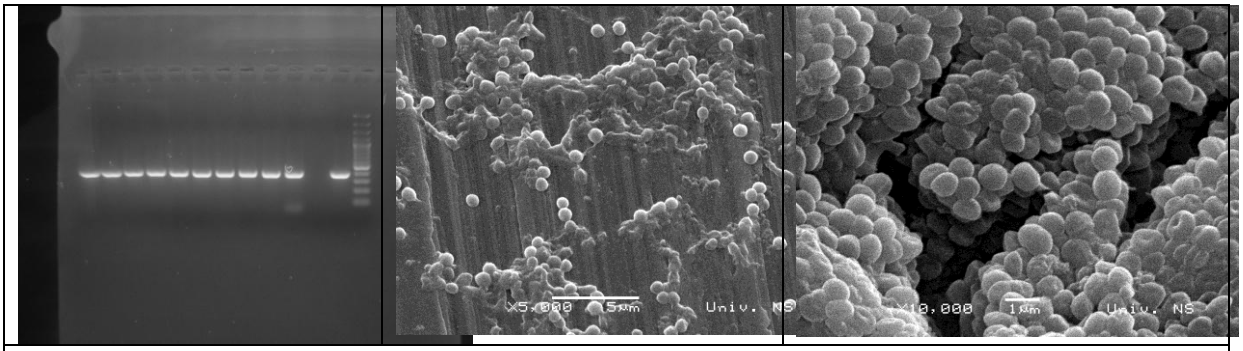

Figure 2: icaD gene (381bp) (unpublished results, NIV NS) identified in bovine mastitis isolates that did not produce biofilm in vitro (photo in the middle) and in strong biofilm producers (photo on the right) (Milanov et al., 2010). SEM photo: Bokorov Miloš, PMF, Novi Sad.

Some recent researches of the pathogenesis of bovine mastitis caused by *Streptococcus uberis* and *Escherichia coli* indicated some analogy with *S. aureus*. *Streptococcus uberis* and some *Escherichia coli* strains can provoke persistent udder infections, and biofilms can provide suitable condition for the persistence of the bacteria at this site (Fernandes *et a*l., 2011; Melchior *et al*., 2006). *E. coli* strains that cause persistent infections manifest ability of internalization of epithelial mammary cells (Dogan *et al*., 2006). The internalization of uropathogenic strains of *E. coli* in epithelial cells results in formation of intracellular bacterial communities (IBCs), which manifest biofilm behaviour and similar mechanism is likely to underlie the pathogenesis of bovine mastitis (Fernandes *et al*., 2014). In bovine mastitis isolates of *E. coli* good capacity of *in vitro* biofilm production was confirmed (Fernandes *et al*. 2011) as well as the presence of virulence genes responsible for biofilm formation, i.e. *fim*A (large subunit of the fimbriae type 1) and *csg*A (large subunit of curli fimbriae) (Silva *et al*., 2013). Clonal persistent *E. coli* intramammary infections (IMI) make some 5-24% of all *E. coli*-associated mastitis (Dogan *et al*., 2006), which suggests adaptation of particular strains to bovine mammary environment (Bradley and Green, 2001). These findings radically change the knowledge on the pathogenesis of coli mastitis, which have traditionally been considered transient infections resulting in elimination of the agent from the tissue (bacteriological healing). In spite of substantial decrease in the rate of severe cases of clinical mastitis, the prevalence of subclinical mastitis in cows is still very high (Melchior *et al.,* 2006). Udder infections associated with species *Streptococcus* (*S. uberis*, *S. dysgalactiae*) as well as coagulase negative *Staphylococcus* spp.*,* often result in recurrent and chronic infections despite good *in vitro* antibiotic susceptibility of the agent. *Streptococcus uberis* is classified into the category of environmental agents; however, it can be found in the tonsils, genital tracts, rumen and coat of a cow. Development of persistent intramammary infections (IMI) implicates a range of cell-surface proteins of *S. uberis*: plasminogen activator protein, adhesion molecule SUAM, lipoprotein receptor antigen MtuA and the oligopeptide transport system OppA (Varhimo *et al*., 2011), which mediate its initial attachment and cell internalization. The isolates of *S. uberis* from milk of cows demonstrated ability of biofilm production *in vitro* , and the process can be enhanced by the addition of milk or individual milk caseins, such as α-, β- and κ-casein, to the growth medium (Varhimo *et al.,* 2011).

The results of laboratory antibiotic susceptibility tests (disc diffusion or dilution tests) of bovine mastitis agents cannot guarantee the effectiveness of the therapy in infections caused by bacteria organized in a biofilm inside the udder tissue. A number of researches demonstrated drastic variations in antimicrobial susceptibility between planktonic population (MIC – minimum inhibitory concentration) and in vitro formed biofilms of the same bacterial isolates (MBEC – minimal biofilm eradication concentration). Some of the results are displayed in Table 2.

Table 2: In vitro research confirmed different antimicrobial susceptibility of the planktonic and biofilm population of bacteria that cause mastitis

		Pen G	Cloxacill.	Streptom	Ceftiofur	Tetrac.	Ampicil	Ref.
T.pyogenes	MIC	<2	<2	4	<2	<2	<2	Olson, 2002
	MBEC	>1024	>1024	256	>1024	>1024	500	
S.aureus	MIC	2	<2	128	<2	<2	32	
	MBEC	>1024	512	>1024	256	512	128	
		Pen G	Novobioc	Oxytetr	Ceftiofur	Gentam	Eroflox	Ref.
S. aureus	MIC	<2	8	<2	4	<2	<2	Ceri *et al*., 1999
	MBEC	>1024	16	256	1024	16	256	
Sc. uberis	MIC	<2	<2	<2	<2	<2	<2	
	MBEC	<2	>1024	128	128	<2	<2	
		Ampicil	Cefazolin	Cefotax	Ciproflox	Gentam	Trim/Sulfa	
E.coli	MIC	2	1	0.06	0.004	2	0.06	
	MBEC	>1024	>1024	256	8	16	64	

The research of bacterial biofilms strongly suggests a radical declining the general attitude on bacteria as unicellular organisms. Current knowledge on bacteria that persist in various organs and tissues organized into multicellular functional and structural bacterial communities known as biofilm impose the need for a new approach to understanding of the pathogenesis of some chronic infections and discovering novel prophylactic, control and therapeutic strategies.

ACKNOWLEDGEMENTS

The research is supported by a grant from the Ministry of Education and Science, Republic of Serbia, Project number TR31071.

REFERENCES

Bradley, A.J., Green, M.J. (2001). Adaptation of *Escherichia coli* to the bovine mammary gland. Journal of Clinical Microbiology, 39, 1845-1849.

Ceri H., Olson M. E., Stremick C., Read R.R. D. Morck D., Buret A.: The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms, Journal of Clinical Microbiology, 37 (6), 1771-1776, 1999.

Clutterbuck, A.L., Woods, E.J., Knottenbelt D.C., Clegg, P.D., Cochrane, C.A., Percival, S.L. (2007). Biofilms and their relevance to veterinary medicine. Veterinary Microbiology, 121, 1-17.

Costa, J.C.M., Espeschit, I.F., Pieri, F.A., Benjamin, L.A., Moreira, M.A.S. (1999). Increased production of biofilms by *Escherichia coli* in the presence of enrofloxacin. Veterinary Microbiology,160, 488–490.

Costerton, J.W., Stewart, P.S., Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. Science, 284, 1318-1322.

Cucarella, C., Tormo, M.A., Ubeda, C., Trotonda, M.P., Monzon, M., Peris, C., Amorena, B., Lasa, I., Penades, J.R. (2004). Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. Infection and Immunity, 72 (4), 2177-2185.

Dogan, B., Klaessig, S., Rishniw, M., Almeida, R.A., Oliver, S.P., Simpson, K., Schukken, Y.H. (2006). Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. Vet Microbiol, 116, 270-282.

Donlan, R.M., Costerton, J.W. (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clinical Microbiology Reviews, 15 (2), 167-193.

Fernandes, J.B.C., Zanardo, L.G., Galvão, N.N., Carvalho, I.A., Nero, L.A., Moreira, M.A.S. (2011). *Escherichia coli* from clinical mastitis: serotypes and virulence factors. Journal of Veterinary Diagnostic Investigation 2011, 23 (6): 1146-1152.

Fox, L., Zadoks, R., Gaskins, C. (2005). Biofilm production by *Staphylococcus aureus* associated with intramammary infection. Veterinary Microbiology, 107, 295-299.

Francey, T., Gaschen, F., Nicolet, J., Burnens, A.P. (2000), The role of *Acinetobacter baumannii* as a nosocomial pathogen for dogs and cats in an intensive care unit. J Vet Intern Med, 14, 177-183.

Gilbert, P., Das, J., Foley, I. (2007). Biofilms susceptibility to antimicrobials. Science, 11, 160-167.

http://www.eurekalert.org/pub_releases/2008-04/vt-vcr040308.php: Veterinary college researchers explore function of biofilm in bovine respiratory disease, Virginia Tech.

Jin, H., Zhou, R., Kang, M., Luo, R., Cai, X., Chen, H. (2006). Biofilm formation by field isolates and reference strain of *Haemophilus parasuis*. Veterinary microbiology, 118, 117-123.

Mah, T.F.C., O'Toole, G. (2001). Mechanisms of biofilm resistance to antimicrobial agents, *TRENDS in Microbiology*. 9 (1), 34-39.

McAuliffe, L., Ayling, R., Ellis, R., Nicholas, R. (2008). Biofilm-grown Mycoplasma mycoides subsp. Mycoides SC exhibit both phenotypic and genotypic variation compared with planktonic cells. Veterinary Microbiology, 129, 315-324.

Melchior, M.B., Vaarkamp, H., Fink-Gremmels, J. (2006). Biofilms: A role in recurrent mastitis infections? The Veterinary Journal, 171, 398-407.

Milanov, D., Lazić, S., Vidić, B., Petrović, J., Bugarski, D., Šeguljev, Z. (2010). Slime Production and Biofilm Forming Ability by *Staphylococcus aureus* Bovine Mastitis Isolates. Acta Veterinaria, 60 (2-3), 217-226.

Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G., Read, R.R. (2002). Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res, 66, 86-92.

Oliveira, M., Bexiga, R., Nunes, S.F., Carneiro, C., Cavaco, L.M., Bernardo, F., Vilela, C.L. (2006). Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. Veterinary Microbiology, 118, 133-140.

Parsek, M.R. and Singh, P.K. (2003). Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol, 57, 677–701.

Parsek, M.R. and Greenberg, E.P. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. Trends In Microbiology, 13, 27–33.

Romero, F.A., Romero, N.A.P., Aparicio, E.D., Castro, R.H. (2010). Bacterial biofilms: Importance in animal diseases. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 700-703; http://www.formatex.info/microbiology2/700-703.pdf

Sandal, I., Shao, J., Annadata, S., Apicella, M., Boye, M., Jensen, T., Inzana, J. (2009). Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory shallenge. Microbes and Infection, 11, 254-263.

Singh, A., Walker, M., Rousseau, J., Weese, J.S. (2013). Characterization of the biofilm forming ability of Staphylococcus pseudintermedius from dogs. BMC Veterinary Research 2013, 9:93 http://www.biomedcentral. com/1746-6148/9/93

Vaneechoutte, M., Devriese, L.A., Dijkshoorn, L., Lamote, B., Deprez, P. (2000). *Acinetobacter baumannii* infected vascular cateter collected from horses in an equine clinic. J Clin Microbiol, 38, 4280-4281.

Varhimo, E., Varmanen, P., Fallarero, A., Skogman, M., Pyörälä, S., Iivanainen, A., Sukura, A., Vuorela, P., Savijoki, K. (2011). Alpha- and β-casein components of host milk induce biofilm formation in the mastitis bacterium Streptococcus uberis. Veterinary Microbiology, 149, 381-389.

Vasudevan, P., Nair, M.K.M., Annamalai, T., Venkitanarayanan, K.S. (2003). Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Veterinary Microbiology, 92, 179-185.

THE OCCURRENCE AND EFFECTS OF AFLATOXINS IN NATURALLY CONTAMINATED COMPLETE FEED FOR FATTENING TURKEYS

Miloš Kapetanov*, Igor Stojanov, Milica Živkov Baloš, Dragana Ljubojević, Željko Mihaljev, Jasna Prodanov Radulović

Scientific Veterinary Institute „Novi Sad", Novi Sad, Rumenački put 20, Serbia

*Corresponding author:
E-mail address: dragana@niv.ns.ac.rs

ABSTRACT

Aflatoxins are products of numerous fungi from *Aspergillus* genus. The most significant aflatoxins producers are *A. flavus* and *A. parasiticus*. Feed contamination can occurs on field, at harvest or during postharvest operations as well as during the feed proccesing. Among poultry, ducks are the most sensitive to aflatoxins, followed by geese, turkeys and pheasants, while chickens show considerable resistance.

Five consecutive fattening cycles of turkeys that were fed with complete diet naturally contaminated with aflatoxins were included in one year study, from 2012 to 2013. The flock size was of approximatelly 2500 to 3000 turkeys. Clinical signs, pathological changes and flock performance, here presented, were diverse and depended on aflatoxins level and birds age. The level of aflatoxins contamination were ranged from below 10 µg/kg to over 45 µg/kg. In one particular flock, due to prolonged exposition and the highest level of aflatoxins, the cycle ended prematurely.

***Keywords**: aflatoxins, broiler turkeys, complete diet*

INTRODUCTION

Mycotoxins are fungal secondary metabolites of different chemical structure and biological effects (Janković et al., 2006; Jakšić et al., 2012). Besides some known chemical compounds that are useful for protection from pathogens, substances with toxic effects that are synthesized by fungi require more attention. There are more than 400 mycotoxins described in literature (Berthiller et al., 2007). For poultry industry, aflatoxins represent one of the most important group of mycotoxins, which are mainly produced by *Aspergillus* spp., predominantly *A. flavus* and *A. parasiticus*.

Feed contamination may occur on field and during different harvesting and postharvesting operations or feeding regimens, (Binder et al., 2007). The amount of aflatoxins accumulated on, for example corn, wheat, rice, cotton and peanut, varies seasonally due to climate and natural physical factors (Jakšić et al., 2012; Kos et al., 2013). In general, high temperature and low humidity of air favor the growth of aflatoxins producing fungi (Zorzete et al., 2013).

In animals, consumption of contaminated feed affects gastrointestinal, reproductive, nervous system and immunity, and causes functional damage of tissues (Gabal et al., 1998; Applegate et al., 2009; Yarru et al., 2009; Yunus et al., 2011). Furthermore, aflatoxins accumulate in final products, such as eggs and meat (Ortatatli et al., 2005; Pandey et al., 2007; Hussain et al., 2010; Yang et al., 2012). Clinical signs and pathological changes depend on species and production type (Feng et al., 2011), aflatoxins content as well as exposition time. Duck is the most sensitive poultry species to aflatoxins, in comparison to goose, turkey and pheasant. The chicken is considered to be the most resistant to aflatoxins. During acute course, neural signs are present, including uncoordinated movement, paresys and paralysis, followed by haemorrhagic diarrhea, icterus, coma and death. Continuous consumption of contaminated feed for one week or longer, leads to significant weight loss, decreased consumption and poor feed conversion, egg drop and lower hatchability. Lethal outcome is often seen in cases of chronic intoxication (Applegate et al., 2009; Manafi et al., 2011).

MATERIAL AND METHODS

The clinical investigations and analyses of feed were conducted during the 2013 year. Clinical examination was done in five consecutive flocks of broiler turkeys (F1-5). Flock size comprised approximately 2500 to 3000 birds. Carcasses and samples of complete feed mixtures were analysed at the Scientific Veterinary Institute, Novi Sad. Pathological *post mortem* examinations were done on a representative number of turkeys. The complete mixtures, including three of starter and two of finisher, were prepared and inoculated on Saburo agar for microbiological investigations to determine the presense of fungi, Quinn (1998). The aflatoxins content was determined using commercial *ELISA* test (Ridascreen®, R-Biofarm, Deutchland, Art.No.R:3801) with detection limit of 3.5 µg/kg. Interpretation of the results was done according to national regulation by regulation on feed quality for animal nutrition (2010).

RESULTS AND DISCUSSION

Health problems and poor production occured on a farm of fattening turkeys of XL hybrid, that were imported at age of one day. The first disease outbreak was recorded on a farm of fattening turkeys at the age of 4 week (F1) with signs of acute aflatoxicosis. In two additional flocks (F3 and F5) similar clinical signs were observed after the third week of age. The disease started after ninth week of age in two intermediate flocks (F2 and F4). Disease was first observed in more advanced birds, that promptly reflected to flock performance, i.e. weight loss and decreased flock uniformity. In all flocks high mortality, poor conversion, and lower body weight were recorded, that resulted in shortening of the cycle for approximately two weeks (Table 1). The outbreaks with clinical signs of aflatoxicosis at fourth week of age was due to *ad libitum* feeding regimen in young turkeys. In two flocks that suffered at older age (after week 9), secondary infection partially masked the intoxication (F4), and also there is a correlation between the concentration of aflatoxins, exposition and deposition in eggs and meat, which is visually confirmed in this study as well as in some previous studies (Pandey et al., 2007; Wu et al., 2011).

Table 1. Results of flock performance in fattening turkeys.

Variable*	Mortality (%)		Feed conversion (%)		Fattening cycle (week)		Finishing body weight (kg)	
Technology	♂	♀	♂	♀	♂	♀	♂	♀
	3		2.5	2.7	20	16	20.9	10.9
Achieved	9.4-100**		>3		18	14	10.9-12.2*	8.1-8.9*

** Results are summarized for four flocks exept for mortality since the turn in flock 3 ended prematurely at week 7 (table 2)*

*** Mortality and body weight at the end of a turn are expressed by variation interval*

The feed samples were collected and investigated immediately after section. However, aflatoxins was confirmed in concentration that exceeded the permitted level of 10 µg/kg (Table 2), as instructed by the regulation on feed quality for animal nutrition (2010). The most severe signs were registered in F3, which consumed feed mixture contaminated with aflatoxins in concentration greater than 45 µg/kg. Because of that rearing of that flock were terminated prematurely at the age of 7 weeks. As for the remaining four flocks, low yield and high condemnation rate were found at slaughter.

Table 2. Aflatoxin content in complete mixtures for fattening turkeys.

Flock	F1	F2	F3	F4	F5
Feed type	starter	finisher	starter	finisher	starter
Aflatoxin content (µg/kg)	12.69	>45	>45	12.39	15.82

Nervous signs were recorded during the clinical examination, including painful peeping, uncoordinated movements, paresys and paralysis. On section, birds were in typical position with prostrate neck, outstreched legs with that lossed skin coloration, and dirty feathers, particularly around vent (Figure 1). Pathological changes included: enlargement and adipose distrophy of liver (Figure 2 and 3), bile stasys, catarrhal and hemorrhagic enteritis in small intestines, undigested feed particles in colon, kidneys with oedema, dylated ureters and uricosis, hyperemic meninges with extravasation. Typical pathological changes that were found in turkeys were in close relation to the level of aflatoxins determined in feed, and were also reported by Al-Sadi and Al-Attar (2000), Ortatatli et al. (2005), Okiki et al. (2010).

Figure 1. Typical position of turkey carcasses

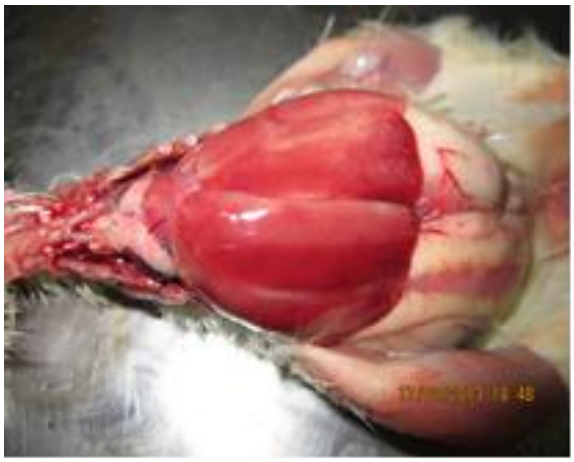

Figure 2. Adipose distrophy of liver

Figure 3. Spotily yellowish liver tissue

Kos et al. (2013) have published a study on natural contamination of maize with aflatoxins in Serbia for the period from the year 2009 to 2012. It is among the most susceptible crop to aflatoxins contamination which occurs in a cyclic manner. Hot and dry summer seasons and infestation with European corn borer (*Ostrinia nubialis*) were registered in the year 2012. Furthermore, extreme weather conditions followed with high temperature, long dry periods and average humidity below 18%, also influence aflatoxins contamination of corn in the state of Missoury, USA in the period 2006-2007 (Wu et al., 2011). Seasonal changes in aflatoxins contamination rate of corn and high aflatoxins concentration that has caused mortality in poultry were reported by Azziz-Baumgartner et al., (2005). Recently, Streit et al. (2012) published mycotoxins contamination of animal feed in the Europe. Studies of Kapetanov et al. (2011, 2011a) on the effects of the global warming has shown changes in disease outbreaks in poultry, indicating more frequent even more severe extreme climate.

CONCLUSIONS

Aflatoxicosis is a significant, severe disease of poultry, that can cause high mortality and poor flock performance. The losses occur if aflatoxins concentration exceeds permitted level. When signs of the disease are present and also if signs of diseases are not present, it seems rational to use tests such as *ELISA* to detect aflatoxins, particularly if the mycological examination indicates low fungal contamination.
It is important to monitor feed quality continuously, in order to avoid negative effects of intoxication in poultry flocks, and accumulation of aflatoxins in meat and eggs.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31071, financed by the Ministry of Science and Technological Development, Republic of Serbia.

REFERENCES

Al-Sadi, H.I., Al-Attar, M.Y. (2000). Outbreaks of aflatoxicosis in broilers. Iraqi Journal of Veterinary Sciences, 13, 93-106.

Applegate, T.J., Schatzmayr, G., Pricket, K., Troche, C., Jiang, Z. (2009). Effect of aflatoxin culture on intestinal function and nutrient loss in laying hens. Poultry Science, 88, 1235–1241.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Rogers, H.S., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L.F., Misore, A., DeCock, K., Rubin, C., Slutsker, L. (2005). The aflatoxin investigative group case control study of an acute aflatoxicosis outbreak, Kenya, 2004. Environmental Health Perspectives, 113, 1779-1783.

Berthiller, F., Sulyok, M., Krska, R., Schuhmacher, R. (2007). Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. International Journal of Food Microbiology, 119, 33–37.

Binder, E.M., Tan, L.M., Chin, L.J., Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Animal Feed Science and Technology, 133, 149.

Gabal, M.A., Azzam, A.H. (1998). Interaction of aflatoxin in the feed and immunization against selected infectious diseases in poultry. II. Effect on one-day-old layer chicks simultaneously vaccinated against Newcastle disease, infectious bronchitis and infectious bursal disease. Avian Pathology, 27, 290-295.

Hussain, Z., Khan, M.Z., Khan, A., Javed, I., Saleemi, M.K., Mahmood, S., Asi, M.R. (2010). Residues of aflatoxin B1 in broiler meat: Effect of age and dietary aflatoxin B1 levels. Food and Chemical Toxicology, 48, 3304-3307.

Jakšić, S., Abramović, B., Jajić, I., Živkov Baloš, M., Mihaljev, Ž., Despotović, V., Šojić, D. (2012). Co-occurrence of fumonisins and deoxynivalenol in wheat and maize harvested in Serbia. Bulletin of Environmental Contamination and Toxicology, 89, 615-619.

Janković, V., Škrinjar, M., Vukojević, J., Karan, D., Radmili, M. (2006). Mikotoksini potencijalna opasnost za žive organizme. Tehnologija mesa, 47, 97-103.

Kapetanov, M., Orlić, D., Potkonjak, D. (2011). Najčešće nutriopatije u živinarskoj proizvodnji. Arhiv veterinarske medicine, 4, 45-53.

Kapetanov, M., Potkonjak, D., Milanov, D., Stojanov, I., Živkov Baloš, M., Prunić, B. (2011a). Investigation of dissemination of aspergillosis in poultry and possible control measures. Zbornik Matice srpske za prirodne nauke, 120, 269-278.

Kos, J., Mastilović, J., Janić Hajnal, E., Sarić, B. (2013). Natural occurrence of aflatoxins in maize harvested in Serbia during 2009-2012. Food Control, 34, 31-34.

Manafi, M. (2011). Effect of feeding different levels of aflatoxin in the diets on production performance of broiler breeders. Advances in Environmental Biology, 5, 3843-3848.

Okiki, P.A., Ojeizeh, T.I., Ogbimi, A.O. (2010). Effects of feeding diet rich in mycotoxins on the health and growth performances of broiler chicken. International Journal of Poultry Science 9, 1136-1139.

Ortatatli, M., Oguz, H., Hatipoglu, F., Karaman, M. (2005). Evaluation of pathological changes in broilers metabolites in liver and skeletal muscles of chicken during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. Research in Veterinary Science, 78, 61-68.

Pandey, I., Chauhan, S.S. (2007). Studies on production performance and toxin residues in tissues and eggs of layer chickens fed on diets with various concentrations of aflatoxin B1. British Poultry Science, 48, 713-723.

Pravilnik o kvalitetu hrane za životinje (2010). Službeni Glasnik RS, 4/2010.

Quinn, P.J. (1998). Clinical veterinary microbiology, Mosby, London, UK.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P. (2012). Current situation of mycotoxin contamination and co-occurrence in animal feed—Focus on Europe. Review Toxins, 4, 788-809.

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., Paul, P., Payne, P, Takle, E. (2011). Climate change impacts on mycotoxin risks in US maize. World Mycotoxin Journal, 4, 79-93.

Yang, J., Bai, F., Zhang, K., Lv, X., Bai, S., Zhao, L., Peng, X., Ding, X., Li, Y., Zhang, J. (2012). Effects of feeding corn naturally contaminated with AFB1 and AFB2 on performance and aflatoxin residues in broilers. Czech Journal of Animal Science, 57, 506–515.

Yarru, L.P., Settivari, R.S., Antoniou, E., Ledoux, D.R., Rottinqhaus, G.E. (2009). Toxicological and gene expression analysis of the impact of aflatoxin B1 on hepatic function of male broiler chicks. Poultry Science, 88, 360-371.

Yunus, A.W., Ghareeb, K., Abd-El-Fattah, A.A.M., Twaruzek, M., Böhm, J. (2011). Gross intestinal adaptation in relation to broiler performance during chronic aflatoxin exposure. Poultry Science, 90, 1683-1689.

Zorzete, P., Baquião, A.C., Atayde, D.D., Reis, T.A., Gonçalez, E., Corrêa, B. (2013). Mycobiota, aflatoxins and cyclopiazonic acid in stored peanut cultivars. Food Research International, 52, 380-386.

THE INFLUENCE OF PRESENCE OF ZINC IN DIET ON PRODUCTION TRAITS OF GOATS

Nurgin Memiši[1]*, Jovanka Lević[2]; Nebojša Ilić[2]

[1]AD Mlekara – Subotica, Tolminska 10, 24000 Subotica, Serbia
[2]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: memisin@mlekara.rs

ABSTRACT

Animals require microelements in small quantities, and they play a role in virtually all physiological and biochemical processes. Microelements are provided to animals in food, by special supplementation (premixes), or in water.
Zinc is a little different from some of the other well-known minerals. Whilst some of these have a well-known, identifiable function familiar to us, such as calcium for bone strength and Fe for healthy red blood cells, zinc has no single clear action but instead performs a number of important functions in the body. This is because zinc is an essential component of around 200 enzymes that are involved in a range of actions within the body. Zinc is needed for a healthy immune system as it is involved with immune cell (T-cell) production in the thymus gland. Along with copper and manganese, zinc is a precursor of the antioxidant enzyme superoxide dismutase (SOD). Zinc is needed for protein synthesis and is important in wound healing and growth. It plays an important role in the repair and renewal of skin cells. A diet marginally lacking in zinc can lead to problems such as frequent infections, delayed wound healing, reduced appetite, decreased sense of taste and smell (sometimes also associated with low iron levels), poor skin condition and, sometimes, white flecks on the nails.
Supplemental zinc is usually added to animal diets in the form of zinc oxide or zinc sulfate. A level of 45 to 75 ppm zinc should be used in the total diet of goats until their zinc requirements are met. Manganese is a mineral element which is in the body of goats essential for normal skin and increase in bone growth; additionally it participates in the activation of a number of enzymes.
Keywords: *goat, minerals, zinc, production traits, health status*

INTRODUCTION

Animals need microelements in small quantities, and these microelements play an important role in virtually all physiological and biochemical processes, from bone structure to maintaining the structure of proteins and lipids. Microelements are provided to animals in food, by special supplementation (premixes) or in water. In intensive production, their addition is obligatory, since this is the only way to provide them in sufficient quantities required for optimum health and production results (Memiši *et al.* 2004).
Minerals activate enzymes and they are essential cofactors of metabolic reactions, function as carriers of proteins, regulate digestion, respiration, water balance, muscle response, the neural transmissions, influence and maintain skeletal strength, balance pH, and even mental balance, protect against disease, are antagonists or synergists of other elements and play a vital role in the resistance, adaptation and evolution of new races and lines (Lamand, 1981; Anke and Szentmihalyi, 1986; Haenlein, 1987; Kessler, 1991).
Regardless of the fact that certain microelements are present in sufficient quantities in food, subclinical or clinical signs of their deficit appear, because their availability varies, or the microelement is present in a form that can not be used. It was established that the presence of certain substances in food (phytic acid and oxalic acid), as well as interaction with other nutrients in the digestive tract influences resorption mechanisms. Resorption of microelements does not depend only on their content in food, but also on the animal's age, on electrochemical reactions in the intestine, and on the form of the microelement. Salts of minerals are most frequently used, namely oxides, carbonates, chlorides, and sulfates. Today, in addition to inorganic forms of minerals, the use of so-called "chelate" forms, i.e.

organically bonded microelements is becoming more frequent (Memisi and Bauman, 2002, 2003a, 2003b and 2007).

GOAT REQUIREMENTS IN ZINC

In feedmeals for goats belonging to various categories, care must be taken to satisfy a specific relationship between various minerals. The quantity of a certain mineral in food is not as important as its availability for the animal. This availability varies to a high degree, depending on numerous factors such as: their form in the food, the phase of development of plants, the presence of other minerals and components in the foods which bind them and make them unavailable, as well as the age and sex of the animal. In order to prevent deficit of mineral elements in goat nutrition, there are various possibilities and certain procedures and methods to prevent this, primarily: treating fodder with various preparations, addition of elements when preparing land for fodder production, use of a premixed and complete mineral mixture in nutrition, and ultimately bodily reserves of the animal – goat can also be increased by using slow release or sustained release injections or capsules.
Zinc is an element that should be administered continuously in the diet of goats, because it is not stored in the body. It is a regular component of several enzyme systems and is involved in the metabolism of proteins, carbohydrates and nucleic acids.
The need for Zn by most animals is based on its influence on enzymes and proteins and their activities, that are linked to vitamin A synthesis, carbon dioxide (CO_2) transport, collagen fiber degradation, free radical destruction, membrane stability of red blood cells, metabolism of essential fatty acids, carbohydrate metabolism, protein synthesis, metabolism of nucleic acids, among others (Powell, 2000; McCall *et al*., 2000; Stefanidou *et al*., 2006; Rubio *et al*., 2007,).
Legumes contain higher levels of zinc than grasses, but its content in them decreases with advance of maturity of the plants. Analysis of the composition of nutrients showed a large variation in the content of Zn, depending on the soil composition (Szentmihalyi et al., 1985). Red clover and other species of the family Fabaceae have contained more Zn than grass, and the contents therein are declining steadily moving away from their maturity and with clover by 23% and by rocking for 51%. The beet leaves had twice the content of the field grass (173 mg Zn to 88 mg Zn / kg DM), while in the grains it is much less frequent. Adding zinc (especially organically bound in combination with vitamin E and selenium) helps to reduce the appearance of occurrence and cure mastitis, stimulate reproduction in males, helps in wound healing, prevention of parakeratosis (sores on the skin that are most visible on the neck and around the nostrils) and hobble because of the fissure foot. In females lack Zn exerts a negative impact at all stages of the production cycle.
In research of Pavlata *et al*., (2011) long-term supplementation of different forms of zinc (Zn) in nutrition of goats (control group A, Group B (n = 9) with zinc oxide, Group C (n = 9) with zinc lactate and Group D (n = 9) with zinc chelate), had no influence on their content in hair, plasma and whole blood. At the end of the trial the Zn concentrations in plasma and whole blood were without major differences between the groups.
The plasma concentration of Zn did not increase from the initial value at the start of the trial. The average concentration of Zn in hair was 95.2–100.0 mg/kg in all groups (Table 1). Zn also plays a key role in maintaining the integrity of the epithelia of the reproductive organs, which is necessary for embryo implantation (Hostetler *et al*., 2003; Robinson *et al*., 2006), besides, adequate concentrations of Zn in the serum and in the diets, are vital for uterine involution, tissue repair, after parturition, and particularly, the return to estrus. In general, Zn affects the reproductive events in goats, directly on events as the manifestation of estrus, embryo implantation, and reduced spermatogenesis, or indirectly affecting the health of livestock. Usually the limited quantity of Zn is available to the body except from ingested in the diet. According to that, this element must be continually supplemented.

Table 1. Basic statistical characteristics of Zn concentration values (mean (–x) ± standard deviation (s), minimum (min), maximum (max)) in various biological materials after three months of the experiment in individual groups of goats supplemented with various Zn forms and control group (without Zn supplementation) (Pavlata et al., 2011)

Biological materials	Parameters	A – control n = 10	B–Zn oxide n = 9	C–Zn lactate n = 9	D–Zn chelate n = 9
Plasma (μmol/l)	x ± s	11.3 ± 1.4	10.3 ± 1.1	9.9 ± 1.6	9.1 ± 1.6
	min	8.6	7.7	8.0	6.1
	max	13.0	12.0	12.9	11.3
Whole blood (μmol/l)	x ± s	35.6 ± 6.2	34.4 ± 7.4	34.1 ± 9.1	36.3 ± 5.3
	min	27.1	27.8	26.9	27.8
	max	46.6	48.1	55.2	47.1
Hair (mg/kg)	x ± s	97.9 ± 10.1	97.9 ± 7.0	95.2 ± 5.1	100.0 ± 8.9
	min	85.5	87.0	87.7	88.0
	max	120.9	108.6	104.6	113.5

The deficit of Zn in the body causes the increase in the Cu content, especially in the brain, liver and uterus, in males and in females (Gruen *et al*. 1986). Similar relationships between the minerals are also observed in Cu deficiency, but they are less pronounced, which means that the absorption of Cu increases in the deficit of Zn, but that the converse is not true (Memisi *et al.*, 2012). Different relationships between minerals absorption were observed with the goats received bentonite which increased the absorption of Fe but has decreased absorption of Cu and Zn (Schwarz and Werner, 1987).

Absorption of zinc occurs throughout the small intestine and usually ranges from 5% to 40% of the intake. Transfer of zinc out of the intestinal mucosal cells to the plasma is regulated by metallothionein. Zinc absorption is reduced whenever diets are high in calcium or phytate.

Studies worldwide have shown that in some countries as regards the presence of certain minerals in the soil, there are different variations in terms of their deficit and surplus, which can help in directing and creating a program for resolving the problem. Such examinations of soil and the plants should be placed in connection with the specific characteristics of the metabolism of various animal species, and accepts that the analysis of animal tissue is definitely the right diagnostic measure (Memisi *et al*., 2008), although different tissues have different preferences according to macro-and micronutrients, and some of them do not have it at all (Memisi *et al*., 2005 and 2007a), and thus their value as indicators differ (Table 2).

Mineral concentrations in liver are the best indicator of the endogenous mineral status of the animal (Humann-Ziehank *et al*., 2008). Nonetheless, blood analysis is more frequently used, because blood samples are easily taken and is also considered a non invasive procedure (Kincaid, 2000). Trace elements deficiencies are expressed in the animal by diverse forms, since these elements form molecule complexes important for the metabolism of proteins, lipids and carbohydrates, where they play key roles as components transcription factors (Zn) (McDowell, 2003; Underwood and Suttle, 2003). Based on the before mentioned information, the mineral status of the animal has effects on every phase of the reproductive cycle (Bedwal and Bahuguna, 1994; Smith and Akinbamijo, 2000; Robinson *et al*., 2006).

Table 2. Significant differences Zn in tissue contents in goats (Anke et al., 1988).

Minerals	Tissue	Controls mg/kg DM (N=31)	Deficient mg/kg DM (N=22)
Zn	Ribs	80	58
	Hair	117	93
	Testicles	74	55

Samples of blood, milk and hair can't help determine the zinc status of an animal, where the only Zn content in the ribs is a good indicator of the supply of animals with this trace element. Since goats to a large extent browse on brush, shrub, and trees, as well as weeds not belonging to grasses and not studied to any greater extent, it is indispensable to know their chemical composition (Devendra*,* 1990). When browse from lower quality grasses is used for goat nutrition, one should keep in mind that many plants have limited value due to one or more inhibitors, that can bind, or in some other way prevent utilization of nutrients (primarily minerals) contained in them. This fact forms the basis for obligatory supplementation of minerals in the nutrition of domestic animals (goats included), which has to a large extent resulted in better milk yield, reproduction, food intake, and reduced stress due by heat and other reasons (Harris*,* 1991; Memisi *et al*., 2008a).
Some studies in sheep, have proposed that Zn requirements are less than those for bovines, suggesting the sheep require less than 8 parts per million (ppm), than that required for calves, for normal growth (Vasquez-Armijo *et al.,* 2011). Consumption in goats fed rations with 6-7 ppm, do not show clinical signs of deficiencies, under this feeding regime, clinical signs of Zn deficiency are observed during the lactation, affecting only, Zn concentration in milk by 50%, but not affecting milk production. In male goats, clinical signs of Zn deficiencies appear when they are fed rations containing 4 ppm of Zn (Vasquez-Armijo *et al*., 2011).
Based on concrete experiments on goats the formulation of minerals depends to an ever decreasing degree on experiments carried out on sheep and cattle (Kessler*,* 1991) (Table 3). A level of 45 to 75 ppm zinc should be used in the total diet of goats until their zinc requirements are met.

Table 3. The latest minimum mineral requirements of goats (Kessler,1991)

Minerals	**Requirements (mg/kg DM/day)**
Fe	30 - 40 - 100
Cu	8 - 10 - 23
Co	0.1 - 0.15
J	0.1 - 0.4 - 0.6 - 0.8
Mn	20 - 40
Zn	10 - 50
Se	100 - 200

Table 4. The recommended amounts of trace elements and threshold toxicity (mg/kg of ration DM-INRA, 1978).

Microelements	**Limit of deficiencies**	**Recommended amount**	**Toxicity limit**
Cu	7	10	20
Co	0.07	0.1 – 0.3	10
J	0.15	0.2 – 0.8	8
Mn	45	50	1000
Zn	45	50	250
Se	0.1	0.1	0.5
Mo	0.2	0.2	3
Fe	15	30	

CONCLUSIONS

High production and milk yield in goats require also more macro– and microelements, and increase needs. Their adequate balancing in feedmeals for goats is more difficult due to the specific nutrition of goats, i.e. due to the use of feeds with insufficiently studied nutritive value. Zn deficit in nutrition of offspring and adult goats can have an unfavorable effect on growth, but can also decrease yield, and may cause health problems.. Therefore, adequate

goat nutrition requires maximum balancing of all nutrients in feedmeals, which is achieved by using various feeds and mineral premixes.

AKNOWLEDGMENTS

The results from this paper are part of the Project III, No 46009: "Improvement and development of hygienic and technological procedures in production of animal originating foodstuffs with the aim of producing high-quality and safe products competitive on global market" funded by the Ministry of science of Serbia

REFERENCES

Anke, M. and SZentmihalyi, S. (1986). Principles of supply and metabolism of trace elements in ruminants. Proc. Macro- and Trace Element Seminar, University Leipzig-Jena, Germany, Dec. 1-2, 87.

Anke, M., Groppel, B., Krause, U., Angelow, L., Regius, A., Masaoka, T., Kosla, T. and Langer, M. (1988). Diagnostic possibilities of the status of zinc, manganese, copper, iodine, selenium, molybdenum, cadmium, nickel, lithium and arsenic. Proc. Macro- and Trace Element Seminar, University Leipzig-Jena, Germany, Dec. 20-21, 368.

Bedwal, R.S., Bahuguna, A. (1994). Zinc, copper and selenium in reproduction. Experientia, 50, 626-640.

Devendra, C. (ed.) 1990. Shrubs and tree fodders for farm animals. Proc. Intern. Dev. Res. Ctr. Workshop, Denpasar, Indonesia, July 24-29, 349.

Gruen, M., Anke, M. and Hennig, A. (1986). Cu-Zn interrelationships during deficiency in studies with goats. Proc. Macro- and Trace Element Seminar, University Leipzig-Jena, Germany, Dec. 1-2, 142.

Haenlein, G.F.W. (1987). Mineral and vitamin requirements and deficiencies. Proc. IVth Intern. Conf. Goats, Brasilia, Brazil, March 8-13, 1249.

Harris, B.Jr. (1991). Extra minerals may increase milk production. Lancaster Farming, 7/6:D2.

Hostetler, C.E., Kincaid, R.L., Mirando, M.A. (2003). The role of essential trace elements in embryonic and fetal development in livestock. The Veterinary Journal, 166, 125-139.

Humann-Ziehank, E., Ganter, M., Hennig-Pauka, I., Binder, A. (2008). Trace mineral status and liver and blood parameters in sheep without mineral supply compared to local roe deer (*Capreolus capreolus*) populations. Small Ruminant Research, 75, 185-191.

INRA. (1978): Alimentation des Ruminants. INRA Publications, Versailles. Itovic (1979): Elevage des jenes caprins. Speoc 127.

Kessler, J. (1991). Mineral nutrition of goats. In: P. Morand-Fehr (ed.), Goat Nutrition, Pudoc Publ., Wageningen, Netherlands, EAAP Publ. No. 46, 104.

Kincaid, R.L. (2000). Assessment of trace mineral status of ruminants: A review. Journal of Animal Science, 77, 1-10.

Lamand, M. (1981). Metabolism and requirements of microelements by goats. Proc. ITOVIC-INRA Intern. Symposium Nutrition Systems Goat Feeding, Tours, France, May 12-15, I:210.

MCcall, K.A., Huang, C., Fierke, C.A. (2000). Function and mechanism of zinc metalloenzymes. The Journal of Nutrition. 130, 1437-1446.

McDowell, L.R. (2003). Minerals in Animal and Human Nutrition, Second Edition. Elsevier Science B. V., Amsterdam, The Netherlands.

Memiši, N., Frida Bauman (2002) Goat. Agricultural library. Belgrade.

Memiši, N., Frida Bauman (2003a). Ishrana koza u periodu bremenitosti. Poljoprivredne aktuelnosti, 1-2, 75-86.

Memiši, N., Frida Bauman (2003b). Ishrana koza u laktacionom periodu. Poljoprivredne aktuelnosti, 1-2, 87-98.

Memiši, N., Frida Bauman (2007). Ishrana koza. Admiralbook. Beograd.

Memiši, N., Frida Bauman, Biserka Pavlov, (2005). Značaj selena u ishrani koza. Zbornik naučnih radova XIX Savetovanja agronoma, veterinara i tehnologa, 11, 3-4, 153-160.

Memiši, N., Frida Bauman, Biserka Pavlov, (2007a): Značaj mikroelemenata u ishrani koza na njihove proizvodne rezultate. Zbornik naučnih radova XXI Savetovanja agronoma, veterinara i tehnologa, 13, 3-4, 143-150.

Memiši, N., Frida Bauman, Mekić, C., Bogdanović, V. (2004). Značaj mineralne ishrane za proizvodnju i zdravstveno stanje koza. Biotehnologija u stočarstvu, 18 (5-6), 81-85.

Memiši, N., Skalicki, Z., Mekić, C., Bogdanović, V. (2008a). The influence of deficience copper and molybdenum in nutrition on health and production traits of goats. Biotehnology in Animal Husbandry, 24, 491-500.

Memiši, N., Tomić, Z., Žujović, M., Ivanović, S., Pavlović, I. (2012). Importance of copper and selenium in nutrition on health and production traits of goats. 6th Central European Congress on food. 23-26 May, p. 1520-1524.

Memiši, N., Žujović, M., Bogdanović, V., Tomić, Z., Petrović, M.P. (2008) The influence of presence of cadmium and arsenic in feedmealon production and reproduction traits of goats. Biotehnology in Animal Husbandry, 24, 3-4, 39-48.

Pavlata, M. CHomat, A. Pechova, L. Misurova, R. (2011). DvorakImpact of long-term supplementation of zinc and selenium on their content in blood and hair in goats. Veterinarni Medicina, 56, (2), 63–74.

Powell, S.R. (2000). The antioxidant properties of zinc. The Journal of Nutrition. 130, 1447-1454.

Robinson, J.J., Ashworth, C.J., Rooke, J.A., Mitchell, L.M., Mcevoy, T.G. (2006). Nutrition and fertility in ruminant livestock. Animal Feed Science and Technology, 126, 259-276.

Rubio, C., González, D., Martín-Izquierdo, R.E., Revert, C., Rodríguez, I., Hardisson, A. (2007). El zinc: oligoelemento esencial. Nutrición Hospitalaria, 22, 101-107.

Schwarz, T. and Werner, E. (1987). The effect of long term bentonite application upon the metabolism of selected trace elements in dwarf goats. Proc. Macro- and Trace Element Seminar, University Leipzig-Jena, Germany, Dec. 21-22, 99.

Smith, O.B., Akinbamijo, O.O. (2000). Micronutrients and reproduction in farm animals. Animal Reproduction Science, 60-61, 549-560.

Stefanidou, M., Maravelias, C., Dona, A., Spiliopoulou, C. (2006). Zinc: a multipurpose trace element. Archives of Toxicology, 80, 1-9.

Szentmihalyi, S., Siegert, E., Hennig, A., Anke, M. and Groppel, B. (1985): Zinc contents of flora in relation to age, geology of soil and plant species. Proc. Macro- and Trace Element Seminar, University Leipzig-Jena, Germany, Dec. 2-3, 466.

Underwood, E.J., Suttle, N.F. (2003). Los minerales en la nutrición del ganado, Tercera Edición. Editorial Acribia, Zaragoza, España.

Vázquez-Armijo1, J. F., Rojo1, R., A.Z.M. Salem, A.Z.M., López, D., Tinoco, J.L., González, A., Pescador, N., Domínguez-Vara, I.A. (2011). Trace elements in sheep and goats reproduction: A review. Tropical and Subtropical Agroecosystems, 14, 1-13.

INFLUENCE OF NUTRITION ON GOAT MILK PRODUCTION TRAITS

Nurgin Memiši[1]*, Slavica Moračanin[2], Nebojša Ilić[3], Tibor Könyves[4]

[1]AD Mlekara – Subotica, Tolminska 10, 24000 Subotica, Serbia
[2]Institute of Meat Hygiene and Technology, Kačanskog 13, 11000 Belgrade, Serbia
[3]University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[4] Megatrend University, Faculty of Biofarming Backa Topola, Marsala Tita 39, 24300 Backa Topola, Serbia

*Corresponding author:
E-mail address: memisin@mlekara.rs

ABSTRACT

This paper describes the effects of nutrition on milk traits and milk fat content in goat herds. Herds of goats from four private breeders, were graying during the growing season at different locations and altitudes for investigation of the effect of grazing on pastures with different botanical composition on milk production. The study was performed on the Balkan goat herds (four herds), from private farms, in the two-year period, with a total of 569 animals.
Winter feeding of goats has been consisted of concentrate and meadow hay. Bulky food was given in small quantities (about 300 grams) three times a day and concentrate in the morning and evening. All goat breeders have applied a semi-intensive system of rearing (sheds-pasture system). Period of summer-autumn nutrition of examined herds, has been based on grazing, with no supplemental feed, even during goats mating period.
Grazing of examined herds (1, 2 and 3) was based on the use of pastures and herbaceous vegetation growing in the area of thermophilic oak forest (500-800 m above sea level), while the fourth herd was grazed at higher altitudes (1200-1850 m), i.e. in the height range of beech forest and above it, where the influence of a community of Nardus stricta is dominant.
Based on the conducted research on the effects of nutrition on milk production and milk fat content in the population of domestic Balkan goat, the statistically significant effect ($P<0.01$) was registered in all studied traits of milk production (lactation length, amount of milk, daily milk production), while the difference in milk fat content was significant at $P<0.05$. Better nutrition of goats during high pregnancy and in the first period of lactation, including both concentrate and bulky food derived from the sown meadows, had a positive effect on milk production in the fourth herd.
Keywords: *goat, nutrition, milk production, milk fat, herd*

INTRODUCTION

In Serbia goats are in most cases grown extensively, although there is a trend towards intensification of production. Mostly they are grown by poor households in passive areas or those that do not have food for cows and goats using modest requests that come to the value of food for their needs. The interest of individual farm for raising goats is increasing (Žujović *et al.,* 2011). During the previous breeding goats in our country it has not been applied any selection work. Goats are generally consistently been left to the selection and seldom to discretion of growers (in the case of growing a small number of goats). Hence the production of milk in the domestic goat genotypes differ depending on the level of services, nutrition and care (Memiši and Žujović, 2010; Memiši and Tomic, 2010; Memiši *et al*., 2009). Production of our goats is directed toward milk-meat production, where the milk usually has priority. This is especially true for those farms where milk goat production is more acceptable then breeding cows or sheep, although goats milk (as opposed to other types of milk), has not been yet under the protection of certain legal incentive measures.
Important regions where Balkan goat today is raised are primarily mountains and mountainous areas, where the goats live on a meager crop of vegetation and debris from the household. There is usually raised a small number of goats, that is exactly as much necessitates the need for milk and meat goats for household nutrition. Where is sheep reared in large herds, often there are goats to be milked and their milk processed together

with the sheep for the development of local cheeses. The main objective of this research was to investigate the effect of nutrition of goats before and during the lactating period on milk composition, as well as the basic parameters of milk traits. The reason for this is in the examination of the influence of grazing during the growing period at different altitudes and pastures with different botanical composition on milk production.

MATERIAL AND METHODS

Investigations were carried out in the villages in the municipality of Prizren in Balkan goats' herds of private farmers during the two year period (2008-2009). As a material for research served 4 herds of Balkan goats accommodated at different altitudes and different locations. After the election of the herd, it was attempted that numerous representation goats covered are controlled by lactation affiliation similar in each herd. So, that all animals in controlled herds on average were between 2 and 3 of lactation.
During both years of investigation meals for feeding goats in the winter were in all herds nearly identical. Winter meal was used in the period from October to the beginning of the month of April, while the herd during the vegetation period (May-October) was graying at locations near the farm on which they are reared.

Goat nutrition during the winter period

The amount of food in the winter was controlled in certain intervals (for two weeks) and thereby it have been registered any changes in the quantity of added food in the rations, whereby thus came to the determination of the average meal for test herds for the entire winter season diet. The chemical composition of the diet (concentrate and hay) and the amount of administered food in the winter with herds of goats is shown in Table. 1. Calculation of nutritive value is made on the basis of recommendations by Obračević (1990).

Table. 1. Nutrition of goats per head during the winter period, kg

Feed	Herd			
	S_1	S_2	S_3	S_4
Meadow hay (medium)	0.900	1.000	1.000	-
Sown meadow (a mixture of perennial ryegrass + cocksfoot)	-	-	-	1.200
Corn	0.350	0.450	0.350	0.300
Wheat bran	0.100	0.150	0.150	0.150
Wheat	-	-	-	0.200
Dry sugar beet pulp	0.150	-	-	-
Salt, g	7.0	7.0	7.0	7.0
Nutritive Parameters				
DM consumed in kg/day	1.28	1.37	1.28	1.58
Oat Nutrient Units / kg	1.14	1.17	1.10	1.23
Digestible crude protein / g	79.6	91.1	83.3	108.4
Ca, g	6.46	6.27	6.25	5.30
P, g	4.27	5.13	4.83	5.94

Hay that goats of the first three herds received as a meal was from the nearby village meadows. It was not meliorated except where it is in the fall thrown manure (every second or third year). In the the yield of grass or hay about two thirds were blade grasses (mostly *Agrostis vulgaria, Festuca rubra, Alopecurus partenisis, Dactylis glomerata, Bromus moulgaria, Bromus mollis* et al.). Leguminosae were very little represented (2% and usually *Trifolium alestre, Trifolium montanum, Trifolium repens, Lotus corniculatus* et al.). A high proportion (about one third) of grass that the cattle have used was of poor quality.

The chemical composition of the concentrates and hay in the diet of goats is such that, on the one hand, does not fully meet the needs of all herds goats in nutrients (especially proteins), and on the other hand, indicates a disparity in diet between herds. Bulky food was given in small quantities (about 300 grams) three times a day and concentrate in the morning and evening. As for the schedule of administration of certain nutrients, all breeders were first giving concentrates and forages (hay), both in the morning and evening feeding. Generally speaking, intake goat herds during the winter period did not significantly differ in both diet and amount of added food, except for goats in the herd 4 (and partly in herd 2) that received a larger amount to concentrate and forage crop, and hence favorable share of nutrients (especially proteins) in a meal.
The transition from winter to the spring diet was mostly gradual and depending on the vegetation development whereby the meal partially changed. Specifically, the amount of concentrate was gradually reduced and supplemented by pasture while the hay percentage remained largely the same (depending on the year and the amount of hay being provided). Taken as a whole, at least as far as the meadow hay is concerned, feeding goats during the whole winter period was very uniform, only the portion of concentrate feed has to be reduced or increased, depending on the physiological needs of goats (during the second half of pregnancy and immediately after kidding).

Goat nutrition during the vegetation period

During the vegetation period, efforts were made to determine the difference in the quality of grass mass i.e. representation of some plant species in grass cover in different localities, as well as high altitude zones where Summer-autumn grazing goats has been performed. When it comes to pasture and meadow vegetation of the test location, part of mountain Sarplanina, then it is imperative that it is assembled by high-altitude zones based on the propagation of forest vegetation in these areas. In the zone of thermophilic forests (450-1.100 m altitude), a herbaceous vegetation types are present like *Chrysopogonetum grylli* and *Agrostidetum vulgaris*. In these areas, herbaceous vegetation is widespread.
In the zone of beech forests (1.100-1.500 m altitude), present are meadow type *Bromo-Cynosuretum* on deeper and moist soils and type *Festucetum vallesiacae* on shallow and rocky terrain. This community is exclusively pasture and has a dominant two species *Bromus arvensis L*. and *Cynosurus cristatus L*. a dominant species belong to this community in terms of food herbs with very high marks, and next to them are represented primarily grass (graminea) and *fabaceae* with something low levels of abundance but high grade of nutritional value. These are the grass *Dactylis glomerata, Festuca pratensis, Bromus inermis Anthoxantum odoratum* and others, and of *fabaceae Cioronilla varia, Lotus corniculatus, Trifolium repens, T. montanus* and others.
At altitudes above 1.500 m and up to the highest ridges of the area, pastures have been developed in which a dominant role is played by grass Nardus stricta. From pasture plant communities *Nardus strictae* most widespread is *Nardus stricta - Festuca fallax* which occupies vast areas in the lower mountain area of about 1,800 m above sea level, and associations *Nardus strictae - Festuca halleri* is widespread in the upper mountain zone from 2.000 up to 2.300 m above sea level.
Otherwise, for all breeders of goats it was applied semi-intensive system of education (sheds-pasture system), except Growers (flock 4), whose flock during the vegetation period (May-October) stayed on the mountain shed which is located at an altitude of 1.520 m where grazed goats.
Period of summer-autumn feeding herds of goats was characterized in that it was based only on the use of grazing, without any recharge concentrates are given even in the period of goats gestation. The following table presents the number of goats per flock, the altitude at which there were locations of private farmers farms, as well as the altitude zone in which the individual performed grazing herds during the growing season.

Table. 2. Altitude at which they are located private farms

Herd	Number of goats in lactation	Farm location (altitude)	Highlands grazing zone in vegetation period, m
S_1	78	640	450 – 960
S_2	93	826	560 – 1.300
S_3	214	912	600 – 1.520
S_4	193	1.271	1.200 – 1.850
Total of heads	569		

Generally speaking, grazing herds of goats (1, 2 and 3) was based on the use of pastures, as well as herbaceous vegetation grazing which has been developed in the area of thermophilic oak forest, while a flock 4 in the vegetation period grazed at high altitudes (1.200 – 1.850 m), i.e. in the height range of beech forest and above it, where is the dominant influence of the community *Nardus stricta*. Average nutritive value of pasture for all four herds during these tests has not been established.

Milk production

Control of' milk production was performed on two occasions at equal time intervals (morning around 7 am and in the evening, about 7 pm), and in intervals of 28-32 days. The animals were in A control. Measuring the amount of milked milk was carried out in a graduated cylinder; with the lowest digit of 10 ml. Control milk output covered a total of 569 animals (Table 2). Determination of fat content was performed by the method according to Gerber, in laboratory of dairy "Progres-export" in Prizren.

Statistical analysis

The statistical processing of the results pertinent to the milk production traits was performed on a personal computer, using the LSMLMW program (Harvey, 1990). The following processing model was created to estimate the milk production traits of the domestic Balkan goat, with reference to the herds:

$$\mathbf{Y_{ijkl} = \mu + S_i + eijkl}$$

where :

$\mathbf{Y_{ijkl}}$ = the phenotypic value of the particular traits included in the anlysis,

μ - an overall average value,

$\mathbf{S_i}$ - the fixed farm effect, i.e. the herd effect (i = 1,...4),

eijkl - the other undetermined effects (an accidental error).

Data processing for milk fat content was done by standard analysis. The statistical significance of the effect considered was evaluated by means of the variance analysis at the level $P<0.05$ and $P<0.01$. The variations between each mean value were also tested by applying the t-test.

RESULTS AND DISCUSSION

Data on the average mean values and the variability of milk production traits for the test population of domestic Balkan goat, are shown in Table 3. Based on the data set forth in Table 3 it is evident that the average duration of lactation was longest in goats in the third heard (242.14 days) and shortest for a goat herd in the fourth (227.33 days), the difference in the duration of lactation was statistically significant at $p<0.01$.

A higher value for the total quantity of milk was found in the third herd (183.39 kg) while with the other three it was at approximately the same level and ranged from 165.33 kg in the second to 171.43 kg in the fourth herd.

Table 3. Average values and variability of milk production depending from herds

Herds	Parameters				
	LSM	SE	$\bar{x}$	SD	CV (%)
Total milk yield, kg					
S_1	175.28a^{AB}	3.67	168.06	46.78	27.83
S_2	172.47^{bA}	3.47	165.33	35.03	21.18
S_3	184.21^{cB}	2.74	183.39	48.74	28.43
S_4	178.28^{aAB}	2.86	171.43	46.39	25.29
Average daily milk yield, kg					
S_1	0.729^{aA}	12.07	0.709	0.169	23.85
S_2	0.722^{bA}	11.40	0.700	0.107	15.31
S_3	0.736^{cA}	9.02	0.704	0.153	21.82
S_4	0.786^{dB}	9.41	0.804	0.144	17.92
Days of lact.					
S_1	239.36^{abA}	2.35	236.58	20.58	8.70
S_2	237.20^{aA}	2.22	236.34	22.91	9.69
S_3	246.46^{bA}	1.76	242.14	28.72	11.86
S_4	227.59^{cB}	1.83	227.33	27.46	12.08
% fat (n=18)					
S_1	3.74^{aA}	0.064		0.264	7.07
S_2	3.67^{aA}	0.064		0.266	7.25
S_3	3.58^{bB}	0.048		0.197	5.52
S_4	3.74^{aA}	0.057		0.240	6.42

^{a,b,c,d}*Means within the same column with different superscripts differ significantly (p<0.05)*
^{A,B,C,D,}*Means within the same column with different superscripts differ significantly (p<0.01)*

Change in milk yield during the lactating period of herds, is shown in Figure 1. From the chart from the first it could be observed that, third, first and second flock had quite monotonous stream of lactation curve at the controls, while the fourth herd has it was significantly higher during the first two control and then rapidly decreases, and in the course of the fourth and subsequent control of all herds had more or less about the same flow lactation curve to drying out point.

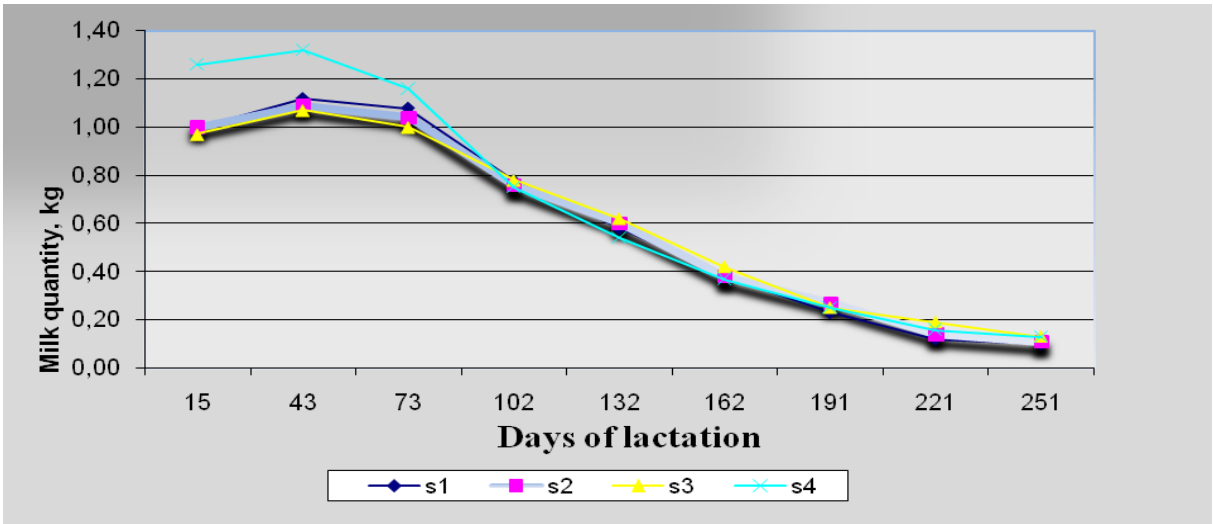

Figure 1 Changes in milk yield per days of control during lactation

The reason for higher milk production achieved in the first control periods in goats in the fourth herd should be sought in better preparation of goats during pregnancy as high and better nutrition in the first period of lactation (first three months), both of concentrated and bulky foods that are from sown meadow pastures, which has greatly affected the increase in milk production in goats in the herd.Standard deviation and coefficient of variation values in observed herds show great variation in milk production in the studied population of goats. Therefore, it has a high potential for improvement of milk productivity, but under better conditions, nutrition and care and using suitable selection measures. As in the case for the total quantity of milk, the results of analysis of variance showed a significant effect between herds ($P<0.01$) when it comes to the average daily milk yield. The lowest average daily milk yield, viewed by herds was found in second herd (0.700 kg), while a slightly higher daily milk yield than the general average of the population had a goat in the fourth herd (0.804 kg). The statistical significance of the difference of the average daily milk yield between individual herds were found between the first and second ($t_{exp} = 2.61$), while it was between the third and fourth ($t_{exp} = 46.08$) and the fourth and second herd ($t_{exp} = 55.02$), was statistically highly significant.

Mean values for fat content in milk ranged from 3.58% in the third herd, to 3.74% in the fourth and the second herd. The differences between the third and the other herds were statistically highly significant (t_{exp} of 4.83 to 9.93), while the differences between the first and second ($t_{exp} = 3.04$), and the fourth and second ($t_{exp} = 3.30$) was just significant.

Comparing the results for the length of lactation and the total quantity of milk established in this research to the values of this trait in domestic and foreign literature cited by some authors, our results are comparable with results that are obtained by Adzic and Ljumović (1981) on the domestic Balkan goat from karst area Cuce in Montenegro, by Jančić et al. (1987) at the domestic Balkan goat farm "Ponikve" near Severin na Kupi and crosses with Sanska breed goats and by Markovic (1997) on the domestic Balkan goat with two different genotypes (red and colored goats), raised on the territory of Montenegro.

The obtained values for the length of lactation and the total quantity of milk in the studied population of domestic Balkan goat grown in the area of northwest part of Sharplanina, are higher than those cited by Hatzininaogly et al. (1983) in their studies for local goat breeds that have been bred in the area of northern Greece, by Tuncel et al. (1983) for local goat breeds in Turkey and their crosses with imported Saanen goat, by Khan et al. (1983) for jamnapari goat and by Mittal (1984) for the Indian dairy type goat named parbastar and for goat race marvari as autochthonous goat breeds and population reared in Southern Italy (AIA, 2005).

There were higher values for average daily milk yield, which was in average 2.7 kg in their studies on 30 goats of color improved breed was cited by Bernacki, (2006), as well as Ataşoğlu et al. (2009), in Saanen goats (2.26 kg). The total quantity of milk determined in these tests is lower compared to the values that are established at the two populations goats that are raised in Italy ((Montefalcone and Valfortorina goat) found by Casamassima et al. (2007) which amounted to 275 kg of and 258 kg, throughout 180 days of lactation.

From these presented data on total milk production, reported by different authors above for the other races, crosses and goat populations it may be observed that data are much higher when it comes to local and primitive races of goats and their crosses with European races , grown in warmer areas in conditions of poor nutrition and care, and with the effect of a number of factors that influence the occurrence of important production traits, and lower than those established by the European noble goat breeds that have a higher milk production, as a result better nutrition and care.

CONCLUSIONS

Based on the conducted research on the effects of diet on herds being raised at different altitudes on milk production and milk fat content in the population of domestic Balkan goat, there was a statistically significant effect ($P<0.01$) on all the characteristics of milk production (lactation length, the amount of milk, daily milk yield, while milk fat content was significant at

P<0.05. The lowest average daily milk yield, observed in herds was found in second herd (0.700 kg), while a slightly higher daily milk yield than the general average of the population had a goat in the fourth herd (0.804 kg).
The higher milk production in the initial period of lactation in goats in the fourth herd is the result of better prepared goats during high pregnancy and better nutrition in the first period of lactation (first three months), both of concentrated and forage feeds used in ration which was derived from sown meadows, which largely influenced the increased milk production in the herd.

AKNOWLEDGMENTS

The results from this paper are part of the Project III, No 46009: "Improvement and development of hygienic and technological procedures in production of animal originating foodstuffs with the aim of producing high-quality and safe products competitive on global market" funded by the Ministry of science of Serbia.

REFERENCES

AIA – Associazione Italiana Allevatori (2005). Controlli della produttività del latte in Italia 2004.Statistiche ufficiali, ATEL Roma

Ataşoğlu, C., Uysal-Pala, Ç., and Karagül-Yüceer, Y. (2009). Changes in milk fatty acid composition of goats during lactation in a semi-intensive production system. Archiv Tierzucht 52, 6, 627-636.

Adžić, N., Ljumović, M., (1981): Morphological and productional characteristics of autohtonic goat from the rocky soil in Montenegro. 32. Annual Meeting EAAP, Zagreb

Bernacka,H. (2006). Cytological quality of goat milk on the basis of the somatic cell count Journal of Central European Agriculture, 7, 4, 773-778.

Casamassima, D., Palazzo, M., Pizzo, R. (2007). Evaluation of milk production and some blood parameters in lactating autochthonous goat extensively reared in Molise region. Ital.J.Anim.Sci. . 6, 1, 615-617.

Gall, C. (1980). Relationships between body conformation and production in dairy goats. Journal Dairy Science 63, 10, 1768-1781.

Harvey, W.R. (1990). Mixed Model Least Squares and Maximum Lidelihood Computer Program. User`s Guide for LSMLMW and MIXMDL.

Hatziminaoglu, J., Zervas, N., Boyazoglu, J., Loridas, L. (1983): First approach of the evaluation of local goat breeds in Northern Greece. 34th Annual Meeting European Associationfor Animal Production, Madrid.

Jančić, S., Miletić Silvija, Antunac, N. (1987). Proizvodnja kozjeg mleka – varijacije količina i sastava u toku laktacije. Zbornik sa IX Savetovanja na temu »Ovčije i kozije mljekarstvo«, Vlašić, Serbia, 77-79,

Kompan, D., Brežnik, S., Birtič, D., Drobnič, M. (1998). Production and composition of sheep and goat milk in Slovenia. 6 th International Symposium «Animal Science Days», Portorož, 16 -18 September, Slovenia.

Khan, B.U., Sahni, K.L. (1983): Non – genetic sources of variation on milk – production traits in Jamnapari goats under semiarid farm conditions. Indian Journal of Animal Sciences, 53 (7) 776-778.

Margetin, M., Milerski, M. (2000). The effect of nongenetic factors on milk yield and composition in goats of white short-haired breed. Czech Journal of Animal Science, 45, 501-509.

Marković, Božidarka. (1997): Proizvodne i reproduktivne osobine važnijih varijeteta domaće balkanske koze u Crnoj Gori. Magistarski rad, Beograd

Memiši, N., Jovanka Lević, Đorđević, N. (2009). New recommendations and contemporary achievements in preparing feeds for goats feeding. XIII International Feed Technology Symposium.October 2009, Novi Sad, Serbia,153-163.

Memiši, N., Tomić, Z. (2010). Normative of protein for goat in intensive production. XIV International Feed Technology Symposium.October 2010, Novi Sad, Serbia, 367-373.

Memiši, N., Žujović, M. (2010). Specific nutrition of goats in production cycle on the basis of body condition scoring. XIV International Feed Technology Symposium.October 2010, Novi Sad, Serbia, 360-366.

Mittal, J.P. (1984). Goat production under arid conditions. Indian farming 34 (3) 33-34,

Obračević, Č. (1990). Tablice hranljivih vrednosti stočnih hraniva i normativi u ishrani preživara. Naučna knjiga, Beograd

Pavlicek J., Antunovic, Z., Sencic, DJ., Marcela Speranda (2006). Proizvodnja i hemijski sastav kozijeg mlijeka u zovisnosti od redoslijda i stadiju laktacije. Poljoprivreda, 12, 2, 52-57.

Tuncel, E., Bayindir, S. (1983). Genetic improvement of goats in Turkey (Meat, Milk, Hair and Skin). Internacional Symposium on production of Sheep and goat in mediterranean area, 17-21 Oct., 1983, Ankara, Turkey: 33-45.

Zeng, S.S., Escobar, E.N., Popham, T. (1997). Daily variations in somatic cell count, composition, and production of Alpine goat milk. Small Ruminant Research, 26, 253-260.

Žujović, M., Memiši, N., Ivanović, S. (2011). Present status, possibilities and perspective of development of goat production in republic of Serbia. 3rd International congress- New perspectives and challenges of sustainable livestock production. Biotehnology in Animal Husbandry, 27, 3, 431-443.

PCR TECHNIQUE FOR DETECTION OF MEAT AND BONE MEAL IN FEED

Ksenija Nesic

Institute of Veterinary Medicine of Serbia, Autoput 3, 11070 Belgrade, Serbia

* Corresponding author:
E-mail address: ksenija_n@yahoo.com

ABSTRACT

Since bovine spongiform encephalopathy (BSE) was proven to be a "feed-borne" disease, the ban of processed animal proteins (PAP) in feed was introduced for farmed animals. This measure led to a significant reduction of the number of new cases. Although optical microscopy has been the only reference method for detection of PAP for years, the EU legislation also foresees that other techniques may be applied in addition to the microscopy, if they provide appropriate information about the origin of the animal constituents present in feed. That would lead to an easing of rigorous prohibitions, which was set to become a reality in the European Union from the 1st of June 2013, when meat and bone meal (MBM) was reintroduced in fish feed. Further relaxation is yet to come for all EU members, as well as for Serbia in the process of harmonization and accession.
This paper presents the use of polymerase chain reaction (PCR) for detection of fish meal and bovine, poultry, pig and mixed meat and bone meal in cattle feed. It was also combined with classical light microscopy in order to create more accurate analytical system. The obtained results show certain inconsistency, which is also the proof that feed is specific matrix which often requires a special approach and specific methodology different from that used for human food. Therefore, development of parallel techniques fit for feed control is necessary and from Serbian analysts requires narrower and more intensive cooperation with the European Reference Laboratory for Animal Proteins engaged in research and validation of new methods.
***Keywords**: animal proteins, feed, microscopy, PCR*

INTRODUCTION

Early after the outbreak of bovine spongiform encephalopathy (BSE) it was concluded that disease was "feed borne". Within the European Union a ban of processed animal proteins (PAP), including meat and bone meal (MBM), as feed ingredient for food-producing animals, was introduced and paralleled by extensive official control activities (European Commission, 2001; 2002; 2003). For years classical light microscopy was the only official method for the detection of PAP in compound feed in the EU, but the method offered very limited species differentiation properties (Van Raamsdonk et al., 2007).
Rejection of PAP for a number of years, which are excellent sources of essential amino acids and valuable proteins, and which, on the other hand, require special measures of safe disposal, increases the need for their re-use in feed, but in a safe and controlled manner. This includes a prohibition on use of proteins derived from the same animal species, so-called "intra-species recycling". By loosening the feed ban with the reintroduction of MBM into the fish feed, since 1st of June 2013 a feed control system, able to achieve sensitivity to detect PAP at the level of 0.1% (EFSA, 2011) and also to identify species from which the PAP originated, has become necessary. New EU Regulation (European Commission, 2013), however, states that PCR (Polymerase Chain Reaction) can be used as an alternative method to gain more information about the origin of the PAP.
Although a real-time PCR test for the detection of ruminant DNA in aquatic feed was validated by European reference laboratory for animal proteins in feedingstuffs, there is a large number of research results which suggests combining PCR with other methods, to achieve higher sensitivity in determining species, primarily with classical and near-infrared microscopy (Toyoda et al., 2004; Fumiere et al., 2010; Huby-Chilton et al., 2010). Therefore, the aim of this study was to investigate the possibility of combining classical microscopy and conventional PCR, as for some laboratories more affordable then real-time PCR and to

compare PCR results obtained from the samples treated at different temperatures (MBM, raw and cooked meat).

MATERIAL AND METHODS

The comparison and the combining of the classical light microscopy and conventional PCR were done on the samples of commercial concentrate for dairy cows with 18% of crude protein. Under laboratory conditions, this compound feed was spiked in various concentrations: 0.1%, 0.5%, 1%, 2%, 5% and 10% with bovine, pig, poultry and mixed MBM and fish meal, which originated from EU and were produced by sterilization process with steam pressure at 133 ^{0}C, 3 bar and 20 min. In this way 6 samples of different concentrations for each of 5 types of MBM were obtained. Appropriate negative and positive controls were used.

Two laboratory methods were applied to detect the constituents of animal origin in all samples: classical light microscopy (ISO 17025 accredited method according to the protocol prescribed by Commission Regulation (EU) No 51/2013 (European Commission, 2013)) and PCR method. Individual PCR protocols were applied to determine DNA presence of various species (cattle, pig, poultry and fish) in the samples of spiked feed. Extraction of DNA was performed using QIAamp DNA Mini Kit, tissue protocol. For the amplification reaction volume of 50 µl was used, which consisted of 25 µl HotStar taq Master Mix, 5 µl of the extracted DNA and 10 µl of each species specific primer: cattle (Kusama et al., 2004), poultry (Lahiff et al., 2001), fish (Nomura et al., 2006) and pig (Yoshida et al., 2009) (concentration of primers in the reaction mixture was 200 nmol/l). Electrophoresis was performed in 2.5% agar gel, during 1.5 h, at 100V in 0.5xTBE buffer, applying a loading buffer and staining with ethidium bromide concentration 1 mg/l. Visualization was done on the UV-transilluminator comparing with the positive controls and the adequate molecular marker. The same PCR protocols were also used to detect animal species (cattle, pig, poultry and fish) in different types of raw and cooked meat.

The combination of microscopy and PCR methods was also performed in order to use the sensitivity of the microscopy method and complement it with the benefits of PCR to identify animal species in feed. Samples of feed mixed with different MBM in concentration 0,1 %, after the preparation for microscopy method in tetrachlorethylene (European Commission, 2013), were used as the material for PCR protocols. Bones were concentrated, together with other mineral particles in the sediment and then, under stereo microscope at the magnification 50x, visible parts were carefully separated and took out from it and afterwards used for DNA extraction and PCR procedures described above.

Statistical analysis for qualitative methods was conducted and the efficiency (accuracy), sensitivity, specificity and limit of detection were determined (Isenberg, 2004).

RESULTS AND DISCUSSION

As shown in Table 1 the classical light microscopy met the required limit of detection of 0,1 % MBM in all types of samples. At the same time, this method achieved the maximum accuracy, with 100% of the samples correctly detected. It also achieved the highest sensitivity and specificity, because all positive and all negative samples were properly identified. Positive findings were based on bone and muscle particles and also on other tissues, such as feathers, hair and fish scales. This is consistent with literature claims (Van Raamsdonk et al., 2007). The amount of detected particles of animal origin was proportional to the level of contamination of the samples, regarding bone structures in the sediment and muscle fibers in the flotating layer. However, neither complete nor accurate quantification was possible, which is consistent with the literature that mentioned only attempts to improve classical light microscopy in this direction (Veys and Baeten, 2010). In examined samples the difference between fish meal and MBM meal derived from terrestrial animals was

successfully noted, which is in accordance with previous research (Van Raamsdonk et al., 2007).

The results obtained by PCR (Table 1) show strong variability in relation to the DNA that was detected. Protocols applied for the detection of pig and poultry DNA gave a limit of detection at the level of microscopy – 0,1 % MBM in compound feed, which was in accordance to the requirements of the EFSA risk assessment (EFSA, 2011). In these two tests also accuracy, sensitivity and specificity were 100%. However, PCR protocols which were applied on the samples spiked with bovine, fish and mixed MBM showed weaker results. Even the samples fortified with 10 % MBM gave no positive response, which was not in agreement with the original literature sources (Kusama et al., 2004; Nomura et al., 2006). Only 14.3% of samples were correctly detected for bovine, fish and mixed MBM (sensitivity). There were no false-positive results, but the percentage of false negatives was high, and the achieved accuracy (efficiency) was 25%. Such low percentage might be due to a small amount of the samples used for the extraction, as prescribed by the kit producer. It could be also a consequence of DNA fragmentation caused by temperature, pressure and duration of sterilization during MBM manufacturing. An additional aggravating circumstance, when it came to fish meal, was the sample inhomogeneity, i.e. complexity of composition which was caused by various species of fish from which fish meal was produced. This could also be the cause of insufficient sensitivity of PCR protocol applied to the samples with the addition of mixed MBM in which identified DNA originated from cattle and pigs. Inconsistency of PCR results might be as well explained by the possible existence of other PCR inhibiting factors, such as chemical substances and solvents used during the processing of animal waste (Prado et al., 2004, Martin et al., 2007; Myers et al., 2010).

Table 1. Results of compound feed analysis by classical light microscopy and PCR

Level of MBM conc. [%]	**Meat and bone meal**									
	bovine		**pig**		**poultry**		**fish**		**mixed**	
	Micros	PCR	Micros	PCR	Micros	PCR	Micros	PCR	Micros	PCR
0,0	-	-	-	-	-	-	-	-	-	-
0,1	+	-	+	+	+	+	+	-	+	-
0,5	+	-	+	+	+	+	+	-	+	-
1,0	+	-	+	+	+	+	+	-	+	-
2,0	+	-	+	+	+	+	+	-	+	-
5,0	+	-	+	+	+	+	+	-	+	-
10,0	+	-	+	+	+	+	+	-	+	-
100,0	+	+	+	+	+	+	+	+	+	+ P,B

Determined target material (+); Not determined target material (-); P-pig DNA; B-bovine DNA

The same PCR protocols applied in combination with microscopy, by isolation of particles from the sediment of the samples contaminated with 0,1 % fish meal and mixed MBM, showed a complete non-sensitivity (Table 2). This result could be explained by the mixed and unknown composition of MBM, what is consistent with the findings of Myers et al. (2010) who has seen inhomogeneity and diverse composition of MBM as a potential source of false-negative results. Fish species present in fish meal used in the experiment, and all land animal species in the composition of mixed MBM, were not known, as well as the origin of the particles which were randomly isolated from the samples solely on the basis of their size. Also the ratio of bone and muscle fragments within these feedingstuffs, which could be highly variable and dependent on the raw materials from which MBM was produced, determine the amount of material that could be isolated from the samples and prepared for PCR analysis (Myers et al., 2010). On the other hand, it is also showed in Table 2 that classical light microscopy and conventional PCR was absolutely successful combination when the subject of detection was MBM derived from one animal species (bovine, pig and poultry, in concentration 0,1 %). But this approach is still very much dependent on the size and the

possibility to physically separate enough particles of animal origin from the sediment and use them for DNA extraction.

Table 2. Results of PCR applied on the bones collected from tetrachlorethylene treated samples

Bovine	Pig	Poultry	Fish	Mixed
+	+	+	-	-

Species determined (+); Species not determined (-)

The Table 3 shows the results of different meat samples testing. All analyzed kinds of meat (beef, pork, poultry and fish), both untreated raw meat as well as cooked at 100^0C for 15 minutes, yielded positive reactions.

Tabela 3. Results of PCR testing of different types of meat

Meat	Bovine	Pig	Poultry	Fish
Raw	+	+	+	+
Cooked	+	+	+	+

Species determined (+); Species not determined (-)

Although all of the results were positive, the literature about human food, processed at lower temperatures in comparison with feed, also contains information on the impact of temperature on the results of the PCR tests (Spychaj et al., 2009). Such data confirms that the effect of thermal treatment is large regardless of the type of matrix, but especially high conditions for the production of MBM (133 ^{0}C, 3 bar, 20 min) are a limiting factor in analytics. This is also stated by other authors (Van Raamsdonk et al., 2007; Fumiere et al., 2009) who dedicated their work to the development of methods for feed control.

CONCLUSIONS

Further relaxation of feed ban is yet to come for all EU members, as well as for Serbia in the process of harmonization and accession. That is the reason for implementation of new PCR protocols for detection of DNA origine of animal particles in feed, although classical light microscopy shows appropriate sensitivity. Here presented results confirm the fact that feed is specific matrix which requires development of methods especially designed for feed control. A simple transfer of method intended for food testing is not always useful. Therefore, due to the need to comply with European regulations, but at the same time to avoid waste of time and money that we lack, dealing with such complex analytical problem actually requires closer and more intensive cooperation with the European Reference Laboratory for Animal Proteins engaged in research and validation of new methods and the establishment of the corresponding National Reference Laboratory.

ACKNOWLEDGEMENTS

This paper is part of the project III 46009 of the Ministry of Education, Science and Technological Development of the Republic of Serbia.

REFERENCES

EFSA. (2011). Panel on Biological Hazards (BIOHAZ), Scientific Opinion on the revision of the quantitative risk assessment (QRA) of the BSE risk posed by processed animal proteins (PAPs), EFSA Journal 9 (1), 1947.

European Commission (2001). Regulation (EC) No 999/2001 of the European Parlament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. *Off. J. Eur. Communities*, L147, 1-40.

European Commission (2002). Regulation (EC) No 1774/2002 of the European Parlament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. *Off. J. Eur. Communities*, L273*, 1-95.*

European Commission (2003). Regulation (EC) No 1234/2003 of 10 July 2003 amending Annexes I, IV and XI to Regulation (EC) No 999/2001 of the European Parlament and of the Council and Regulation (EC) No 1326/2001 as regards transmissible spongiform encephalopathies and animal feeding. *Off. J. Eur. Union*, L173*, 6-13.*

European Commission (2013). Regulation (EC) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed. Off. J. Eur. Union, L020, 33-43.

Fumiere, O., Marien, A., Fernandez Pierna, J. A., Baeten, V., Berben, G. (2010). Development of a real-time PCR protocol for the species origin confirmation of isolated animal particles detected by NIRM. Food Additives and Contaminants, 27 (8), 1118-1127.

Fumiere, O., Veys, P., Boix, A., von Holst, C., Baeten, V., Berben, G. (2009). Methods of detection, species identification and quantification of processed animal proteins in feedingstuffs. Biotechnology, Agronomy, Society and Environment, 13(S), 59-70.

Huby-Chilton, F., Murphy, J., Chilton, N. B., Gajadhar, A. A., Blais, B. W. (2010). Detection of prohibited animal products in livestock feeds by single-strand conformation polymorphism analysis. Journal of Food Protection, 73 (1), 119-124.

Isenberg, H. D. (2004). Clinical Microbiology Procedures Handbook. 2nd ed. ASM Press, USA.

Kusama, T., Nomura, T., Kadowaki, K. (2004). Development of primers for detection of meat and bone meal in ruminant feed and identification of the animal of origin. Journal of Food Protection, 67 (6), 1289-1292.

Lahiff, S., Glennon, M., O'Brien, L., Lyng, J., Smith, T., Maher, M., Shilton, N. (2001). Species-specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal (MBM). Molecular and Cellular Probes, 15, 27-35.

Martin, I., Garcia, T., Fajardo, V., Lopez-Calleja, I., Hernandez, P.E., Gonzalez, I., Martin, R. (2007). Species-specific PCR for the identification of ruminant species in feedstuffs. Meat Science, 75, 120-127.

Myers, M. J., Farrell, D., Deaver, C., Mason, J., Swaim, H., Yancy, H. F. (2010). Detection of rendered meat and bone meals by PCR is dependent on animal species of origin and DNA extraction method. Journal of Food Protection, 73 (6), 1090-1096.

Nomura, T., Kusama, T., Kadowaki, K. (2006). Detection of fish DNA in ruminant feed by PCR amplification. Shokuhin Eiseigaku Zasshi, 47 (5), 222-224.

Prado, M., Casqueiro, J., Iglesias, Y., Cepeda, A., Barros-Velazquez, J. (2004). Application of a polymerase chain reaction (PCR) method as a complementary tool to microscopic analysis for the detection of bones and other animal tissues in home-made animal meals. Journal of the Science of Food and Agriculture 84, 505-512.

Spychaj, A., Mozdziak, P. E., Pospiech, E. (2009). PCR methods in meat species identification as a tool for the verification of regional and traditional meat products. ACTA Scientiarum Polonorum Technologia Alimentaria 8 (2) 2009, 5-20.

Toyoda, A., Nakajo, M., Kawachi, M., Yano, H. (2004). PCR detection of bovine mitochondrial DNA derived from meat and bone meal in feed. Journal of Food Protection, 67(12), 2829-2832.

Van Raamsdonk, L.W.D., Von Holst, C., Baeten, V., Berben, G., Boix, A., de Jong, J. (2007). New developments in the detection and identification of processed animal proteins in feeds. Animal Feed Science and Technology, 133, 63-83.

Veys, P., Baeten, V. (2010). New approach for the quantification of processed animal proteins in feed using light microscopy. Food Additives and Contaminants A, 27, 926-934.

Yoshida, T., Nomura, T., Shinoda, N., Kusama, T., Kadowaki, K., Sugiura, K. (2009). Development of PCR primers for the detection of porcine DNA in feed using mtATP6 as the target sequence. Journal of the Food Hygienic Society of Japan, 50 (2), 89-92.

IMPACT OF FISH FEED FATTY ACID COMPOSITION ON OMEGA FATTY ACID PROFILE OF CARP FLESH

Dragan Palić[1]*, Dušica Čolović[1], Radmilo Čolović[1], Đuro Vukmirović[1], Ljiljana Kostadinović[1], Rade Jovanović[2], Olivera Đuragić[1]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Institute for Science Application in Agriculture, Despota Stefana 68b, 11000 Belgrade, Serbia

*Corresponding author:
E-mail address: dragan.palic@fins.uns.ac.rs

ABSTRACT

Fish is a source of proteins, vitamins and minerals, but they are also a rich source of polyunsaturated fatty acids (PUFAs). To PUFAs and in particular long-chain omega-3 fatty acids (n-3 FAs) have been given special attention due to their positive effects on human health. Another essential group of fatty acids are omega-6 acids (n-6 FAs). Besides the level of n-3 FAs, the ratio of n-6/n-3 FAs is also important for human health. The aim of this study was to establish the impact of linseed and fish oil addition to carp feed on omega fatty acids composition of carp flash. Experimental diets were based on mixture of soybean and sunflower meal (18%:18%) with addition of 6% of linseed oil (Diet 1) or fish oil (Diet 2). Fatty acids in lipids of carp feed and carp flash were analyzed by gas chromatography. Both experimental groups had significantly ($P<0.05$) higher total content of n-3 FAs than the control group. The contents of eicosapentaenoic (EPA) and docosahexaenoic (DHA) n-3 FAs were the highest in the flash of carp fed Diet 2, where EPA content was 3.43% and DHA content was 2.30% *vs.* 0.20% and 0.71% in the control group, i.e. content of both fatty acids were significantly ($P<0.05$) increased compared to the control group. Flash of carp fed experimental diets also had significantly ($P<0.05$) better n-6/n-3 ratio than the control group. Addition of both linseed and fish oil to carp feed favourably influenced omega fatty acid composition of carp flash, but addition of fish oil generated slightly better results.
Keywords: *omega fatty acids, carp feed, carp flash*

INTRODUCTION

It has been well documented that inadequate nutrition can be the cause of many health problems in humans. Fish are a source of proteins, vitamins and minerals, but they are also extremely rich source of polyunsaturated fatty acids (PUFAs) for human consumption (Kminkova *et al*., 2001). To polyunsaturated fatty acids (PUFAs) and in particular long-chain omega-3 fatty acids (n-3 FAs) have been given special attention. They are considered as essential fatty acids because they cannot be produced within the body, but must be acquired through diet (Kolanowski and Laufenberg, 2006). There are two sources of essential omega-3 fatty acids: plants and marine sources. Fish and fish oils contain eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids, whereas canola, walnut, soybean and linseed oil contain α-linolenic acid (ALA, 18:3 n-3) (Gogus and Smith, 2010). Positive effects of omega-3 fatty acids on human health have been well established in many studies (Harris and von Schacky 2004; Harris, 2008; Harris *et al*., 2009). It has been proven that EPA and DHA decrease risk of cardiovascular deceases (CVD), and that favourably affect the CVD mortality (Kris-Etherton *et al*., 2003a; Simopoulos, 2008). The mechanism of omega-3 fatty acids role in reducing CVD risk is still being studied, but research results suggest that they can have positive influence on arrhythmias, thrombosis, triglyceride and lipoprotein levels, atherosclerotic plague and inflammatory processes (Kris-Etherton *et al*., 2003b). It has also been reported that omega-3 PUFAs protect from deceases such as Alzheimer's, multiple sclerosis and cancer (Gogus and Smith, 2010). While essential PUFAs such as ALA, EPA and DHA cannot be synthesized in the human body, they can be effectively synthesized by aquatic organisms (Ljubojević *et al*., 2013). Therefore, humans can be supplied by omega-3 acids by consumption of marine and freshwater fishes (Jabeen and Chaudhry, 2011).

Another essential group of fatty acids are omega-6 acids. Linoleic acid (LA, 18:2 n-6) is the primary dietary omega-6 PUFA, which is converted to metabolically important omega-6 PUFA arachidonic acid (ARA, 20:4 n-6) (Harris *et al*., 2009). Besides the level of n-3 fatty acids, the ratio of n-6/n-3 fatty acids is also important for human health (Simopoulos, 2008).
Carps represent one of the largest groups of cultured fish. The principal type of cyprinid production in Serbia (90-95 percent) is the semi-intensive system, with common carp being the major species (FAO, 2011). The essential element of this system is that the protein requirements for the fish are provided through natural food developed in the pond. The carbohydrate part of the diet is ensured through supplementation with cereals, which have low level of essential fatty acids (EFA). According to Csengery (1996), diets deficient in EFAs enhance the synthesis of oleic acid and cause gradual decrease in the levels of n-3 PUFAs in carp flesh. This effect could be overcome by addition of raw materials, which are rich source of omega-3 fatty acids, to fish feed.
The aim of this study was to establish the impact of linseed and fish oil addition to carp feed on omega fatty acids composition of carp flash.

MATERIAL AND METHODS

Feed

The diets were formulated so that the basic carp feed contained the following ingredients: corn (28.7%), wheat (10%), corn gluten (10%), fish meal - with 70% of protein (10 %), soybean meal – with 44% of crude protein (18 %), sunflower meal – with 40% of crude protein (18%), yeast (3%), premix (1%) and lysine (1.3%). Experimental diets were prepared by addition of 6% of linseed oil (Diet 1) or 6% of fish oil (Diet 2) to the basic feed. The control diet was prepared by addition of 6% of soybean oil to the basic feed.
Basic carp feed was produced on twin-screw extruder (Mu Yang MY 90, China) with a screw diameter of 85 mm, length-to-diameter ratio of 20:1, and maximum temperature of 135 °C. Extruder was equipped with diferential diameter conditioner (DDC). Addition of oil was done on laboratory vacuum coater (model F-6-RVC, Forberg International AS, Norway).

Experimental trial

Carp feeding was done according to design reported by Csengeri *et al*. (2013). The feeding experiment was performed in 2.0 m^3 plastic tanks divided into two compartments. There were 12 individuals per compartment. Water inflow into the tanks was regulated to ensure oxygen concentration of the outflow water at least 80 % of saturation. During the experiment, water temperature and oxygen saturation were measured twice per day and water chemistry analyses were also performed twice per day. Feeding was performed by automatic belt feeders during 10 hours per day. The diets were fed to carp for 8 weeks.

Determination of fatty acid composition

Lipids for fatty acid analysis were extracted from feed and carp fillet samples by use of cold extraction process, which involves mixing with chloroform and methanol mixture (2:1). Extracts were purified according to the method of Folch (Folch *et al*., 1957). Lipid samples were trans-esterified using 14% (w/w) boron trifluoride/methanol solution (Sigma Aldrich, MO, USA). Obtained fatty acid methyl esters (FAME) samples were analyzed by gas chromatography in an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector (FID) and auto-injector for liquids. Samples were injected onto a fused silica capillary column (SP-2560, 100 m x 0.25 mm I.D., 0.20 µm; , SUPELCO, Bellefonte, NY, USA) with a split ratio 30:1. Carrier gas was helium (purity > 99.9997 vol %; flow rate = 1.5 ml/min). Applied column temperature regime was: 140 °C for 5 min - 3 °C/min up to 240 °C - hold for 10 min. Identification of fatty acid methyl ester peaks of the samples was done by comparison of their retention times with retention times of peaks from standard fatty acid methyl ester mixure (FAME Mix, Cat. no. 4-7885; SUPELCO, Bellefonte, NY). The fatty acid (FA) composition was calculated from peak areas of FA of the samples and FAME and expressed as g FA per 100 g of total FA.

Data analysis

One-way ANOVA and Tukey HSD test were applied to analyze variations of the results using the statistical data analysis software system STATISTICA (2011). The level of confidence was set at 95%.

RESULTS AND DISCUSSION

The omega fatty acids content of carp diets is presented in Table 1.

Table 1. Omega-3 and omega-6 fatty acids content in carp diets

Fatty acid	% of fatty acid in total fatty acid content		
	C	D1	D2
C18:2 n-6c (LA)	47.31±0.28	32.69±0.28	19.26±0.19
C18:3 n-6	0.11±0.03	ND	0.56±0.07
C18:3 n-3 (ALA)	5.70±0.20	25.62±0.13	2.83±0.15
C20:3 n-6	0.83±0.09	0.84±0.11	4.58±0.21
C20:4 n-6 (ARA)	ND	ND	0.31±0.08
C20:5 n-3 (EPA)	0.51±0.08	0.68±0.03	3.88±0.20
C22:6 n-3 (DHA)	1.30±0.06	1.49±0.19	7.01±0.10
Total n-3	7.51±0.34	27.79±0.35	13.72±0.45
Total n-6	48.25±0.40	33.53±0.39	24.71±0.55

Values are means ± SD of three replicates; ND – not detected; C – control diet; D1 – diet with added linseed oil; D2 – diet with added fish oil

Results showed that the experimental diets D1 and D2 had higher total content of n-3 fatty acids compared to the control diet, whereas the content of total n-6 fatty acids decreased. Regarding individual omega fatty acids, ALA content has been increased with diet D1, whereas EPA and DHA were increased with diet D2. These increases can be ascribed to presence of linseed and fish oil as they are rich in ALA and EPA/DHA respectively (Gogus and Smith, 2010).

The results of omega fatty acids content in fillets of carp fed diets D1 and D2 are presented in Table 2.

Regarding the content of omega fatty acids in carp flash, both experimental groups had significantly ($P<0.05$) higher total content of n-3 fatty acids than the control group. The n-6/n-3 ratio in both experimental groups was significantly ($P<0.05$) lower compared to the control group.

Table 2. Omega-3 and omega-6 fatty acids content in fillets of carp fed different diets

Fatty acid	% of fatty acid in total fatty acid content		
	C carp	D1 carp	D2 carp
C18:2 n-6c (LA)	20.48 ± 0.51^{a}	17.22 ± 1.04^{b}	14.42 ± 0.30^{b}
C18:3 n-6	0.51 ± 0.06^{a}	0.42 ± 0.08^{a}	0.51 ± 0.07^{a}
C18:3 n-3 (ALA)	1.87 ± 0.04^{a}	6.87 ± 0.35^{b}	2.45 ± 0.29^{b}
C20:3 n-6	0.57 ± 0.04^{a}	1.02 ± 0.29^{a}	0.70 ± 0.01^{a}
C20:4 n-6 (ARA)	0.04 ± 0.01^{a}	0.64 ± 0.03^{b}	1.21 ± 0.07^{c}
C20:5 n-3 (EPA)	0.20 ± 0.14^{a}	0.54 ± 0.01^{a}	3.43 ± 0.38^{b}
C22:6 n-3 (DHA)	0.71 ± 0.06^{a}	1.17 ± 0.01^{b}	2.30 ± 0.08^{c}
Total n-3	2.64 ± 0.03^{a}	8.42 ± 0.12^{b}	8.17 ± 0.17^{b}
Total n-6	21.60 ± 0.48^{a}	19.49 ± 0.95^{ab}	16.84 ± 0.46^{b}
n-6/n-3	8.18 ± 0.10^{a}	2.32 ± 0.15^{b}	2.06 ± 0.01^{b}

Values are means ± SD of three replicates; Values in rows with same letters are not significantly different ($P<0.05$); C carp – carp fed control diet; D1 carp – carp fed diet 1; D2 carp – carp fed diet 2;

The recommended n-6/n-3 ratio should be lower than 4 (WHO, 2003; Simopoulos, 2008). Both experimental groups of carp fulfilled this recommendation, while the contol group did not.

EPA and DHA contents were the highest in the experimental group fed Diet 2, where EPA content was 3.43% and DHA content was 2.30%, i.e. content of both fatty acids were significantly ($P<0.05$) increased compared to the control group (0.20% and 0.71% respectively). These findings are in line with the results reported by Jovanović *et al*. (2013).

CONCLUSION

Addition of linseed and fish oil to carp feed favourably influenced omega fatty acid content of carp flash. The total n-3 fatty acids content significanly increased by addition of linseed and fish oil. Also, the health beneficial EPA and DHA fatty acids in the flash of carp fed Diet 1 (with addition of linseed oil) and Diet 2 (with adition of fish oil) significantly increased compared to the control group. The n-6/n-3 ratio in both experimental groups was significanly lowered compared to the control group and was in optimal range for human health, i.e below 4. Both linseed and fish oil showed as beneficial regarding improvement of omega acid profile of carp meat, but fish oil produced slightly better results.

ACKNOWLEDGEMENTS

This paper is a result of research within the projects III 46012 and TR 31011 financed by the Ministry of Education, Science and Technological Development, Republic of Serbia.

REFERENCES

Csengeri, I. (1996). Dietary effects on fatty acid metabolism of common carp, Archives of Animal Nutrition, 49 (1), 73-92.

Csengeri, I., Čolović, D., Rónyai, I., Jovanović, R., Péter-Szűcsné, J., Sándor, Z., Gyimes, E. (2013). Feeding of common carp on floating feeds for enrichments of fish flash with essential fatty acids. Food and Feed Research, 40 (2), 59-70.

FAO (2011). National Aquaculture Sector Overview Serbia. FAO, Rome http://www.fao.org/fishery/countrysector/naso_serbia/en

Folch, J., Lee, M., & Sloane Stanley, G. H. (1957). A simple method for isolation and purification of total lipids from animal tissue. Journal of Biological Chemistry, 226, 497-509.

Gogus, U., Smith, C. (2010). n-3 omega fatty acids: a review of current knowledge. International Journal of Food and Technology, 45, 417-436.

Harris, W.S. (2008). The omega-3 index as a risk factor for coronary heart disease. American Journal of Clinical Nutrition, *87 (suppl),* 1997S–2002S.

Harris, W.S., Mozaffarian, D., Rimm, E., Kris-Etherton, P., Rudel, L.L., Appel, L.J., Engler, M.M.. Engler, M.B., Sacks, F. (2009). Omega-6 Fatty Acids and Risk for Cardiovascular Disease: A Science Advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. Circulation, *119,* 902-907.

Harris, W.S., von Schacky, C. (2004). The Omega-3 Index: a new risk factor for death from coronary heart disease? Preventive Medicine, *39,* 212-220.

Jabeen, F., Chaudhry, A. S. (2011). Chemical composition and fatty acid profile of three freshwater fish species. Food Chemistry, 125, 991-996.

Jovanović, R., Ivanov, D., Čolović, R., Vukmirović, Dj., Palić, D., Lević, J., Djuragić, O. (2013). Influence of fatty acid composition of fish feed on fatty acid composition of carp meat. Conference proceedings of 6th International conference „Water & Fish", Belgrade, Serbia, 50-55.

Kolanowski, W., Laufenberg, G. (2006). Enrichment of food products with polyunsaturated fatty acids by fish oil addition. European Food Research Technology, 222, 472-477.

Kminkova, M., Winterova, R., Kučera, J. (2001). Fatty Acids in Lipids of Carp (*Cyprinus carpio)* Tissues. Czech Journal of Food Sciences, 19 (5), 177-181.

Kris-Etherton, P.M., Harris, W.S., Appel, L.J. (2003a). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Arteriosclerosis Thrombosis Vascular Biology, 23, 20-31.

Kris-Etherton, P.M., Harris, W.S., Appel, L.J. (2003b). Omega-3 Fatty Acids and Cardiovascular Disease: New Recommendations from the American Heart Association. Arteriosclerosis Thrombosis Vascular Biology, 23, 151-152.

Ljubojevic, D., Trbovic, D., Lujic, J., Bjelic-Cabrilo, O., Kostic, D., Novakov, N., Cirkovic, M. (2013). Fatty acid comosition of fishes from inland waters. Bulgarian Journal of Agricultural Science, 19 (Supplement 1), 62-71.

Simopoulos, A. P., (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental Biology and Medicine, 233, 674-688.

STATISTICA (Data Analysis Software System) (2011). v.12.0, Stat-Soft, Inc, USA (*www. statsoft.com*)

WHO (2003). Diet, nutrition and the prevention of chronic diseases. Report of a joint WHO/FAO expert consultation. WHO technical report series 916, Geneva.

LABORATORY EVALUATION OF A BACTERIAL INOCULANT FOR ENSILING ALFALFA

Dragan Palić[1]*, Djuro Vukmirović[1], Radmilo Čolović[1], Miroslav Plavšić[2], Sanja Teodosin[1]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: dragan.palic@fins.uns.ac.rs

ABSTRACT

Effects of a bacterial inoculant on fermentation characteristics and aerobic stability of alfalfa silage were determined under laboratory conditions. For ensiling were used 2.0 dm^3 poly-propylen containers, which were opened on days 9, 18 and 55 for sampling and analysis of pH and content of dry matter, crude protein, ammonia, lactic acid and volatile fatty acids. Aerobic stability of silages was determined on day 55 of ensiling. Dry matter and crude protein were significantly ($P<0.05$) higher in inoculated than in the control samples on all sampling days. There was significantly ($P<0.05$) lower pH in inoculated silage samples on days 9 and 18. Addition of inoculant caused higher ($P<0.05$) lactic acid concentration on days 9 and 18, while concentration of acetic acid was higher ($P<0.05$) in inoculated silage on all sampling days. Inoculated silage was aerobically more stable than the control as indicated by significantly ($P<0.05$) lower CO_2 production.
Keywords: *silage, alfalfa, fermentation, aerobic stability*

INTRODUCTION

In most ruminant production systems, livestock derive 40 to 90% of their nutritional requirements from forages (Charmley, 2001). Therefore the successful storage of forages during the season of abundance for later animal consumption is a crucial matter. Forages are stored in two possible forms, either as hay or as silage (Rizk, 2004).
Ensiling is a preservation method for moist forage crops. It is based on solid-state lactic acid fermentation under anaerobic conditions whereby lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly to lactic acid. As a result the pH decreases, and the moist crop is preserved. Air is detrimental to silage because it enables plant respiration and activity of aerobic spoilage microorganisms such as yeasts and moulds (Weinberg and Ashbell, 2003*).*
The most important crops for ensiling worldwide are whole-crop maize, alfalfa and various grasses (Wilkins *et al.,* 1999). The principle of silage inoculation with LAB has been used for more than 100 years. The LAB comprises a rather large group of bacteria, which are divided into two categories: homofermentative, which under anaerobic conditions produce only lactic acid (LA) from WSC, whereas heterofermentative LAB, in addition to LA, also produce ethanol, acetic acid and carbon dioxide (FTIC, 2011). The number of LAB present on plants at harvest may be too low to ensure rapid and efficient preservation, and therefore silage inoculants have been developed to improve the nutritive value of silages and to reduce risks during ensiling (Henderson, 1993). Inoculant development till mid 1990's focused on homofermentative LAB species (*Lactobacillus plantarum, Lactococcus lactis, Pediococcus pentocaseous, Pediococcus acidilactici, Enterococcus faecium*). Heterofermentative LAB *Lactobacillus buchneri* have been subsequently introduced to improve aerobic stability of silage (Weinberg and Muck, 1996).
The advantages of use of bacterial inoculants have been demonstrated by number of authors, showing beneficial effects on the fermentation and silage quality (Wrobel and Zastawny, 2004*;* Nkosi *et al.,* 2009*;* Čabarkapa *et al*, 2010*;* Palić *et al.,* 2011). Regarding addition of inoculants to alfalfa, some authors (Rizk *et al.,* 2005*;* Filya *et al.,* 2007*;* Hashemzadeh-Cigari *et al.,* 2011) indicated that inoculation of alfalfa silages enhanced the silage fermentation, while some reported lesser effects (Lin *et al.,* 1992). Also, there are few

data about effects of inoculants with homofermentative and heterofermentative LAB on ensiling alfalfa grown under south-eastern European climate conditions. The aim of this study was to determine effects of adding a LAB silage inoculant to alfalfa cultivated in province Vojvodina (north Serbia), on fermentation characteristics and aerobic stability of silage.

MATERIAL AND METHODS

First-cut alfalfa (cultivar Banat) was harvest using a precision silage chopper to obtain a theoretical 20 mm chop length. Used silage inoculant *Bonsilage Plus* (Schaumann, Agri Austria GmbH), contains homofermentative strains of *Lactobacillus plantarum* (DSM 12836), *Pediococcus pentosaceus* (DSM 12834) and *Lactobacillus rhamnosus* (NCIMB 30121) and heterofermentative *Lactobacillus brevis* (DSM 12835) and *Lactobacillus buchneri* (DSM 12856). It was used as an inoculant by mixing 0.15 g with 0.1 dm^3 of water (to provide 2.5×10^5 CFU/gram of fresh material), and spraying over 30 kg of alfalfa. The application rate was in accordance with the level of LAB in the inoculant as determined by the manufacturer. In order to add the same amount of moisture as in the treated alfalfa, the control was treated by spraying 0.1 dm^3 of water on 30 kg alfalfa. The alfalfa (462 g/kg DM) was compacted into 2.0 dm^3 poly-propylene containers equipped with a water valves to enable gas release (Čolović *et al.,* 2010). Each container was filled with approximately 1.1 kg (wet mass) alfalfa without a headspace, and a packing density of 550 kg/m^3 was obtained. Silage treatments included control (with no additive) and experimental, with added inoculant. A total of 18 containers were filled and stored at a temperature of 24–28^{0}C.

On day 0, fresh alfalfa was collected for subsequent chemical analysis. Three silage containers were opened for each treatment on each of days 9, 18, 55 for determination of pH, dry matter (DM), crude protein (CP), ammonia (NH_3-N), lactic acid (LA) and volatile fatty acids (VFA).

The pH, DM and CP were determined following the procedures of AOAC (AOAC, 2000). The NH_3-N, LA and VFA were determined using methods described by Naumann and Bassler (1997). At day 55 silages were subjected to an aerobic stability test conducted according to the procedure of Ashbell *et al.* (1991).

Data was analysed for effects of treatment on the fermentation and aerobic stability of silages in a completely randomized design by ANOVA using GENSTAT (2000). Data was also tested for normality and homogeneous treatment variances, and significance was declared at the 5% probability level.

RESULTS AND DISCUSSION

Evolution of pH in fresh alfalfa and experimental silages is shown in Table 1.

The pH of fresh alfalfa was 6.07, which is optimal for enterobacteria (present in the natural plant microbiota) which produce short-chain volatile fatty acids (acetate, lactate and propionate). This leads to pH drop and establishment of optimal conditions for LAB that are present in higher portion in inoculated samples, while enterobacteria are inhibited by the acids they produce (Seglar, 2003). In the silage samples with added inoculant, pH was significantly lower than in the control samples on days 9 and 18. The extent of the decline in pH reflects the concentration of LAB which were responsible for the fermentation process (McDonald *et al.,* 1991). At day 55, pH increased above 7, both in control and in inoculated samples, with no significant difference between them. In the present study, the biggest drop in pH occurred on day 9 when it fell to 4.88.

Table 1. Evolution of pH in fresh alfalfa and experimental silages

Parameter/Sample	Sampling day			
	0 (Fresh alfalfa)	9	18	55
pH				
C	6.07	5.57± 0.29 [a]	5.63 ± 0.03 [a]	7.50 ± 0.50 [a]
BP		4.88 ± 0.03 [b]	5.03 ± 0.09 [b]	7.29 ± 0.52 [a]

C - without inoculant; BP - with inoculants;
[ab]Means with different superscripts in the same column are significantly different (P<0.05)

A constant drop of DM during ensiling was recorded. DM content (Table 2) was significantly higher in inoculated than in control samples. Similarly, Cai *et al.* (1999) found that inoculating silage with LAB reduced dry DM losses.
In the control sample content of CP (Table 2) was significantly lower than in inoculated samples on all sampling days. This is consistent with the results of Jatkauskas *et al.* (2010).
DM and CP content, as well as fermentation parameters of experimental silages are shown in Table 2.

Table 2. Dry matter (DM), crude protein (CP) content and fermentation parameters of fresh alfalfa and experimental silages

Parameter/Sample	Sampling day			
	0 (fresh alfalfa)	9	18	55
DM (g/kg DM)				
C	462.1	439.4 ± 6.8 [a]	427.1 ± 1.9 [a]	421.5 ± 5.2 [a]
BP		476.4 ± 0.3 [b]	459.4 ± 15.1 [b]	444.3 ± 7.3 [b]
CP (g/kg DM)				
C	88.6	75.1 ± 2.0[a]	74.5 ± 1.7[a]	69.8 ± 2.7[a]
BP		87.3 ± 3.9[b]	86.7 ± 3.2[b]	80.3 ± 5.0[b]
Lactic acid (g/kg DM)				
C	2.2	3.6 ± 0.5 [a]	8.3 ± 0.7 [a]	2.5 ± 1.5 [a]
BP		13.4 ± 0.7 [b]	11.4 ± 0.8 [b]	2.5 ± 0.2 [a]
Acetic acid (g/kg DM)				
C	3.1	10.2 ± 0.1 [a]	10.5 ± 0.6 [a]	9.5 ± 1.0 [a]
BP		12.4 ± 0.7 [b]	13.5 ± 0.9 [b]	12.2 ± 2.2 [a]
Butyric acid (g/kg DM)				
C	ND	ND	ND	ND
BP		ND	ND	ND
NH_3-N (g/kg DM)				
C	0.7	1.2 ± 0.1 [a]	1.7 ± 0.05 [a]	3.1 ± 0.46 [a]
BP		1.1 ± 0.0 [a]	1.6 ± 0.2 [a]	3.5 ± 0.38 [a]

C = without inoculant
BP = with inoculant
ND = not detected
[ab]Means with different superscripts in the same column are significantly different (P<0.05)

Lactic acid concentration was significantly higher in samples with inoculant on days 9 and 18, due to activity of homofermentative strains from inoculant (Nkosi *et al.,* 2009). However, on day 55 day lactic acid concentration was reduced and was not significantly different in inoculated and control samples. Similarly, acetic acid concentration (Table 2) was significantly higher in inoculated samples at days 9 and 18, but contrary to lactic acid, acetic acid remained at high level at day 55, as a result of action of heterofermentative LAB, which

is in line with findings of Danner *et al.* (2003). It has been reported that inoculation of silage with a heterofermentative LAB resulted in a decrease in lactic acid, while increasing acetic acid concentration *(*Ranjit *et al.,* 2002*)*, which corresponds with the results obtained in our study. Butyric acid was also determined and was not detected in any silage sample, which indicated that Clostridial secondary fermentation did not take place *(*FTIC, 2011*)*.
Ammonia nitrogen (NH_3-N) increased through the experiment, with no significant differences between inoculated and control samples. Higher values of pH on day 55 (Table 1) were probably the consequence of higher content of ammonia. Higher ammonia indicates protein brake down from proteolytic enzymatic activity contained within the crop (Seglar, 2003), which corresponds with the drop of CP content at day 55, as presented in Table 2.
Results of aerobic stability test on day 55 showed positive effect of added inoculant on aerobic stability of silage, expressed by released CO_2 upon exposure to air. Amount of released CO_2 in inoculated samples was 13.9 g CO_2/kg DM, being more than three times lower then in the control samples (47.4 g CO_2/kg DM). According to Ranjit *et al.* (2002*)*, microbial inoculants that contain strains of *L. buchneri* are designed to improve the aerobic stability of silages by producing higher concentrations of acetic acid.

CONCLUSIONS

Inoculant with homofermentative and heterofermentative LAB used in this investigation had a positive effect on alfalfa silage characteristics. During the 55-day study, dry matter and crude protein were significantly higher in alfalfa silages with addition of inoculant than in the control samples. Addition of inoculant caused significantly higher lactic acid concentration on days 9 and 18, while concentration of acetic acid was significantly higher in inoculated silage on all sampling days. The aerobic stability of silage, as measured by the release of CO_2 after exposure to air, was significantly improved by addition of inoculant, which corresponds to the significant increase of acetic acid concentration in inoculated silage.

ACKNOWLEDGEMENTS

This study has been supported by the Ministry of Education, Science and Technological Development of Republic of Serbia through the project III46012.

REFERENCES

AOAC (2000). Official Methods of Analysis of AOAC International, 17th edition (Ed. Horwitz, W.), AOAC International, Gaithersburg, USA.

Ashbell, G., Weinberg, Z.G., Azriel, A., Hen, Y., Horev, B. (1991). A simple system to study the aerobic deterioration of silages. Canadian Agricultural Engineering, 33**,** 391–393.

Charmley, E. (2001). Towards improved silage quality - a review. Canadian Journal of Animal Science, 81 (2), 157-168.

Cai, Y., Benno, Y., Ogawa, M., Kumai, S. (1999). Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. Journal of Dairy Science, 82, 520-526

Čabarkapa, I., Palić, D. Plavšić, D., Vukmirović, Dj., Čolović, R. (2010). The influence of a bacterial inoculant on reduction of aerobic microflora during ensiling of alfalfa. Food and Feed Research, 37 (1), 23-26.

Čolović, R., Vukmirović, Dj., Palić, D., Plavšić, M., Glamočić, D., Jajić, I., Jeremić, D. (2010). A simple system for studding the fermentation dynamics of silages. Journal on Processing and Energy in Agriculture, 14, 157-159.

Danner, H., Holzer, M., Mayrhuber, E., Braun, R. (2003). Acetic acid increases stability of silage under aerobic conditions. Applied and Environmental Microbiology, 69 (1), 562–567

Filya, I., Muck, R. E., Contreras-Govea, F. E. (2007). Inoculant Effects on Alfalfa Silage: Fermentation Products and Nutritive Value. Journal of Dairy Science, 90, 5108–5114.

FTIC (2011). Technology of Forage and By-products Preservation. 16. The Microbiology and chemistry of silage. http://ftic.co.il/Forage%20Manual%20Microbiology-en.php

GENSTAT FOR WINDOWS® (2000). Release 4.2, 5th edition. VSN International Ltd., Oxford, UK.

Hashemzadeh-Cigari, F., Khorvash, M., Ghorbani, G. R., Taghizadeh, A. (2011). The effects of wilting, molasses and inoculants on the fermentation quality and nutritive value of lucerne silage. South African Journal of Animal Science, 41 (4), 377-388.

Henderson, N. (1993). Silage additives. Animal Feed Science and Technology, 45, 35–56

Jatkauskas, J., Vrotniakiene, V., Aragon, Y.A. (2010). Fermentation quality and nutritive value of grass-legume silage treated with inoculant BioStabil Plus. Proceedings of 14th International Symposium FORAGE CONSERVATION, Brno, Czech Republic, 45-47

Lin, C., Bolsen, K.K., Bradford, J.E., Brent, B.E., Feyerherm, A.M., Aimutis, W.R. (1992). Effects of Biomate® inoculant and dextrose on the fermentation of alfalfa silages. Conference: Cattlemen's Day, Kansas State University, Manhattan, KS, 120-125.

McDonald, P., Henderson, A.R., Heron, S.J.E. (1991). The biochemistry of silage, 2nd edition. Chalcombe Publications, Bucks, Great Britain.

Naumann K., Bassler R. (1997). Methodenbuch III. Die chemische Untersuchung von Futtermitteln, 4. Ergänzung, VDLUFA-Verlag, Darmstadt, Germany.

Nkosi, B.D., Meeske, R., Palic, D., Langa, T. (2009). Laboratory evaluation of an inoculant for ensiling whole crop maize in South Africa. Animal Feed Science and Technology, 150,144–150.

Palić, D., Vukmirović, Dj., Čolović, R., Kokić, B., Čabarkapa, I., Ivanov, D., Okanović, Dj. (2011). The effect of a bacterial inoculant on fermentation, microbial status and aerobic stability of whole crop maize silage, Romanian Biotechnological Letters, 16 (3), 6282 – 6288.

Ranjit, N.K., Taylor, C.C., Kung, L. JR. (2002). Effect of *Lactobacillus buchneri* 40788 on the fermentation, aerobic stability and nutritive value of maize silage. Grass and Forage Science, 57, 73–81.

Rizk, C. (2004). Effects of inoculation on alfalfa silage quality and its feeding on the performance of dairy cattle, PhD thesis, Mc Gill University, Montreal, Quebec, Canada.

Rizk, C., Mustafa, A. F., Phillip, L. E. (2005). Effects of inoculation of high dry matter alfalfa silage on ensiling characteristics, ruminal nutrient degradability and dairy cow performance. Journal of the Science of Food and Agriculture, 85, 743-750.

Seglar, B. (2003). Fermentation analysis and silage quality testing. Proceedings of the Minnesota Dairy Health Conference, St. Paul, MN, 119-136.

Weinberg, Z.G., Ashbell, G. (2003). Engineering aspects of ensiling. Biochemical Engineering Journal, 13, 181– 188.

Weinberg, Z.G., Muck, R.E. (1996). New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiological Reviews, 19 (1), 53–68

Wilkins, R.J., Syrjala-qvist, L., Bolsen, K.K. (1999). The future role of silage in sustainable animal production. Proceedings of 12th International Silage Conference, Uppsala, Sweden, 23–40

Wrobel, B., Zastawny, J. (2004).The nutritive value and aerobic stability of big bale silage treated with bacterial inoculants. Proceedings of 20th General Meeting of European Grassland Federation, Luzern, Switzerland, 978-980.

TRICHINELLA SPECIES IN DOMESTIC AND SYLVATIC ANIMALS

Petrović Jelena*, Grgić Živoslav, Ivan Pušić

Scientific Veterinary Institute „Novi Sad", Rumenacki put 20, 21000 Novi Sad

*Corresponding author:
E-mail address: jelena@niv.ns.ac.rs

ABSTRACT

Trichinella sp. causes a zoonotic helmintic infection commonly referred to as trichinellosis. Trichinella has a cosmopolitan distribution, infecting people and other mammals all over the world. Infection is endemic in majority of European countries. The research on trichinellosis that has been carried out in Serbia so far, focused at minimizing the risk of trichinellosis transmission on humans and reducing economic losses in pig production. Sylvatic trichinellosis has been poorly investigated and little is known about Trichinella infection of wild animals in Serbia. The aim of the present study was molecular characterization of the *Trichinella sp.* larvae isolated from domestic, synanthrophic and sylvatic animals. Also, the goal was to reveal the specificity of *Trichinella* life cycle in Vojvodina region. Total of 592 samples (domestic and wild pigs, jackals and foxes) were examined, *Trichinella* were isolated from 38 samples (6.42%) by artificial digestion and examined by molecular methods, and single species was determined - *T. spiralis*. Our data suggests that any program for reducing the trichinellosis incidence should include measures for prevention of the transmission of trichinellosis between domestic pigs and sylvatic animals.
***Keywords**: Trichinella, domestic and sylvatic animals*

INTRODUCTION

Trichinellosis is a zoonotic helmintic infection caused by parasitic larvae of genus *Trichinella*. Infection is endemic in majority of European countries. This species is well adapted to domestic and wild pigs (Sus scrofa) which represent the most important reservoir for this parasite. Furthermore, *T. spiralis* has been detected frequently in carnivores and rodents, and less so in horses and dear. In some European countries like Austria, Belgium, Czech Republic, Denmark, Germany, Ireland, Netherlands, Norway, Slovenia, Switzerland, United Kingdom trichinellosis outbreaks in domestic animals, have not been registered in last 25 years. Other countries still struggle with significant number of human trichinelosis (Bosnia and Herzegovina, Bulgaria, Croatia, Georgia, Latvia, Lithuania, Romania, Russia, Spain and Serbia), sometimes with a yearly incidence 12 cases per 100,000 (Pozio, 2007).
Domestic pigs represent the main source of human trichinellosis in Serbia and the infection usually occurs after consumption of raw or undercooked meat. The average annual prevalence of trichinellosis in endemic regions of Serbia for the period 1995-2004 in domestic pigs was 0.42%. In the same period 432 human cases were diagnosed (Tešić et al., 2011). There are several endemic regions for trichinellosis in Serbia: Srem, and the valleys of the Danube, Drina and Kolubara (Čuperilović et al., 1989).
Serbia belongs to a group of countries where *T. spiralis* is present in domestic but also in sylvatic and synanthrophic animals.
The research on trichinellosis that has been carried out in Serbia so far, focused at minimizing the risk of transmission of trichinellosis to humans and reducing economic losses in pig production. Sylvatic trichinellosis has been poorly investigated and little is known about *Trichinella* infection of wild animals in Serbia. The aim of this study was molecular determination of *Trichinella* larvae isolated from domestic, synanthrophic and sylvatic animals. Also, the goal was to reveal the specificity of Trichinella life cycle in Vojvodina region.

MATERIALS AND METHODS

Samples were collected in hunting grounds in Vojvodina from January 2011 year to December 2013.year. Samples originating from domestic pigs were meat products collected by veterinary officials during trichinella outbreaks. Samples originating from sylvatic animals were diaphragms. Totally of 592 samples were examined: meat products from domestic pigs 102, wild pigs 407, foxes 22 and jackals 61. Samples were examined by artificial digestion according to Commission Regulation (EC) No 2075/2005. From the 38 positive samples, larvae were collected and examined, DNA was isolated by standard phenol-chloroform method of extraction with usage of proteinase K (Sambrook et al. 1989.). Determination of isolated muscle larvae was made by PCR method (Appleyard et al. 1999.), four primer sets were used, which enable differentiation of species and genotype in Trichinella genus (Zarlenga i Dame, 1992.).

RESULTS AND DISCUSSION

Table 1. Presence of Trichinella species in samples from domestic and sylvatic animals from Vojvodina region

Animal species	No. of samples	Positive	*Trichinella* species	Maximum no of larvae per g
Domestic swine *Sus scrofa*	102	13 (12.7%)	*T. spiralis*	570
Wild swine *Sus scrofa*	407	12 (2.9%)	*T. spiralis*	1100
Red fox *Vulpes vulpes*	22	1 (4.5%)	*T. spiralis*	1
Golden jackal *Canis aureus*	61	12 (19.7%)	*T. spiralis*	10.5
Total	592	38 (6.4%)	*T. spiralis*	-

Two patterns of maintaining and transmitting the *Trichinella* parasite are distinguished. One refers to the cycle in domestic animals, and the other to sylvatic cycle. Both domestic and sylvatic cycles of *Trichinella* are present in Serbia, with marked differences between Vojvodina and other regions of Serbia. It is questionable whether sylvatic cycle is independent from the cycle in domestic animals, and to which extent the game animals represent a reservoir for *Trichinella* in Vojvodina region. Two different types of pig production system are present in Serbia: hobby herds with backyard pigs which are usually slaughtered at home and large commercial farms with pigs been slaughtered in abattoirs. In some areas in Vojvodina (Kovilj, Titel, Fruška gora, Pančevo etc), backyard pigs are free ranging and contact with wild animals carcasses is highly possible.

The main indicators of *Trichinella* life cycle are: prevalence of trichinellosis in different species of wild and domestic animals, the degree of infestation and species of *Trichinella*.

In our study the samples from domestic animals originate from *Trichinella* outbreaks as suspect samples, so the prevalence is relatively high 12.74% (Table 1). Beside domestic pigs, in Serbia trichinellosis has been detected in horses, rats, wild boars, foxes, jackals, raccoons, wolves, and bears (Cvetkovic et al., 2011, Petrović 2012). In the countries where trichinellosis of domestic animals has been eradicated, such as Denmark, the prevalence of sylvatic trichinellosis is very low (0.001%) (Enmark et al., 2000). Among the wild animals, we have found highest prevalence in jackals (19.67%) (Table 1). According to Campbell (1988), regardless of the etiological agents and geographic region, the main reservoirs of sylvatic *Trichinella* are carnivorous with cannibalistic and scavenging behaviour. According to our study, jackals are the main reservoir for sylvatic trichinellosis in Vojvodina region. Even higher prevalence was found (53.8%) in jackals from Eastern Serbia (Živojinović et al.,

2013). At the early 80-ties of the last century, the jackals coming over the Carpathian Mountain and across the Danube basin first settled in eastern Serbia, and later on expanded to Danube and Sava and on the territory of Vojvodina. Currently the jackal population is large and growing in Serbia and jackals could be considered as synanthrophic animals. Jackals inhabit different terrains and can be found in the hills and lower mountains as well as on open hunting plains. In Greece jackal groups were recorded at the average distance of only 2.61 km from the closest settlement (Giannatos et al., 2005). Synanthrophic animals are species of wild animals that live near and benefit from humans like some species of rodents, pigeons (Pozio, 2007).

The degree of infestation in omnivorous and carnivorous game animals in our country is high, with average of 10.5 larvae/g, but extremely high infestation was detected in one wild boar with a 1100 larvae/g, and one domestic swine with a 570 larvae/g (Table 1). Consumption of the smoked meat product from this domestic swine was the source of severe human *Trichinella* outbreak. If the prevalence of sylvatic trichinellosis in a particular geographical area is high, then the risk of the spread of infection to domestic pigs is significant, especially at the pastures where the animals are commingling.

In Serbia *T. spiralis* and *T. britovi* have been detected in wild animals (Cvetković et al., 2011), but in Vojvodina region only *T. spiralis* has been yet detected (Table 1). Climatic conditions in Vojvodina are favourable to the life cycle of *T. spiralis*. Vojvodina is a flat land with no geographical barriers which clearly separate the sylvatic habitat from the habitat of domestic animals, as is the case in mountainous areas. The incidence of *T. spiralis* directly affects the spatial proximity of habitat in which wild and domestic animals coexist. *T. spiralis* is rarely found in wild animals that live far away from villages and farms, and the presence of *T. spiralis* suggests a human influence in the ecology of sylvatic trichinellosis. Sylvatic *Trichinella*, such as *T. britovi*, can also be found in domestic animals. However, this type of infestation presents the end of a life cycle because sylvatic *Trichinella* can be maintained only within population of wild carnivores that live in natural habitat (Pozio et al., 1998). The main predisposing factor for the occurrence of *Trichinella* in wild carnivores reflects their feeding habits. Animals with cannibalistic and scavenging behaviour in sylvatic habitat are at highest risk of acquiring sylvatic trichinellosis. However, in areas such as Vojvodina, where jackals and foxes livein close proximity to human settlements and feed on the domestic animals carcasses or discarded offals, the risk of infestation with *T. spiralis* is increased. Besides domestic pigs, wild boars are the species that is most susceptible to this type of *Trichinella*. It is believed that the life cycle of *T. spiralis* may include circulation from domestic pigs to wild boars and vice versa. An important indicator of epidemiology in wild boars is the behavioural characteristics of this species. Wild boars can tolerate the presence of humans and often graze in the cultivated areas. An important source of *Trichinella* for wild pigs is improperly disposed animal waste, in the areas with poor veterinary-sanitary control.

Difference between regions in *Trichinella* species distribution was documented in Hungary. North-eastern mountainous region with *T. britovi* in wild boars and red foxes and no trichinella infection in domestic pigs and southern flat region with *T. spiralis* in wild boars, foxes and domestic pigs. This is consistent with the presence of numerous foci of *T. spiralis* in domestic and wild animals of neighbouring countries Croatia, Serbia, Romania (Szell et al., 2012),and Vojvodina is adjacent to the southern region of Hungary.

Direct or indirect (outbreaks of trichinellosis in humans due to pork consumption), evidence on the circulation of *Trichinella* in domestic pigs unmistakably identified *Trichinella*-infected pigs among those originating from poor husbandry conditions. *Trichinella* infections in domestic pigs from commercial farms with high biosecurity standards have not been documented (Pozio 2014). The spread of trichinellosis is influenced by poor socioeconomic conditions, insufficient education of hunters and farmers, insufficient veterinary control and improper disposal of dead animals. Feeding of domestic animals with wild animal carcasses (intentional or intentional- disposed dead animals), feed on pork scarps from pigs slaughtered at the home, cannibalism due to high mortality rate, present a high risk (Pozio, 2014).

CONCLUSION

Prevalence of trichinellosis in domestic swine is high in Serbia which indicates a significant risk for human health, but it is also significant risk for sylvatic animals. Our data suggest that any program for reducing the trichinellosis incidence should include measures for prevention of the transmission of trichinellosis between domestic pigs and sylvatic animals.

ACKNOWLEDGEMENTS

The presented work is part of the research done in scientific project „TR-31084"granted by the Serbian Ministry of Education and Science.

REFERENCES

Appleyard G. D., Zarlenga D. S., Pozio E., and Gajadher A. A. (1999). Differentiation of *Trichinella* Genotypes by polymerasa Chain Reaction Using Sequence-Specific Primers. Journal of Parasitology, 85(3), 556-559.

Cambell W.C. Epidemiology I. (1983). Modes of transmission. In: Cambeill W.C. *Trichinella* and richinosis, Plenum Press, New York, 425-244.

Commision Regulation (EC) No 2075/2005. Laying down specific rules on official controls for *Trichinella* in meat. Official Journal of the European Union L 338/60.

Cuperlovic, K., Lalic, R., Ivanovska, D., Sofronic, Lj. and Djordjevic M. (1989). Epizootiology and prevalence of swine trichinellosis in an endemic locus in Yugoslavia. Acta Veterinaria 39, 235-240.

Cvetković J., Teodorović V., Gianluca M., Vasilev D., Vasilev S., Ćirović D., Sofronić-Mlosavljević Lj. (2011). First report of *Thichinella britovi* in Serbia. Acta Parasitologica, 56(2), 232-235.

Enemark H., Bjorn H., Henriksen S., Nielsen B. (2000). Screening for infection of Trichinella in red fox (Vulpes vulpes) in Denmark. Veterinary Parasitology, 88 (3-4), 229-237.

Giannatos G., Marinos Y., Maragou P., Catsadorakis G. (2005). The status of the Golden Jackal (Canis aureus L.) in Greece,Belgian Journal of Zoology, 135(2),145–149.

Petrović J., Pušić I., Apić J., Milanov D., Grgić Ž., Đorđević V., Matekalo-Šverak V. (2012). Silvatična trihineloza-uloga divljih životinja u ciklusu širenja trihienloze u Srbiji. Veterinarski glasnik, 66, 3-4, 175-83.

Pozio E. (2007). World distribution of Trichinella spp. infections in animals and humans. Veterinary Parasitology, 149, 3–21.

Pozio E. (2014). Searching for Trichinella: not all pigs are created equal. Trends in Parasitology, 30 (1), 4–11

Sambrook I., E. F. Fritsch and Moniatis T. (1989). Molecular Clonning A laboratory manual 2nd.ed. Cold Spring Harbour. New York. Apendix E.3p.

Szell Z., Marucci G., Ludovisi A., Gomez-Morales M.A., Sreter T, Pozio E. (2012). Spatial distribution of Trichinella britovi, T. spiralis and T. pseudospiralis of domestic pigs and wild boars (Sus scrofa) in Hungary. Veterinary Parasitology, 183 (3-4), 393–396.

Tesic, M., Baltic, M., Bozic, D., Stojiljkovic, L., Plavsic, B., Tajdic, N., Mirilovic, M. and Rajkovic, M. (2011). Effects of the application of trichinellosis control program in an endemic area in Serbia. Acta veterinaria 61(1), 77-87.

Zarlenga D., Dame B. (1992). The identification and characterisation of a break within the large subunit ribosomal RNA of T. spiralis; Comparison of gap sequences within the genus. Molecular Biochemical Parasitology, 51(2), 281-289.

Zivojinovic M, Sofronic-Milosavljevic L, Cvetkovic J, Pozio E, Interisano M, Plavsic B, Radojicic S, Kulisic Z. (2013).Trichinella infections in different host species of an endemic district of Serbia. Veterinary Parasitology, 194 (2-4), 136–138.

INFLUENCE OF MYCOTOXINS IN SWINE FEED ON THE HEALTH STATUS OF SWINE BREEDING CATEGORIES

Jasna Prodanov-Radulović[1]*, Radoslav Došen[1], Igor Stojanov[1], Milica Živkov-Baloš[1], Vladimir Polaček[1], Doroteja Marčić[1], Dragica Stojanović[2]

[1]Scientific Veterinary Institute "Novi Sad, Rumenacki put 20, 21000 Novi Sad, Serbia
[2]Faculty of Agriculture, Department for veterinary medicine, University of Novi Sad, Trg Dositeja Obradovica 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: jasna.prodanov@gmail.com

ABSTRACT

Mycotoxins are toxic secondary metabolites of fungi commonly found on grains, which can cause severe impacts on animal health and performance. They are often present in swine feed in amount that can have detrimental impact on reproduction and health of swine breeding categories. Reproductive failure in swine is often a difficult diagnostic problem. Problems are expressed only as alterations of the reproductive cycle, reduced feed intake, slow growth or impaired feed efficiency. Many times, when diagnosis of infectious disease or management problems is not obtained, feed quality and safety may be questioned. This paper include field observations regarding the influence of swine feed containing different mycotoxins on the health status and occurence of the reproductive failure in swine breeding categories (sows, gilts). The material for this research included four swine farms, where certain reproductive disorders and health problems in breeding animals were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: anamnestical and clinical evaluation, gross pathological examination, standard bacteriological testing for detection the precence of aerobic and anaerobic bacteria, and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography. On the basis of the obtained results, it may be concluded that the presence of mycotoxins in swine feed was directly connected to the chronic health disorders (swine dysentery) and reproductive failures in the examined breeding swine categories (vulvovaginitis, endometritis, rebreeding, infertility). The presence of mycotoxins in swine feed have influence on the reproduction and health status of pigs and in the certain conditions may significantly disturb the production process.
***Keywords**: mycotoxins, swine reproductive disorders, breeding*

INTRODUCTION

Mycotoxins are toxic secondary metabolites of fungi commonly found on grains, which can cause severe impacts on animal health and performance (Greinier *et al.*, 2013; Weaver *et al.*, 2013). Feed spoilage by fungi is not a new problem, but due to their great adaptability these microorganisms are posing a serious risk to the animal feed industry (Jakšić *et al.*, 2011; Greinier *et al.*, 2013). At the global level, it is considered that 25% of the world crop production is contaminated by mycotoxins, which may be a risk factor affecting human and animal health (Bouhet and Oswald, 2005; Weaver et al., 2013). A recent study investigated the occurrence of mycotoxins in European feed samples and concluded that 82% of the samples were contaminated with mycotoxins, indicating that mycotoxins are omnipresent (Goossens *et al.*, 2012).

Pigs are considered to be the farm animals which are the most affected by mycotoxins in general (Burel *et al.*, 2013; Wache *et al.,* 2009). Mycotoxins are often present in swine feed in amount that can have detrimental impact on reproduction and health of swine breeding categories (Hueza *et al.,* 2014; Prodanov-Radulović *et al.,* 2011). Reproductive failure in swine is often a difficult diagnostic problem. Problems are expressed only as alterations of the reproductive cycle, reduced feed intake, slow growth or impaired feed efficiency (Diekman and Green, 1992; Kanora and Maes, 2009; Osweiler *et al.,* 1990).

This paper include field observations regarding the influence of swine feed containing different mycotoxins on the health status and occurence of the reproductive failure in swine breeding categories (sows, gilts).

MATERIAL AND METHODS

The material for this research included the samples from four swine farms, where certain reproductive disorders and health problems in breeding animals (sows, gilts) were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: anamnestical and clinical evaluation, gross pathological examination, standard bacteriological testing for detection the precence of aerobic and anaerobic bacteria in the samples of reproductive organs derived from slaughtered sows and gilts, and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography.

RESULTS AND DISCUSSION

On the first examined swine farm, applying control of the anamnestic data of excluded sows, the following reproductive disorders were discovered: rebreeding (27%), infertility (20%), anestrous (10.6%) and frequently observed endometritis. The highest percent of excluison was connected with the first litter sows i. e. with the first and the second parity (43%) and in the herd the maiden gilts were introduced. The most of the rebreedings was in relation to the first and second expected oestrus. The occurence of the increased number of deadborn and mummified piglets was not detected. However, in certain number of boars the high percent litters with small piglets were evident. In some gilts and sows, clinical symptoms included bloody diarrhoea, and sporadically vaginal and rectal prolapses. By gross pathological examination of genital organs (ovaria, uterus) from excluded females in the slaugher-hause, the following lesions were discovered: 24% ovaries in the luteal phase, 16% ovaries in the follicular phase and in 2% the pathological changes (cysts and fibrosis). Also, a significant percent of endometritis (24%) was discovered (presence of liquid muddy content in the uterus with small pieces of destroyd tissue). From examined ovaries, in only 20% ovulation rate 20 and higher was establish. By bacteriological testing on tissue samples from the dams genital organs the presence of *Streptococcus dysgalactiae subsp.equisimilis, Staphylococcus haemolyticus, Escherichia coli, Streptococcus uberis* was detected. Having in mind the clinical and pathological symptoms observed, a justified suspicion on the presence of mycotoxins in feed was made. Applying laboratory testing increase of the total number of fungi in the microbiologically examinated feeds was discovered: corn ($887x10^3$ *Aspergillus*, *Rhisopus*), feed for pregnant sows ($123x10^3$ *Penicillium, Fusarium*) and feed for lactating sows ($526x10^3$ *Aspergillus, Penicillium,Mucor*). The presence of mycotoxins zearalenone and ochratoxin (ZEA 0.72 mg/kg and OCT 0,08 mg/kg) in the feed for pregnant sows was detected.

Many toxigenic strains of molds can occur in grains without the production of mycotoxins, and there is little correlation between spore counts or degree of fungal growth and presence of mycotoxins. Conversely, absence of molds does not mean that a feed is safe from mycotoxins (Kanora and Maes, 2009; Osweiler and Ensley, 2012). It is common that many mycotoxins occur simultaneously in feeds. Combinations of some mycotoxins may potentiate the action of one another, or at least exert an additive effect (Kabak *et al.,* 2006). For known mycotoxins of clinical importance, response is usually subacute or chronic and the presenting signs are often subtle and vague (Prodanov-Radulović *et al.,* 2011). Many times problems are expressed only as alterations of the reproductive cycle, reduced feed intake, slow growth or impaired feed efficiency (Osweiler and Ensley, 2012).

The main mycotoxins encountered in pig reproduction are zearalenone as a major toxin, ergot alkaloids and trichothecenes represented by T-2 (Kanora and Maes, 2009). Zearalenone affects reproduction of livestock most seriously because it possesses

estrogenic activity (Minervini and Aquila, 2008). Pigs are very susceptible to zearalenone which has a hyperestrogenic effect (Kanora and Maes, 2009). The other mycotoxins affect reproduction in livestock via indirect means such as reduced feed intake or reduced growth or by damaging other vital organs of the body (Diekman and Green, 1992; Osweiler *et al.,* 1990).

On the second examined swine farm applying clinical examination of swine, in the piglets of different age the most prominent clinical sign was vulvovaginitis. The litter size varied significantly, whilst the small litter dominated in the recently farrowed sows and gilts. In about 30% of farrowed sows the mastitis metritis agalactiae syndrome was discovered. In weaned piglets and fatteners the vulvovaginitis in the almost all female pigs was noticed. By clinical examination, the impressum was that all animals are in the heat, while the boars were uninterested for the jump (weakened libido). There were no rebreedings and deadborn piglets beacuse there were no pregnant females. By laboratory testing of several corn samples the presence of ZEA (14 – 20 mg/kg) was detected, depend from the sampling place. Applying gross pathological control in the slaughter hause in all examined 25 females the cystic degeneration of ovaries, enlarement of the uterus and frequently, the fluid mucopurulent content in uterus was discovered.

Mycotoxin ZEA has a unique nonsteroidal resorcyclic acid lactone structure. This structure resembles many characteristics of steroid hormones and allows ZEA to bind to estrogen receptors, where it acts as an agonist and partial antagonist to estradiol (Malekinejad *et al.,* 2007; Minervini and Dell Aquila, 2008). In pigs ZEA causes multiple reproductive dysfunctions in the mature, cycling gilt (constant estrus, pseudopregnancy, infertility) and if added to the diet of pregnant sows caused them to farrow smaller litters with smaller offspring (Diekman and Green, 1992; Kanora and Maes, 2009).

Clinical signs of ZEA mycotoxicosis vary with dosage and age of swine exposed. In prepubertal gilts, ZEA cause vulvovaginitis, which is characterized by tumescence and edema of the vulva and vagina and precocious mammary development. As with other estrogens, ZEA is luteotropic in swine and can induce anestrus in sows if consumed during the middle portion of the estrous cycle. Piglets born from sows receiving ZEA may have enlarged external genitalia and uteri (Osweiler and Ensley, 2012). ZEA and its metabolites, alpha and beta ZEA can cross the placenta and are present in milk of exposed sows, causing exposure of the embryo and neonate (Malekinejad *et al.,* 2007) and may contribute to estrogenic effects in piglets (perinatal hyperestrogenic syndrom). Lower conception rate, increased numbers of repeat breeders, decreased litter size, increased numbers of stillbirths are frequently observed (Osweiler and Ensley, 2012). The influence of ZEA on litter size can be explained by negative impact on fertilization, but also by embryonic and fetal death of the piglets. This is probably due to the negative impact on the luteinizing effect (Kanora and Maes, 2009). Clinical signs in neonatal gilts include swelling of the vulva and teats, edematous infiltration of the perineal region, usually accompanied by inflammation and necrosis of the teats. An increase of splayleg and trembling piglets has been reported (Osweiler and Ensley, 2012).

On the third examined farm, in sows gravidity period and farrowing time were prolonged, cases of agalactia (sudden loss of milk and lying on the udder), a small number of stillbirths and mummified piglets were noticed. The newborn piglets were described as weak, nonviable, with diarrhoea. Sporadically, the occurence of splayleg was observed. The sows were in good body condition in all production stages but in the high number (48%) the increace of body temperature after parturition was detected. Applying laboratory feed testing, simultaneous presence of several mycotoxins (ZEA, aflatoxin-AFB1, AFG1, OCT) was established. The mycotoxins were detected in feed for pregnant and lactating sows (ZEA 0.8 mg/kg; AFB1 0.008 mg/kg; AFG1 0.02 mg/kg; OCT 0.2 mg/kg), in corn (ZEA 4 mg/kg; AFB1 0.008 mg/kg; AFG1 0.002 mg/kg; OCT 0.2 mg/kg), sunflower pellets (ZEA 4 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg) and soyabean pellets (ZEA 2.0 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg).

Most fungi are able to produce several mycotoxins simultaneously and the mycotoxins produced depends on the feedstaff and crop growing conditions (Weaver et al., 2013). As it

is a common practice to use multiple grain sources in animal diets, the risk of exposure to several mycotoxins increases with diet complexity (Greinier et al., 2013). Mycotoxins mixtures i.e. the combinations of several micotoxis are likely to occur naturally and they may influence on the immunity in an additive or synergistic manner. Economic losses that occur as a consequence of interaction of several mycotoxins are still unknown because in low concentrations several mycotoxins may interact in way that is difficult to detect (Osweiler and Ensley, 2012). Combinations of several and more moderate concentrations of different mycotoxins, which individually may appear to be too low in level to be a concern, can cause cumulative toxicosis, which affect the ability of the pigs organism to fight diseases (Prodanov-Radulović *et al.,* 2011).

Pigs are highly susceptible to AF. Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Four AF are described, according to their fluorescence at chromatography : B1, B2, G1 and G2 (Jakšić *et al.,* 2011). Aflatoxins B1, G1 and the fifth metabolite M1 can be found in milk of sows that have ingested contaminated feed (Živkov-Baloš *et al.,* 2008). Experimental intoxications have shown damaged lymphocytes and macrophages in piglets, indicating loss of immune-competence due to exposure of sows to AF (Kanora and Maes, 2009).

The health problems and significant reproductive disturbances on the fourth swine farm were noticed. Applying control of the anamnestic data, lately the frequent periods when sows delivery mummified piglets, stillbirths and decreased litter size were observed. Also, the increased number of rebreeding sows, at irregular intervals was discovered. The conception rate has dramatically decreased and the problem with frequent abortions 2 months before was intensified. Beside this, just farrowed piglets are nonviable and despite the medical treatment, they live only 3-4 days after birth. Sporadically, the occurence of clinical signs of vulvovaginitis in just born piglets were evident. After laboratory testing of swine feed samples the simultaneous presence of several mycotoxins was established: ZEA (6.4 mg/kg), AF (0.0064 mg/kg) and OCT (0.032 mg/kg).

The effects of ZEA in mature gilts and sows depend on the time that the contaminated diet is feed in relation to mating, the concentration in the diet and the duration of administration (Kanora and Maes, 2009). Gilts are more sensitive than sows. ZEA ingestion by mature gilts may produce two different effects which are related to estrogenic properties of this mycotoxin. When females are not pregnant, ZEA induces a pseudopregnancy state characterized by uterine hypertrophy and corpora lutea maintenance on ovaries. Females do not cycle and cannot be breed. When fed during gestation, ZEA reduces development of the uterus, placental membranes and fetuses. These effects may induce lower embryonic survival or higher rate of immature piglets at birth, which, are less able to suckle and may die during the few days after farrowing (Etienne and Jemmali, 1982).

CONCLUSIONS

On the basis of the obtained results, it may be concluded that the presence of mycotoxins in swine feed was directly connected to the reproductive failures (vulvovaginitis, endometritis, rebreeding, infertility) and health disorders in the examined breeding swine categories. Clinical symptoms are in many cases not very pronounced. However, the presence of mycotoxins in swine feed may have influence on the reproduction and health status of breeding categories and in the certain conditions may significantly disturb the production process.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, grants TR 31071.

REFERENCES

Bouhet, S., Oswald, I.P. (2007). The intestine as a possible target for fumonisin toxicity. *Mol Nutr Food Res, 51*(8), 925-931.

Burel, C., Tanguy, M., Guerre, P., Boilletot, E., Cariolet, R., Gueguiner, M., Postollec, G., Pinton, P., Salvat, G., Oswald, I.P., Fravalo, P. (2013). Effect of Low Dose of Fumonisins on Pig Health: Immune Status, Intestinal Microbiota and Sensitivity to Salmonella. Toxins, 5, 841-864.

Diekman, M.A. and Green, M.L. (1992). Mycotoxins and reproduction in domestic livestock. *J Anim Sci* 70, 1615-1627.

Etienne, M. and Jemmali, M. (1982). Effects of Zearalenone (F2) on Estrous Activity and Reproduction in Gilts. *J Anim Sci* 55: 1-10.

Greinier, B., Applegate, T.J. (2013). Modulation of Intestinal Functions following Mycotoxin Ingestion: Meta-Analysis of Published Experiments in Animals. Toxins, 5, 396-430.

Goossens, J., Vandernbroucke, V., Pasmans, F., De Baere, S., Devreese, M., Osselaere, A., Verbrugghe, E., Haesebrouck, F., De Saeger, S., Eeckhout, M., Audenaert, K., Haesaert, G., De Backer, P., Croubels, S. (2012). Influence of Mycotoxins and Mycotoxin Adsorbing Agent on the Oral bioavailability of Commonly Used Antibiotics in Pigs. Toxins, 4, 281-295.

Hueza, I.M., Raspantini, P.C.F., Raspantini, L.E.R., Atorre, A.O., Gorniak, S.L. (2014). Zearalenone, an Estrogenic Mycotoxin, Is an Immunotoxic Compound. Toxins, 6, 1080-1095.

Jakšić, S., Stojanov, I., Živkov-Baloš, M., Mašić, Z., Mihaljev, Ž., Abramović, B. (2011). Mycoxicological test from the aspect of the HACCP system and legislation. *Proc. Nat. Sci.*,120, 95-100.

Kabak, B., Dobson, A.D., Var, I. (2006). Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed. *Food Science and Nutrition*, 46, 593-619.

Kanora, A., Maes, D. (2009). The role of mycotoxins in pig reproduction: a review. Veterinarni Medicina, 54, 12, 565-576.

Malekinejad, H., Schoevers, E.J., Daemen, J.J.M., Zijlstra, C., Colenbrander, B., Fink-Gremmels, J.,Roelen, A.J. (2007). Exposure of Oocytes to the Fusarium Toxins Zearalenone and Deoxynivalenol Causes Aneuploidy and Abnormal Embryo Development in Pigs. Biology of Reproduction, 77, 840-847.

Minervini, F. and Dell Aquila, M.E. (2008). Zearalenone and Reproductive Function in Farm Animals. Int. J. Mol. Sci., 9, 2570-2584.

Osweiler, G.D., Stahr, H.M., Beran, G.W. (1990). Relationship of mycotoxins to swine reproductive failure. J Vet Diagn Invest, 2, 73-75.

Osweiler GD and Ensley SM (2012): Mycotoxins in Grains and Feeds. In Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (Eds.), Diseases of Swine, 10th edition (pp. 938-952), Iowa: Wiley-Blackwell Publishing.

Prodanov-Radulović, J., Došen, R., Stojanov, I., Pušić, I., Živkov-Baloš, M., Ratajac, R. (2011). Interaction between the mycotoxins and causative agents of swine infective diseases. *Proc. Nat. Sci.*,120, 251-259.

Wache, Y.J., Valat, C., Postollec, G., Bougeard, S., Burel, C., Oswald, I.P., Fravalo, P. (2009). Impact of Deoxynivalenol on the Intestinal Microflora of Pigs. *Int. J. Mol. Sci.,* 10, 1-17.

Weaver, A.C., See, M.T., Hansen, J.A., Kim, Y.B., De Souza, A.L.P., Middleton, T.F., Kim, S.W. (2013). The Use of Feed Additives to Reduce the Effects of Aflatoxin and Deoxynivalenol on Pig Growth, Organ Health and Immune Stautus during Chronic Exposure. Toxins, 5, 1261-1281.

Živkov-Baloš, M., Mihaljev, Ž., Kovačević, M., Bugarski, D. (2008). Ekskrecija aflatoksina mlekom: rizik za potrošače. Arch. vet. med., 1, 64-68.

BIOACTIVE COMPOUNDS OF GARLIC, BLACK PEPPER AND HOT RED PEPPER

Nikola Puvača[1]*, Dragana Ljubojević[2], Dragomir Lukač[1], Miloš Beuković[1], Ljiljana Kostadinović[3], Sanja Teodosin[3], Vidica Stanaćev[1]

[1]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
[2]Institute of Veterinary Medicine Novi Sad, Rumenački put 20, 21000 Novi Sad, Serbia
[3]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: nikola.puvaca@stocarstvo.edu.rs

ABSTRACT

Goal of this review is to show the most important bioactive compounds in herbal plants such as garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.) and red hot pepper (*Capsicum annuum* L.) and its modes of action. Allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate) is thought to be the principal bioactive compound present in aqueous garlic extract or raw garlic homogenate. When garlic is chopped or crushed, allinase enzyme, present in garlic, is activated and acts on alliin (present in intact garlic) to produce allicin. Other important sulfur containing compounds present in garlic are allyl methyl thiosulfonate, 1-propenyl allyl thiosulfonate and γ-Lglutamyl-S-alkyl-L-cysteine. Piperine is an alkaloid responsible for the pungency of black pepper, along with chavicine (an isomer of piperine). The active compound in black pepper is piperine (1-piperoyl piperidine) which is responsible for bio enhancing effect. It has been found that piperine bioavailability enhancing property may be attributed to increased absorption, which may be due to alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine. Capsinoids is a family of compounds that are analogues of capsaicin, which is the pungent component in hot chilli peppers. Capsinoids are widely present at low levels in chilli pepper fruit, it includes capsiate, dihydrocapsiate and it has a very favourable safety profile. Capsaicin (8-methyl-N-Vanilly-6-nonenamide) is the active substance responsible for the irritating and pungent effects of various species of hot pepper. These biological responses, among all of the bioactive compounds, have been largely attributed to reduction of risk factors for cardiovascular diseases and cancer, stimulation of immune function, enhanced detoxification of foreign compound, hepatoprotection, cholesterol content reduction, antimicrobial effect, antifungal effect, antiinflammatory effect and antioxidant effect.
***Keywords**: garlic, black pepper, hot red pepper, allicin, piperine, capsaicin*

INTRODUCTION

Herbs and spices, which are important part of the human diet, have been used for thousands of years in traditional medicine and to enhance the flavour, colour and aroma of foods. In addition to boosting flavour, herbs and spices are also known for their preservative (Neilsen and Rios, 2000), antioxidative (Shobana and Naidu, 2000), and antimicrobial (Salie *et al.*, 1996) roles. Aromatic plants have also been used therapeutically to improve the health and wellbeing of animals, most were given for prophylactic purposes and to improve growth rate and feed conversion ratio efficiency. In many countries as well in Serbia consumer pressure is pushing the poultry industry to rear animals without antibiotics as growth promoters (Dibner and Richards, 2005; Castanon, 2007). Removal of antibiotics as growth promoter has led to animal performance problems, feed conversion ratio increases, and to a rise in the incidence of certain animal diseases (Wierup, 2001). The alternatives to antibiotics as growth stimulators from the group of prebiotics, probiotics, organic acids, essential oils, medicinal plants or parts of plants such as thyme, basil, oregano, pepper and plenty of others are numerous (Simon, 2005; Kostadinović and Lević. 2012; Puvača *et al.*, 2013). Garlic is one of the most traditionally used plants as a spice and herb. Garlic has been used for a variety of reasons where the most of them has been approved scientifically. Garlic preparations and extracts have been shown to exhibit: antiatherosclerotic, antimicrobial, hypolipidemic,

antithrombosis, antihypertensive, antidiabetes effects and etc. (Mansoub, 2011). There are a lot of active components in garlic like ajoene, s-allyl cysteine, diallyl sulphide and the most active one allicine (Rahmatnejad and Roshanfekr, 2009). Allicine possibly reduces LDL, triglyceride and cholesterol in serum (Alder and Holub, 1997) and tissues (Stanaćev *et al.*, 2012), and it has been used for cardiovascular diseases (Tanamai *et al.*, 2004). Garlic has been found to decrease serum and liver cholesterol levels (Qureshi *et al.*, 1983; Crespo and Steve-Garcia, 2003), inhibit bacterial growth (Griffin *et al.*, 1992), inhibit platelet growth and reduce oxidative stress. In broilers, it was reported that garlic, as a natural feed additive improves broiler growth and feed conversion ratio, and decreased mortality rate (Stanaćev *et al.*, 2010). Improvement of broilers performance and carcass traits can be achieved by supplementation of diets with garlic powder (Konjufca *et al.*, 1997; Lin *et al.*, 2000; Demir *et al.*, 2003; Puvača *et al.*, 2013). It was reported that feeding garlic powder at levels of 1.5, 3 and 4.5% had no effect on poultry performance, but caused a significant reduction in poultry serum, liver cholesterol and white, red and skin cholesterol content. Black pepper (*Piper nigrum* L.) is a flowering vine of Piperaceae family and has been a prized spice in many cultures all over the world. This herb is a known spice which improves digestibility (Moorthy *et al.*, 2009). It is a common medicinal herb used in human diet. The volatile oil of pepper has been shown to have antimicrobial activity (Dorman and Deans, 2000). Black pepper has many medicinal properties for treatment of vertigo, asthma, indigestion, congestion, fever, diarrhea (Turner, 2004). When it is used in broiler chicken nutrition it is found that very small addition of black pepper in the diet, about 0.5 to 1.0% significantly reduces cholesterol levels in meat (Al-Kassie *et al.*, 2011). Hot red pepper (*Capsicum annum* L.) is one of the most important herbs widely used in human nutrition. Beside the pungent effect, in poultry nutrition is added in small amount between 0.25 and 1.0% because of the important role in decreasing deposition of cholesterol and fat in the body which contributes to decrease levels of triglycerides and support the vascular system in the body. Hot red pepper is also rich in vitamin C which has a considerable impact in improving production through the reduction of heat stress on a fact that poultry consumption of hot red pepper induces a considerable change in energy balance (Al-Kassie *et al.*, 2012).
Aim of this paper is to show the most important bioactive compounds in herbal plants such as garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.) and hot red pepper (*Capsicum annuum* L.) and its modes of action.

Garlic (*Allium sativum* L.) and its bioactive constitutes

Garlic (*Allium sativum*) has long been used both for flavoring and for the potential benefits of preventing and curing ailments in many cultures (Rivlin, 2001). Epidemiological, clinical, and preclinical studies have shown the close relation between dietary habits, including garlic intake, and the occurrence of disease. Garlic has been investigated extensively for health benefits and it is considered one of the best disease-preventive foods, based on its potent and varied effects. The chemistry of the Allium species has been dominated by many sulfur-containing compounds that give them a characteristic flavor. However, a variety of components, including nonsulfur compounds, work synergistically to provide various health benefits. Because of the complex chemistry in Allium plants, variations in processing yield quite different preparations (Amagase *et al.*, 2001). Garlic exhibits hypolipidemic, antiplatelet, and procirculatory effects. It prevents cold and flu symptoms through immune enhancement and exhibits anticancer and chemopreventive activities. The major sulfur-containing compounds in intact garlic are γ-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin). Both are abundant as sulfur compounds, and alliin is the primary odorless, sulfur-containing amino acid, a precursor of allicin (Stoll and Seebeck, 1948), methiin, (+)-S-(trans-1-propenyl)-L-cysteine sulfoxide, and cycloalliin (Fujiwara *et al.*, 1958). These sulfoxides, except cyloalliin, are converted into thiosulfinates (such as allicin) through enzyme reactions when raw garlic is cut or crushed. Thus, no thiosulfinates are found in intact garlic. Gamma-Glutamyl-S-allyl-L-cysteine is converted into S-allylcysteine through an enzymatic transformation with g-glutamyltranspeptidase when garlic is extracted with an aqueous

solution (Matsuura, 1997). S-allylcysteine a major transformed product from Gamma-Glutamyl-S-allyl-L-cysteine, is a sulfur amino acid detected in the blood that is verified as both biologically active and bioavailable. The disruption of garlic bulbs causes the formation of thiosulfinates such as allicin through the enzymatic reaction of sulfur-substituted cysteine sulfoxides, compartmentalized in the cytoplasma with alliinase in the vacuole, via sulfur-substituted sulfenic acids as a highly reactive intermediate. Allicin easily reacts with amino acids and proteins, creating an SH group. Allicin binds to protein and fatty acids in the plasma membrane, are thus trapped before absorption, and cannot circulate in the blood (Freeman and Kodera, 1995). Typical volatiles that have been identified in crushed garlic and garlic essential oil include DAS, DADS, diallyl trisulfide, methylallyl disulfide, methylallyl trisulfide, 2-vinyl-4H-1, 3-dithiin, 3-vinyl-4H-1, 2-dithiin, and (E,Z)-ajoenes. Over 20 sulfides have been identified in steam-distilled garlic oil and oil-soluble extract of garlic, and many of them, especially sulfides having an allyl group, are responsible for the characteristic smell and taste after ingesting garlic. The major sulfides in garlic oil include DAS (57%), allylmethyl (37%), and dimethyl (6%) mono- to hexasulfides, in some cases, together with a small amount of allyl 1-propenyl and methyl 1-propenyl di-, tri-, and tetrasulfides (Lawson *et al.*, 1991). Diallyl trisulfide is the most abundant in fresh garlic oil, but commercially available garlic oil products have an increased amount of DADS (Jirovetz *et al.*, 1992). Table 1 shows main components and characteristics of some garlic products.

Table 1. Components and characteristics of garlic powder and garlic essential oil

Form of garlic	***Main compounds and characteristics***	***References***
Garlic powder	Alliin and a small amount of oil-soluble sulfur compounds	Freeman and Kodera., 1995. Amagase, 2006.
	Results on cholesterol levels in blood and tissues	
Garlic essential oil	About 1% of Oil-soluble sulfur compounds (DAS and DADS) in 99% vegetable oil	
	No water-soluble fraction	

Black pepper (*Piper nigrum* L.) piperine and piperic acid

Black pepper or *Piper nigrum* is one of the most popular spice products in oriental countries (mostly in Southeast Asia). *Piper nigrum* is a plant of the *piperaceae* family, largely used as a flavouring agent in foods. Its characteristic aromatic odour is due to the volatile oils in the cells of the pericarp (Murthy and Bhattacharya, 2008). It has been traditionally used for the treatment of malaria in India and the epilepsy in China (Majeed and Prakash, 2000). Moreover, black pepper has an anti-inflammatory activity manifested by stimulating the production of an anti-inflammatory cytokine like (IL-10). On the other hand, black pepper inhibits the expansion of genes encoding the nitric oxide synthase (iNOS) and the cyclooxygenase-2 (COX-2). The iNOS and COX-2 stimulate the production of many pro-inflammatory mediators such as (Interleukin-4 (IL-4), Interleukin-10 (IL-10), Interleukin-13 (IL-13), interferon-alpha (α-IFN)), and the transformation growth factor b-TGF (Hanada and Yoshimura, 2002; Makarov, 2000). Piperine (1-piperoyliperidine), which belongs to the alkaloid family, represents the major component in the dry fruit of *Piper nigrum*. Piperine has been reported to have several pharmacological effects such as anti-diarrhoeal and hepatoprotective (Koul and Kapil, 1993; Bajad *et al.*, 2000). Some studies have shown that piperine possesses an anti-inflammatory and an analgesic effect (Gupta *et al.*, 2000). In addition, it has a high antioxidant activity and is used for the treatment of Alzheimer diseases (Chonpathompikunlert *et al.*, 2010; Selvendiran *et al.*, 2003). The chemical modification in the structure of piperine to piperic acid was confirmed by the appearance of the carboxyl group during the hydrolysis. Piperic acid has a high anti-hyperlipidemic activity (Han *et al.*, 2008). Recently, piperine and its derivatives have been evaluated for their inhibitory effects

against epimastigote and amastigote (Ribeiro *et al.*, 2004; Da Silva Ferreira *et al.*, 2008). Table 2 gives a preview of some important roles of *Piper nigrum* fruits and piperine.

Table 2. Selected roles of Piper nigrum and piperine

Effects	***References***
Protection against oxidative stress	Iwashita *et al.*, 2007.
Decreased mitochondrial lipid peroxidation	Vijayakumar *et al.*, 2004.
Stimulation of digestive enzymes	Awen *et al.*, 2010.
Increase gastric acid secretion	Manoharan *et al.*, 2009.
Growth stimulatory activity	Pattanaik *et al.*, 2009.

Hot red pepper (*Capsicum annum* L.) alkaloid compound

Capsicum is a genus of plant under the family of *Solanaceae*, and this capsicum has varieties of names according to their location and type. The most familiar peppers names are chili, bell, red, green or just called as pepper (Faustino *et al.*, 2007). Chili (*Capsicum annum* L.) is fruit-vegetable commonly found in humans daily food menu. They are extremely popular for the huge content of vitamin C and total soluble phenolics higher than other vegetables commonly recognized as a source of this substance (Kumar *et al.*, 2009). There are many properties that differ chili from other fruit-vegetables, such as in their shape, size, colour, flavour and heat, or they can be hot, sweet, fruity, earthy, smoky and floral. Varieties and stages of maturity also have great influence on chilies quantity (Sanatombi and Sharma, 2008). Scientific research has proven that, *capsicum annum*, is the only crop that produce alkaloid compound called capsaicinoids, which is responsible for the hot taste. Capsaicinoids are alkaloids that are important in the pharmaceutical industry for their neurological effectiveness (Hayman and Kam, 2008). Pepper that are fresh is known as the very good source of vitamin C and E as well as provitamin A and carotenoids, and its also known for antioxidant properties (Serrano-Martinez *et al.*, 2008). Vitamin C, including ascorbic acid and its oxidation product (dehydroascorbic acid), has many biological activities in the human body due to its antioxidant properties. In human consumption, peppers and bell peppers are an important source of nutrient such as in providing carotenoids, phenols, vitamin C, foliates. In peppers, there are phytochemical property that have many biochemical and pharmacological properties which includes antioxidants, anti-inflammatory, antiallergenic and anti-carcinogenic activities (Lee *et al.*, 2005). It also has been proven that, ripe red peppers can reduce the risk of cancer (Nishino *et al.*, 2009). In addition, peppers also express antimicrobial property (Wahba *et al.*, 2010). Table 3 gives the content review of some selected compounds of hot red pepper.

Table 3. Content of selected compounds of hot red pepper

Compounds	***Amaunt***	***References***
Capsaicin	309.30 µg/g	Othman *et al.*, 2011.
Dihydrocapsaicin	238.20 µg/g	
Vitamin C	120.25 mg/100	Ranajit *et al.*, 2013.
Carotenoids	1060.24 µg/g	
Total Phenols	2150.25 µg/g	
Total Flavonoids	1.60 µmol/g	
Antioxidant capacity	150.25 µmol/g	

ACKNOWLEDGEMENTS

This paper is a part of the project III 46012 which is financed by Ministry of Education, Science and Technological Development of Republic of Serbia.

REFERENCES

Alder, A.J. Holub, B.J. (1997). Effect of garlic and fish-oil supplementation on serum lipid and lipoprotein concentrations in hypercholesterolemic men. American Journal of Clinical Nutrition, 65, 445-450.

Al-Kassie, G.A.M., Al-Nasrawi, M.A.M., Ajeena, S.J. (2011). Use of black pepper (Piper nigrum) as feed additive in broilers diet. Research Opinions in Animal and Veterinary Sciences, 1, 169-173.

Al-Kassie, G.A.M., Ghassan, Y., Butris, S.J.A., Ajeena, J. (2012). The potency of feed supplemented mixture of hot red pepper and black pepper on the performance and some hematological blood traits in broiler diet. International Journal of Advanced Biological Research, 2, 53-57.

Amagase, H. (2006). Clarifying the Real Bioactive Constituents of Garlic. The Journal of Nutrition, 136 (3), 716S-725S.

Amagase, H., Petesch, B., Matsuura, H., Kasuga, S., Itakura, Y. (2001). Intake of garlic and its bioactive components. The Journal of Nutrition, 131 (95), 5S–62S.

Awen, B.Z., Ganapati, S., Chandu, B.R. (2010). Influence of sapindus mukorossi on the permeability of ethyl cellulose free film for transedermal use. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 1, 35-38.

Bajad, S., Bedi, K. L., Singla, A. K., Johri, R. K. (2001). Antidiarrhoeal activity of piperine in mice. Planta Medica, 67, 284-287.

Castanon, J.I.R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. Poultry Science, 86, 2466-2471.

chemical and biological studies. Bioorganic & Medicinal Chemistry, 16 (6), 2984-2991.

Chonpathompikunlert, P., Wattanathorn, J., Muchimapura, S. (2010). Piperine, the main alkaloid of Thai black pepper, protects against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of Alzheimer's disease. Food and Chemical Toxicology, 48 (3), 798-802.

Crespo, N., Steve-Garcia, E. (2003). Polyunsaturated fatty acids reduce insulin and very low density lipoprotein levels in broiler chickens. Poultry Science, 82, 1134-1139.

Da Silva Ferreira, W., Freire-de-Lima, L., Saraiva, V. B., Alisson-Silva, F., Mendonça-Previato, L., Previato, J. O. (2008). Novel 1,3,4-thiadiazolium-2- phenylamine chlorides derived from natural piperine as trypanocidal agents:

Demir, E., Sarica, S., Ozcan M.A., Suicmez, M. (2003). The use of natural feed additives as alternatives for an antibiotic growth promoter in broiler diets. British Poultry Science, 44, S44-S45.

Dibner, J.J., Richards, J.D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. Poultry Science, 84, 634-643.

Dorman, H.J., Deans, S.G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology, 88, 308-316.

Faustino, J.M.F., Barroca, M.J., Guine, R.P.F. (2007). Food Bioproduct Process, 85, 163-170.

Freeman, F., Kodera, Y. (1995). Garlic chemistry: stability of S-(2-propenyl)-2-propene-1-sulfinothiate (allicin) in blood, solvents and simulated physiological fluids. Journal of Agricultural and Food Chemistry, 43, 2332–2338.

Fujiwara, M., Yishimura, M., Tsuno, S., Murakami, F. (1958). ''Allithiamine,'' a newly found derivative of vitamin B1. IV. on the alliin homologues in the vegetables. The Journal of Biochemistry, 45, 141–149.

Griffin, H.D., Guo, K., Windsor, D., Butterwith, S.C. (1992). Adipose tissue lipogenesis and fat deposition in line broiler chicken. Journal of Nutrition, 122, 363-368.

Gupta, S. K., Bansal, P., Bhardwaj, R. K., Velpandian, T. (2000). Comparative antinociceptive, anti-inflammatory and toxicity profile of nimesulide vs nimesulide and piperine combination. Pharmacological Research, 41, 657-662.

Hanada, T., Yoshimura, A. (2002). Regulation of cytokine signaling and inflammation. Cytokine & Growth Factor Reviews, 13, 414-421.

Hayman, M., Kam, P.C.A. (2008). Current Anaesthesia & Critical Care, 19, 338-343.

Iwashita, M., Saito, M., Yamaguchi, Y., Takagaki, R., Nakahata, N. (2007). Inhibitory effect of ethanol extract of Piper longum on rabbit platelet aggregation through antagonizing thromboxane A2 receptor. Biological and Pharmaceutical Bulletin, 30, 1221-1225.

Jirovetz, L., Jager, W., Koch, H.P., Remberg, G. (1992). Investigation of volatile constituents of the essential oil of Egyptian garlic (Allium sativum) by means of GCMS and GC-FTIR. Zeitschrift für Lebensmittel-Untersuchung und Forschung, 194, 363–365.

Konjufka, V., Pesti, G., Bakalli, R. (1997). Modulation of cholesterol levels in broiler meat by dietary garlic and copper. Poultry Science, 76, 1264-1271.

Kostadinović, Lj., Lević, J. (2012). Use of phytogenic products for pig and broiler diseases. Proceedings of 15th International Feed Technology Symposium, Novi Sad, Serbia, pp. 64-74.

Koul, I. B., Kapil, A. (1993). Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. Planta Medica, 59, 413-417.
Kumar, A.O., And, S., Subba, T. (2009). Notulae Scientia Biologicae,1, 50-52.
Lawson, L.D., Wang, Z.J., Hughes, B.G. (1991). Identification and HPLC quantitation of the sulfides and dialk(en)yl thiosulfinates in commercial garlic products. Planta Medica, 57, 363–370.
Lee, J.J., Crosby, K.M., Yoo, K.S., Lescobar, D.I. (2005). Scientia Horticulturae, 106, 341-352.
Lin, M.C., Wang, E.J., Lee, C., Chin, K.T., Liu, D., Chiu, J.F., Kung, H.F. (2000). Garlic inhibits microsomal triglyceride transfer protein gene expression in human liver and intestinal cell lines and in rat intestine. Journal of Nutrition, 8, 1165-1168.
Majeed, M., Prakash, L. (2000). The medical uses of pepper. International Pepper New, 25, 23-31.
Makarov, S. S. (2000). NF-kB as a therapeutic target in chronic inflammation: recent advances. Molecular Medicine Today, 6, 441-448.
Manoharan, S., Balakrishnan, S., Menon, V.P., Alias, L.M., Reena, A.R. (2009). Chemopreventive efficacy of curcumin and piperine during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. Singapore Medical Journal, 50, 139-146.
Mansoub, N.H. (2011). Comparative effects of using garlic as probiotic on performance and serum composition of broiler chickens. Annals of Biological Research, 2, 486-490.
Matsuura, H. (1997). Phytochemistry of garlic horticultural and processing procedures. In: Lachance PA, editor. Neutraceuticals: designer foods III. garlic, soy and licorice. Trumbull, CT: Food and Nutrition Press, pp. 55–69.
Moorthy, M., Ravikumar, S., Viswanathan, K., Edwin, S.C. (2009). Ginger, pepper and curry leaf powder as feed additives in broiler diet. International Journal of Poultry Science, 8, 779-782.
Murthy, C. T., Bhattacharya, S. (2008). Cryogenic grinding of black pepper. Journal of Food Engineering, 85, 18-28.
Neilsen, P. V., Rios, R. (2000). Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. International Journal of Food Microbiology, 60, 219-229.
Nishino, H., Murakosh, M., Tokuda, H., Satomy, Y. (2009). Archives of Biochemistry and Biophysics, 483, 165-168.
Othman, Z.A.A., Ahmed, Y.B.H., Habila, M.A. (2011). Determination of capsaicin and dihydrocapsaicin in capsicum fruit samples using high performance liquid chromatography. Molecules, 16, 8919-8929.
Pattanaik, S., Hota, D., Prabhakar, P., Pandhi, P. (2009). Pharmacokinetic interaction of single dose of piperine with steady-state carbamazepine in epilepsy patients. Phytotherapy Research, 23 (9), 1281-1286.
Puvača, N., Stanaćev, V., Glamočić, D., Lević, J., Perić, L., Stanaćev, V., Milić, D. (2013). Beneficial effects of phytoadditives in broiler nutrition. World's Poultry Science Journal, 69, 27-34.
Qureshi, A.A., Abuirmeileh, N., Din, Z.Z., Elson, C.E., Burger, W.C. (1983). Inhibition of cholesterol and fatty acid biosynthesis in liver enzymes and chicken hepatocytes by polar fractions of garlic. Lipids, 18, 343-348.
Rahmatnejad, E., Roshanfekr, H. (2009). Evaluation the effect of several non-antibiotic additives on growth performance of broiler chickens. Journal of Animal and Veterinary Advances, 8, 1757-1760.
Ranajit, K.S., Shafiqur, R., Afandi, A. (2013). Bioactive compounds in chilli peppers (Capsicum annuum L.) at various ripening (green, yellow and red) stages. Annals of Biological Research, 4 (8), 27-34.
Ribeiro, T. S., Freire-de-Lima, L., Previato, J. O., Mendonça-Previato, L., Heise, N., de Lima, M. E. F. (2004). Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of Trypanosoma cruzi. Bioorganic & Medicinal Chemistry Letters, 14, 3555-3558.
Rivlin, R. (2001). Historical perspective on the use of garlic. The Journal of Nutrition, 131 (95), 1S–4S.
Salie, F., Eagles, P. F. K., Leng, H. M. J. (1996). Preliminary antimicrobial screening of four South African Asteraceae species. Journal of Ethnopharmacology, 52, 27-33.
Sanatombi, K. Sharma, G.J. (2008). Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 36 (2), 89-90.
Selvendiran, K., Vijeya Singh, J. P., Krishnan, K. B., Sakthisekaran, D. (2003). Cytoprotective effect of piperine against benzo[a]pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in Swiss albino mice. Fitoterapia, 74, 109-115.
Serrano, M., Zapata, P.J., Castillo, S., Guillen, F., Martinez-Romero, D., Valero, D. (2010). Food Chemistry, 118, 497-503.
Shobana, S., Naidu, K. A. (2000). Antioxidant activity of selected Indian spices. Prostaglandins Leukotienes and Essential Fatty Acids, 62, 107-110.
Simon, O. (2005). Micro-organisms as feed additives-probiotics. Advances in Pork Production, 39, 161-167.
Stanaćev, V., Glamočić, D., Milošević, N., Perić, L., Puvača, N., Stanaćev, V., Milić, D., Plavša, N. (2012). Influence of garlic (Allium sativum L.) and copper as phytoadditives in the feed on the content of cholesterol in the tissues of the chickens. Journal of Medicinal Plants Research, 6, 2816-2819.
Stanaćev, V., Milošević, N., Plavša, N., Bjedov, S., Stanaćev, V., Puvača, N., Arapović, Ž. (2010). Phyto additives (Allium sativum L.) in the diet of fattening chickens. Proceedings of the 14th International Symposium of Feed Technology, Novi Sad, pp. 295-302.
Stoll, A., Seebeck, E. (1948). Allium compounds. I. Alliin the true mother compound of garlic oil. Helvetica Chimica Acta, 31, 189–210.

Tanamai, J., Veeramanomai, S., Indrakosas, N. (2004). The efficacy of cholesterol-lowering action and side effects of garlic enteric coated tablets in man. Journal of the Medical Association of Thailand, 87, 1156-1161.

Turner, J. (2004). Spice: Black pepper and White pepper. The History of a Temptation. London, Vintage Books.

Vijayakumar, R.S., Surya, D., Nalini, N. (2004). Antioxidant efficacy of black pepper (Piper nigrum L.) and piperine in rats with high fat diet induced oxidative stress. Redox Report, 9 (2), 105-110.

Wahba, N.M., Ahmed, A.S., Ebraheim, Z.Z. (2010). Foodborne Pathogens and Disease, 7, 411-418.

Wierup, M. (2001). The Swedish experience of the 1986 ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microbial Drug Resistance, 7, 183–190.

EFFECT OF SPICE HERBS IN BROILER CHICKEN NUTRITION ON PRODUCTIVE PERFORMANCES

Nikola Puvača[1]*, Dragomir Lukač[1], Vidica Stanaćev[1], Ljiljana Kostadinović[2], Miloš Beuković[1], Dragana Ljubojević[3], Slađana Zec[4]

[1]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
[2]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[3]Institute of Veterinary Medicine Novi Sad, Rumenački put 20, 21000 Novi Sad, Serbia
[4] Perutnina Ptuj – Topiko a.d., Petefi brigade 2, 24300 Bačka Topola, Serbia

*Corresponding author:
E-mail address: nikola.puvaca@stocarstvo.edu.rs

ABSTRACT

Aim of this paper was to investigate the effect of dietary spice herbs such as garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.) and hot red pepper (*Capsicum annuum* L.) in broiler chicken nutrition on productive performances. For biological research eight treatments with 1200 broiler chickens of hybrid line Hubbard in total were formed, with four replicates. Control treatment (T1) was fed with commercial mixtures of standard composition and quality based on corn flour and soybean meal. Experimental treatments were fed with same commercial mixtures only with addition of spice herbs as follows: garlic 0.5 (T2) and 1.0 g/100g (T3), black pepper 0.5 (T4) and 1.0 g/100g (T5), hot red pepper 0.5 (T6) and 1.0 g/100g (T7) and mixture of garlic, black pepper and hot red pepper (1:1:1) in total of 0.5 g/100g (T8). Chickens were fed with starter mixtures diet without addition of spice herbs for first two weeks, after which chickens are fed with grower and finisher mixtures according the plan till the end of experiment which lasted 42 days. At the end of experiment and on the basis of gained results it can be concluded that the chickens at experimental treatments T6 and T7 achieved statistically significant ($p<0.05$) higher final body masses (2460.6 and 2442.4 g) compared to the chickens at control and other treatments. Feed conversion ratio for the entire fattening period ranged from 1.8 kg/kg (T2, T5) to 2.1 kg/kg (T1) with no statistically significant differences ($p>0.05$). European broiler index (EBI) was lowest in treatment T1 (220.4) and the highest in treatment T6 (298.6) with statistically significant differences ($p<0.05$). It can be concluded that the chickens which received dietary hot red pepper 0.5 and 1 g/100 g achieved better performance compared with other experimental treatments as well as with control treatment.
***Keywords**: garlic, black pepper, hot red pepper, nutrition, chickens*

INTRODUCTION

Besides of important role of medicinal herbs, aromatic plants and spices in daily human nutrition for enhancement of taste, aroma and color of food, these additives have been usefully used in animal nutrition for improvement of health and animal wellbeing. With the ban of antibiotics use in animal nutrition due to the emergence of microbe resistance, alternative growth promoters must be founded. Removal of antibiotics as growth promoters has led to animal performance problems, feed conversion ratio incensement, and a rise in the incidence of certain animal diseases (Wierup, 2001). The alternatives to antibiotics as growth stimulators are numerous (Simon, 2005; Kostadinović and Lević, 2012; Stanaćev *et al.*, 2011; Puvača *et al.*, 2013). Plant derived additives used in animal nutrition to improve performance have been called "phytogenic feed additives" (Windish *et al.*, 2008). This form of feed additives has recently gained interest for use in poultry with increasing numbers of scientific publications since the ban of antibiotics growth promoters in 1999. The primary mode of action of these growth promoting feed additives can be attributed mainly to the stabilization of feed hygiene and also from the beneficial effect on the gastrointestinal microbiota through controlling pathogens (Roth and Kirchgessner, 1998). A component in blends of essential oils such as thymol, eugenol, curcumin and piperin reduces *Clostridium perfringens* concentrations in both the intestinal tract and faeces of broilers through the entire

growing period (Mitch *et al.*, 2004). In commercial broiler production mainly powder forms or essential oils of oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), garlic (*Allium sativum*), black pepper (*Piper nigrum*) and chilli (*Capsicum annum*) are used singly or in combination as feed additives (Grashorn, 2010; Puvača *et al.*, 2013; Puvača *et al.*, 2014).

Garlic is one of the most traditionally used plants as a spice herb. Garlic has been used for a variety of reasons which most of them has been approved scientifically: antiatherosclerosis, antimicrobial, hypolipidemic, antithrombosis, antihypertension, antidiabetes and etc. (Mansoub, 2011). There are a lot of active components in garlic like ajoene, s-allyl cysteine, diallyl sulphide and the most active one allicine (Rahmatnejad and Roshanfekr, 2009). Allicine possibly reduces low density lipoprotein (LDL), triglycerides and cholesterol in serum (Alder and Holub, 1997) and tissues (Stanaćev *et al.*, 2012; Puvača *et al.*, 2014), and it has been used in treatments against cardiovascular diseases (Tanamai *et al.*, 2004). In broilers, it was reported that garlic, as a natural feed additive have improved broiler growth and feed conversion ratio, and decreased mortality rate (Stanaćev *et al.*, 2010). Garlic also manifests hypocholesterolemic effects on chickens through inhibition of the most important enzymes that participate in the synthesis of cholesterol and lipids (trihydroxy-tri-methylglutaryl coenzyme A reductase, cholesterol-7α hydroxylase and the synthesis of fatty acids). In addition, this additive has a relatively low market price, is added in small amounts of 0.2 to 2%, thus not increase production costs, which is of particular importance to manufacturers (Zekić *et al.*, 2014).

Black pepper is known as spices due to its pungent quality. Black pepper is found to improve feed digestibility (Moorthy *et al.*, 2009). Pepper efficiency compounds are consisting of capsaicin, capsisin and capsantine that some of them allay rheumatic aches. Black pepper is rich in glutathione peroxidase and glucose-6-phosphate dehydrogenase, and it has been shown that piperine can dramatically increase absorption of selenium, vitamin B complex, β carotene and curcumin as well as other nutrients (Khalaf *et al.*, 2008; Tazi *et al.*, 2014). Piperine enhances the thermogenesis of lipids and accelerates energy metabolism in the body and also increases the serotonin and β-endorphin production in the brain (Al-Kassie *et al.*, 2011). Pepper has been found to have antioxidant properties and anticarcinogenic effect, especially when is combined with chili (Nalini *et al.*, 2006). Among its chemical and biological activities, piperine exhibits antimicrobial (Reddy *et al.*, 2004), antinflammatory (Pradeep and Kuttan, 2004) and antioxidant (Mittal and Gupta, 2000) properties.

Hot red pepper plays an important role in decreasing the deposition of cholesterol and fat in the body and contributes to decrease levels of triglycerides and work to support the vascular system in the body. Hencken, (1991) explained that hot red pepper is rich in vitamin C which have a considerable impact in improving production through contributes the reduction of heat stress on a fact that birds consumption of hot red pepper induce a considerable change in energy balance when individuals are given free access of feed (Yoshioka *et al.*, 2001). A recent studies involved in poultry performance showed that blends of active compounds for hot red pepper causes chemo preventive and chemotherapeutic effects.

Aim of this study was to investigate the effect of dietary spice herbs such as garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.) and hot red pepper (*Capsicum annuum* L.) on productive performances of broiler chicken.

MATERIAL AND METHODS

Biological tests were carried out under production conditions at the experimental farm "Pustara" in property of the Faculty of Agriculture, Department of Animal Science in Novi Sad. At the beginning of experiment, eight treatments of 150 one day-old broiler chickens of hybrid line Hubbard per treatment in four repliction on a total of 1200 chickens were formed. For nutrition of chicks three mixtures were used, starter, grower and finisher with 22, 20 and 18% of crude protein, respectively. The first 14 days, during the preparatory period, chicks were fed with starter mixture. Following the preparation period, chicks were fed the next 21 days with grower mixtures, and then the last 7 days of fattening period with finisher mixtures

according the experimental desing given in Table 1. During the experiment, chicks were fed and watered *ad libitum*, and microclimate conditions were regularly monitored. Chickens were on the floor holding system. Control of body weight and feed consumption was performed weekly.
The results were expressed as least squares means (LSM) values and standard errors of least squares means (SE_{LSM}). The data were submitted to analysis of variance (ANOVA) and Fisher's LSD post-hoc test of significance within the statistical software STATISTICA 12.

Table 1. Experimental design

Experimental treatmens		Concetration of additives in chicken diets		
		In starter, g/100g	In grower, g/100g	In finisher, g/100g
		1 – 14 days	15 – 35 days	36 – 42 days
T1	Control treatment	0.0	0.0	0.0
T2	Garlic powder	0.0	0.5	0.5
T3	Garlic powder	0.0	1.0	1.0
T4	Black pepper powder	0.0	0.5	0.5
T5	Black pepper powder	0.0	1.0	1.0
T6	Hot red pepper powder	0.0	0.5	0.5
T7	Hot red pepper powder	0.0	1.0	1.0
T8	Mixture of garlic, black pepper and hot red pepper (1:1:1)	0.0	0.5	0.5

RESULTS AND DISCUSSION

Based on the obtained results it can be noted that the addition of garlic, black pepper and hot red pepper in the diet of broiler chickens led to a statistically significant ($p>0.05$) differences in body weight. From the preparatory period chickens have exit with uniform body weight with no statistical significant differences ($p>0.05$).
At the end of the third week, chickens in treatment T2 achieved the highest body weight (818.5 g) with statistically significant differences ($p<0.05$) compared to the treatments T1, T4 – T8. Almost the same tendency was observed at the end of fourth week where the highest body masses was recorded in chickens with dietary addition of 0.5 and 1.0 g/100g of garlic powder (1202.3 and 1204.9 g) with statistically significant differences ($p<0.05$) compared with T1, T4 and T5, while the significant differences with treatments T6, T7 and T8 was absent. At the end of second fattening period, addition of hot red pepper in treatments T6 and T7 exerted their stimulating effect and led to statistically significant differences ($p<0.05$) in body weight in relation to control and other experimental treatments. After the completion of experimental period the highest achieved body weight of chicken was at treatment T6 (2460.6 g) which was followed by treatment T7 (2442.4 g) with statistically significant differences ($p<0.05$) compared to other treatments. Treatments with addition of garlic powder achieved final body masses of 2371.1 and 2336.1 g which was statistically significantly ($p<0.05$) higher than masses of chickens at treatments T1 (2075.8 g), T4 (2076.5 g) and T5 (2077.5 g). Addition of black pepper led to a statistically significant ($p<0.05$) lover body weight compared to other experimental treatments but without significant differences ($p>0.05$) compared to a control treatment. Similar results with the use of spice herbs in broiler chicken nutrition were reported by the other researchers (Moorthy *et al.*, 2009; Al-Kassie *et al.*, 2011; Mansoub, 2011; Stanaćev *et al.*, 2012; Puvača *et al.*, 2014; Tazi *et al.*, 2014).

Table 2. Body weight of chickens, g

Experimental treatments		Age of chickens						
		1 day	7 days	14 days	21 days	28 days	35 days	42 days
T1	LSM	42.8^{a}	162.7^{a}	388.6^{a}	785.6^{bc}	1162.4^{b}	1643.8^{c}	2075.8^{d}
	SE_{LSM}	0.47	1.52	3.64	8.38	11.84	12.2	24.23
T2	LSM	42.1^{a}	160.2^{a}	389.7^{a}	818.5^{a}	1202.3^{a}	1743.1^{b}	2371.1^{b}
	SE_{LSM}	0.47	1.63	3.84	8.41	11.8	12.16	23.96
T3	LSM	42.2^{a}	159.7^{a}	386.4^{a}	804.6^{ab}	1204.9^{a}	1737.2^{b}	2336.1^{bc}
	SE_{LSM}	0.47	1.64	3.79	8.5	11.75	11.94	23.43
T4	LSM	42.4^{a}	159^{a}	384.2^{a}	754.1^{d}	1117.1^{c}	1577.8^{d}	2076.5^{d}
	SE_{LSM}	0.47	1.62	3.79	8.41	11.8	12.39	24.42
T5	LSM	42.4^{a}	160.4^{a}	386.6^{a}	727.5^{e}	1055.6^{d}	1503.7^{e}	2077.8^{d}
	SE_{LSM}	0.47	1.62	3.86	8.35	11.75	12.16	23.96
T6	LSM	42.5^{a}	162.5^{a}	385.3^{a}	770.5^{cd}	1193.6^{ab}	1815.1^{a}	2460.6^{a}
	SE_{LSM}	0.47	1.63	3.86	8.29	11.84	12.25	24.05
T7	LSM	42^{a}	161.6^{a}	385.1^{a}	762.4^{cd}	1183.6^{ab}	1812.1^{a}	2442.4^{a}
	SE_{LSM}	0.47	1.6	3.87	8.38	11.84	12.2	24.33
T8	LSM	41.8^{a}	163.2^{a}	384.9^{a}	778.6^{c}	1178.7^{ab}	1717.5^{b}	2297.8^{c}
	SE_{LSM}	0.47	1.6	3.81	8.38	11.71	12.02	23.78

Treatments with different letter indexes in the same column are statistically significantly different (p<0.05)

The feed conversion ratio is given in Table 3. In preparation period of chicken feed conversion ratio was uniform and ranged between 1.3 and 1.4 kg of feed per kg of gain, without statistically significant (p>0.05) differences. In the grower phase the lowest achieved feed conversion ratio was in treatment T2 (1.7 kg/kg) and the highest in T4 and T5 (1.9 kg/kg) treatments.

Table 3. Chicken feed conversion ratio, kg/kg

Experimental treatments		Periods of nutrition			
		Starter phase	Grower phase	Finisher phase	Entire period
		1 – 14 days	15 – 35 days	36 – 42 days	1 – 42 days
T1	LSM	1.3^{ab}	1.8^{ab}	3.0^{a}	2.1^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T2	LSM	1.4^{ab}	1.7^{b}	2.3^{b}	1.8^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T3	LSM	1.4^{ab}	1.8^{b}	2.5^{b}	1.9^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T4	LSM	1.4^{ab}	1.9^{a}	2.5^{b}	1.9^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T5	LSM	1.3^{b}	1.9^{ab}	2.3^{b}	1.8^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T6	LSM	1.4^{a}	1.8^{ab}	2.4^{b}	1.9^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T7	LSM	1.4^{ab}	1.8^{b}	2.6^{b}	1.9^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15
T8	LSM	1.4^{ab}	1.8^{b}	2.6^{b}	1.9^{a}
	SE_{LSM}	0.01	0.05	0.14	0.15

Treatments with different letter indexes in the same column are statistically significantly different (p<0.05)

Feed conversion ratio in finisher phase was higher in control treatment T1 (3.0 kg/kg) with statistically significant ($p<0.05$) differences compare to the other treatments. The lowest feed conversion ratio of 2.3 kg/kg was recorded in T2 and T5 treatments, followed by 2.4 kg/kg in T6, 2.5 kg/kg in T3 and T4, and 2.6 kg/kg in T7 and T8. Lower feed conversion ratio in experimental treatments shows that addition of garlic, black pepper and hot red pepper and their mixture have positive influence on feed utilization and efficiency. For the entire experimental period, feed conversion ratio was lowest in treatments T2 and T5 (1.8 kg/kg) and the highest in control treatment T1 (2.1 kg/kg), without statistically significant ($p>0.05$) differences.

Table 4 gives the overview in European broiler index (EBI) and chicken mortality rate. Addition of these feed additives led to a statistically significant ($p<0.05$) increase in values of EBI of experimental treatments in compare to a control treatment T1. The highest mortality rate (5.1 %) and the EBI (220.4 %) were recorded in control treatment. Mortality rate of 0.0% was recorded in treatment T8 and 279.5 % EBI which was significantly ($p<0.05$) higher compare to treatments T1, T4 and T5.

Table 4. European broiler index and chicken mortality, %

Experimental treatments		EBI	Mortality
T1	LSM	220.4^{g}	5.1^{a}
	SE_{LSM}	2.77	0.96
T2	LSM	295.1^{ab}	3.2^{ab}
	SE_{LSM}	2.77	0.96
T3	LSM	283.7^{cd}	1.3^{bc}
	SE_{LSM}	2.77	0.96
T4	LSM	244.4^{f}	1.3^{bc}
	SE_{LSM}	2.77	0.96
T5	LSM	260.4^{e}	0.6^{bc}
	SE_{LSM}	2.77	0.96
T6	LSM	298.6^{a}	2.6^{ac}
	SE_{LSM}	2.77	0.96
T7	LSM	288.6^{bc}	2.6^{ac}
	SE_{LSM}	2.77	0.96
T8	LSM	279.6^{d}	0.0^{c}
	SE_{LSM}	2.77	0.96

Treatments with different letter indexes in the same column are statistically significantly different (p<0.05)

The highest recorded EBI values was 298.6 % in treatment T6 and 295.1 % in treatment T2 without significant ($p>0.05$) differences between themselves, but with significant ($p<0.05$) differences with other experimental treatments.

CONCLUSIONS

Based on the obtained results, it can be concluded that the addition of garlic, black pepper and hot red pepper in broiler chicken nutrition have positive effect on productive performances. Addition of hot red pepper in amount of 0.5 g/100g has led to the highest final body weight, lower feed conversion ratio and higher feed utilization, with the highest percentage of European broiler index. Therefore the general conclusion would be that the additions of these dietary spice herbs have positive influence on chicken productive performance, but the further investigation of their mode of action is still necessary.

ACKNOWLEDGEMENTS

This paper is a part of the project III 46012 which is financed by Ministry of Education, Science and Technological Development of Republic of Serbia and project 114-451-4685/2013-03 financed by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina. Also the realization of one part of this experiment was supported by the Perutnina Ptuj – Topiko a.d., Petefi brigade 2, 24300 Bačka Topola, Serbia.

REFERENCES

Alder, A.J., Holub, B.J. (1997). Effect of garlic and fish-oil supplementation on serum lipid and lipoprotein concentrations in hypercholesterolemic men. American Journal of Clinical Nutrition, 65, 445-450.

Al-Kassie, G.A.M., Al-Nasrawi, M.A.M., Ajeena, S.J. (2011). Use of black pepper (*Piper nigrum*) as feed additive in broilers diet. Research Opinions in Animal and Veterinary Sciences, 1, 169-173.

Grashorn, M.A. (2010). Use of phytobiotics in broiler nutrition– an alternative to infeed antibiotics? Journal of Animal and Feed Sciences, 19, 338–347.

Hencken, H. (1991). Cooling the burn from hot peppers. Journal of the American Medical Association, 266, 2766-2770.

Khalaf, A.N., Shakya, A.K., Al-Othman, A., El-Agbar, Z., Farah, H. (2008). Antioxident activity of some common plants. Turkish Journal of Biology, 32, 51-55.

Kostadinović, Lj., Lević, J. (2012). Use of phytogenic products for pig and broiler diseases. Proceedings of 15th International Feed Technology Symposium, Novi Sad, Serbia, pp. 64-74.

Mansoub, N.H. (2011). Comparative effects of using garlic as probiotic on performance and serum composition of broiler chickens. Annals of Biological Research, 2, 486-490.

Mittal, R., Gupta, R.L. (2000). In vitro antioxidant activity of piperine. Methods and Findings in Experimental and Clinical Pharmacology, 122, 271-274.

Moorthy, M., Ravikumar, S., Viswanathan, K., Edwin, S.C. (2009). Ginger, pepper and curry leaf powder as feed additives in broiler diet. International Journal of Poultry Science, 8, 779-782.

Nalini, N., Manju, Y., Menon, V. (2006). Effect of spices on lipid metabolism in 1, 2-dimethylhydrazine-induced rat colon carcinogenesis. Journal of Medicinal Food, 9, 237-45.

Pradeep, C.R., Kuttan, G. (2004). Piperine is a potent inhibitor of nuclear factor‐kB (NF‐kB), c‐Fos, CREB, ATF‐2 and pro‐inflammatory cytokine gene expression in B16F‐10 melanoma cells. International Immunopharmacology, 4, 1795‐1803.

Puvača, N., Stanaćev, V., Beuković, M., Ljubojević, D., Kostadinović, Lj., Džinić, N. (2014). Effect of phytogenic feed additive (*Allium sativum* L.) on broiler chicken nutrition on breast meat quality and tissues cholesterol content. Proceedings of International Scientific Conference, Vukovar, Croatia, 7, 75-80.

Puvača, N., Stanaćev, V., Glamočić, D., Lević, J., Perić, L., Stanaćev, V., Milić, D. (2013). Beneficial effects of phytoadditives in broiler nutrition. World's Poultry Science Journal, 69, 27-34.

Rahmatnejad, E., Roshanfekr, H. (2009). Evaluation the effect of several non-antibiotic additives on growth performance of broiler chickens. Journal of Animal and Veterinary Advances, 8, 1757-1760.

Reddy, S.V., Srinivas, P.V., Praveen, B., Kishore, K.H., Raju, B.C., Murthy, U.S., Rao, J.M. (2004). Antibacterial constituents from the berries of Piper nigrum. Phytomedicine, 11, 697‐700.

Roth, F., Kirchgessner, X. (1998). Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. Journal of Animal Feed Science, 8, 25-33.

Simon, O. (2005). Micro-organisms as feed additives-probiotics. Advances in Pork Production, 39, 161-167.

Stanaćev, V., Glamočić, D., Milošević, N., Perić, L., Puvača, N., Stanaćev, V., Milić, D., Plavša, N. (2012). Influence of garlic (*Allium sativum* L.) and copper as phytoadditives in the feed on the content of cholesterol in the tissues of the chickens. Journal of Medicinal Plants Research, 6, 2816-2819.

Stanaćev, V., Glamočić, D., Milošević, N., Puvača, N., Stanaćev, V., Plavša, N. (2011). Effect of garlic (*Allium sativum* L.) in fattening chicks nutrition. African Journal of Agricultural Research, 6, 943-948.

Stanaćev, V., Milošević, N., Plavša, N., Bjedov, S., Stanaćev, V., Puvača, N., Arapović, Ž. (2010). Phyto additives (*Allium sativum* L.) in the diet of fattening chickens. Proceedings of the 14th International Symposium of Feed Technology, Novi Sad, pp. 295-302.

Tanamai, J., Veeramanomai, S., Indrakosas, N. (2004). The efficacy of cholesterol-lowering action and side effects of garlic enteric coated tablets in man. Journal of the Medical Association of Thailand, 87, 1156-1161.

Tazi, S.M.E., Mukhtar, M.A., Mohamed, K., Tabidi, M.H. (2014). Effect of using black pepper as natural feed additive on performance and carcass quality of broiler chicks. International Journal of Pharmaceutical Research and Analysis, 4, 108-113.

Wierup, M. (2001). The Swedish experience of the 1986 ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microbial Drug Resistance, 7, 183–190.

Windish, W., Schedle, K., Plitzner, C., Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of animal Science, 86, 140-148.

Yoshioka, M., Doucet, E., Drapeau, V., Dionne, I., Tremblay, A. (2001). Combined effects of red pepper and caffeine consumption on energy balance in subjects given free access to foods. British Journal of Nutrition, 85, 203-211.

Zekić, V., Puvača, N., Milić, D., Beuković, M., Glamočić, M., Vukelić, N., Lukač, D., Zekić, S. (2014). Effect of garlic powder in broiler chicken nutrition: Emphasis on production economic efficiency coasts and chicken meat quality. Custos e @gronegócio, 10 (2), 86-98.

RAGWEED (*AMBROSIA ARTEMISIIFOLIA* L.) – DETERMINATION OF PHYTOESTROGEN ACTIVITY, BASIC NUTRIENT CONTENT AND ITS POTENTIAL AS A FORAGE FOR SMALL RUMINANT

Radomir Ratajac[1]*, Aleksandar Milovanović[1], Marina Žekić Stošić[1], Tomislav Barna[1], Željko Mihaljev[1], Jasna Prodanov Radulović[1], Dragica Stojanović[2]

[1]Scientific Veterinary Institute Novi Sad, Rumenački put 20, 21000 Novi Sad, Serbia
[2]Faculty of Agriculture, Department for veterinary medicine, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: radomir.ratajac@gmail.com

ABSTRACT

The aim of this study was to investigate the level of estrogen activity of the ragweed by bioassay in immature female rats and nutritional value of the plant. In bioassay a standardized curve of dose-dependent response of the uterus weight after treatment with various doses of 17-β estradiol was established. Experimental groups of rats were fed with ragweed extract through a gastric tube, and estrogen activity was calculated. There were no clinical signs of disease during treatment, as well as in sections (post-mortem) no changes in the internal organs. Results indicate that ragweed has a weak estrogenic activity, with no statistical significance and is not expected to influence on sexual development of the experimental animals.
Ragweed has been analyzed for the nutrient status. Chemical analysis showed that plant has a high protein concentration that could be used for the production of proteins of animal origin. A high level of ash indicates the presence of mineral matter (micro and macro elements). Short time feeding sheep with ambrosia did not led to the appearance of adverse effects on health and behavior. Animals ate ragweed with pleasure, especially at the stage before flowering.
In rural areas, small ruminants may serve as a biological enemy in controlling the spread of ragweed, either grazing or after mowing. It is necessary to determine whether the products obtained from animals fed with ambrosia, possibly possess residues that may be harmful to people allergic to ragweed pollen.
Keywords: *ragweed, phyto-estrogens, rat, nutritional value, sheep*

INTRODUCTION

Common ragweed (*Ambrosia artemisiifolia* L.) is one of the most important weed species at the time being. The name of this genus Ambrosia is derived from the Greek word for "food of the gods". These are annuals, perennials, shrubs and sub-shrubs with erect, hispid stems growing in large clumps to a height of 75-150 cm. The stems are basally branched. They form a slender taproot or a creeping rhizome. Among Ambrosia species common ragweed (*Ambrosia artemisiifolia* L.) is the most important. *A. artemisiifolia* in the last two decades become the best recognized weed species in Eastern-Europe. This was happened because so many people developed allergies to its airborne pollen. Therefore national governments had to initiate programs to bring attention to this noxious weed (Kazinczi *et al*., 2008).
A. artemisiifolia not only invades a broad range of open disturbed areas, such as waste lands, linear constructions (roadsides, railway tracks, riverbanks), building operations, but also field crops, such as sunflower, soybean and maize. As a result of late emergence of *A. artemisiifolia*, it can also grow during the inter-crop period in rape seed or cereal stubbles, as well as on fallow or set-side land (Chollet *et al*., 1999). There are a lot of reasons which play a considerable role in Ambrosia distribution. The lack of its natural enemies in Europe, incorrect human activities (increasing of waste lands, the lack of proper stubble treatments and that of professional advice towards the farmers, ineffective of the laws and authority arrangements connected with Ambrosia problems), increasing of hobby gardening, inexperience of the inhabitants, crop seeds infested with Ambrosia achenes, expensive weed

control technologies and the appearance of herbicide resistant biotypes are the most important factors in this respect. Beside these, its biological characteristics (morphological-genetic variability, broad ecological amplitude, continuous germination, considerable biomass production, good competition, mineral utilization and drought tolerance ability, allelopathy, effective survival ability under tress conditions) can also greatly contribute to its harmful effect (Tóth *et al.*, 2001, Szentey *et al.*, 2004).

The main problems, caused by *A. artemisiifolia* are: i) harmful effect for the agriculture; ii) export problems (it is a quarantine pest in some countries); iii) human health problems (air pollution by allergic pollens); iv) natural and environmental protection problems (harmful effect on the ecological balance of biocoenosis and the biological diversity); v) touristic problems (*A. artemisiifolia* is abundant near touristic centers in summer times) (Kazinczi *et al*., 2008b).

In Europe, all the highest counts on peak days are reported from the Carpathian Basin, Serbia and Hungary. Novi Sad (northern region of Serbia), the southern part of the Great Hungarian Plains (Szeged) and southwest Hungary (Pécs) have the highest concentrations of *A. artemisiifolia* pollen, not only in the Carpathian Basin itself but in the whole European Continent, although in Serbia the average *A. artemisiifolia* pollen grains was 185 m^3 in 2004, while 657 m^3 in 2003 (Konstantinović *et al.*, 2004).

In contrast to the harmful effects, that are quite well known and researched, the useful effects of this plant are not known or investigated. In the literature, however, some beneficial characteristics of ragweed are mentioned. In Canada the tea of the flowering *A. artemisiifolia* plant was used for stomach disease. Indians used its leaves against insect-bite and inflamed injuries. Decoction of the leaves is good against skin diseases, septic wounds and inflammation of the eye. Its tea with astringent effect is good against gripes, vomitus, pleuritis and constipation. Root decoction alleviates menses complaints (Bremness, 1998).

The seeds of *A. artemisiifolia* serve as an important complementary food for winged game (pheasants and partridges), especially in cool winter times. Its seeds are rich of oils, the food-value is similar to that of soybean, and therefore singing-birds like to eat it (Baldwin and Handley, 1976). Unfortunately, this is favorable for its spreading (Járainé, 2003).

On the basis of some feeding experiments it can be proved that sheep like to eat the fresh Ambrosia plants. The protein content of the plant is considerable and harmonic amino acid composition was found. Sheep cannot digest the seed, therefore the seed can pass intact through the alimentary track (Húsvéth *et al.*, 1999).

On the basis of the above facts the aim of present study was to determine basic nutrient value and the presence of phytoestrogens in the samples of ragweed originating from several regions in Serbia. The palatability and safety of fresh ragweed plant was also examined in the diet of sheep.

Phytoestrogens are plant compounds with estrogen-like biological activity. The estrogenic effect of phytoestrogens was first observed as reproductive disturbances in sheep (Bennetts *et al.*, 1946). In our work we used a rat uterotrophic assay for detection of phytoestrogens, which quantifies increasing of uterine weight upon exposure of the animal to estrogenic substances. A positive result of the tests indicates that the test substance can influence the reproductive system of humans and animals. General opinion is that a negative result of uterotrophic assay excludes any positive result obtained by *in vitro* methods. In recent years, this protocol has been improved by integration of histological parameters, such as epithelial cell height, thereby increasing the number of uterine-based endpoints. In order to get a better understanding of the underlying mechanisms of dietary estrogen exposure, effects studied at the level of morphology and histology were complemented by gene expression data and coregulator recruitment (Heneweer *et al*., 2007).

MATERIAL AND METHODS

The sampling of ragweed was conducted from June to October 2007, at various stages of vegetation. The samples were taken from seven different locations: Temerin (the land cultivated and planted with alfalfa, ragweed was in the stage of growth prior to flowering –

period of early vegetation); Zmajevo (the land after wheat harvest, ragweed was in the stage of growth prior to flowering – period of early vegetation); Odžaci (the land near the road and canal, ragweed was in the stage of growth before flowering – period of secondary vegetation); Novi Sad (the land beside the road, ragweed was in the stage of growth before flowering – period of secondary vegetation); Šabac (the land after wheat harvest, ragweed was in the stage of growth before flowering – period of secondary vegetation); Mali Iđoš (the land along the road, ragweed was in the flowering stage – period of late vegetation) and Palić (the land near the road, ragweed was in the flowering stage – period of late vegetation). The plant was cut and air dried. The dried plant of ragweed with moisture below 9% were milled and subjected to a chemical analysis and extraction.

The content of fat, crude fiber, ash and moisture in the samples was performed by standard methods, while the protein was determined by measuring total nitrogen by total combustion (according to Dumas), as the standard method (AOAC 990.03) on the instrument ,,Elementar Rapid N cube". The content of nutrients was expressed in percentages. The results were statistically analyzed.

Extraction was carried out based on the description by Liu *et al.* (2001) with methanol (methanol p.a., CH_3OH, Centrohem, Novi Sad). A sample of the plant material (100 g) was coated with methyl alcohol (700 mL), and with constant shaking at room temperature was allowed to stand for 24 hours. Triple successive extractions with fresh solvent were made. The extracts were merged and evaporated under reduced pressure. The solvent was removed using the water pump injector, and moisture was removed using a rotary vacuum pump. The maximum temperature of bath was 50°C.

Wistar immature female rats from the farm of laboratory animals of the Scientific Veterinary Institute "Novi Sad" were used for biological assay. The rats were housed in polycarbonate cages in a controlled environment (temperature 20°C ± 2°C with frequent ventilation and an illumination schedule of 12-h light/12-h dark). The experimental animals were given free access to food and tap water. Rats were fed with standard laboratory diet - pellets (Rat formula feed; Veterinarski Zavod Subotica), which has been previously tested for presence of mycotoxins (zearalenone). Sexually immature female rats that were used in the experiment were caged with their mothers until the time of sacrifice. The rat uterotrophic assay was standardized by using dose-dependent response curve of the uterus weight after treatment with various doses of 17-β - estradiol (CAS 57-63-6, Sigma, Germany; three groups, n = 6 rats per each group). From postnatal days 21 to 23, immature female rats (7 experimental groups, n = 6 per each group) received daily plant extract by oral gavage using a stomach tube (5 ml/kg BW/day). Dosages were adjusted according to BW. Clinical signs, and abnormal behaviors were recorded daily throughout the experimental period. All animals were euthanized 24 h after the final treatment by ethyl ether and their weights were measured. On dissection the uteri were prepared (removing ovaries and connective tissue) and weighed on the third decimal scale.

Five adult rams (Württemberg breed, averraging 75 kg of BW, 8-12 month old) were feed with 3 kg per day of row Ambrosia plant for 15 days. Apetite (palatability) and animal health status were monitored daily.

RESULTS AND DISCUSSION

The values of basic nutrients are presented in Table 1. The variability between certain parameters in ragweed samples originated from different sites were not significant. A slightly higher variability was found only for crude fat content (29.33%) and crude ash (31.55%), but this is the result of higher values of these parameters in the samples from only one site.

The average content of crude fat in the ragweed samples (1.50±0.44%) was lower than the fat content in dehydrates alfalfa hay (with 15% prot.) or in unshelled oats: 2.3% and 4.5% respectively (Sinovec and Ševković, 1995). In available literature data it is stated that alfalfa and outs are used as standard in comparing nutritional values of weeds (Marten and Anderson, 1975).

Table 1. Content of the basic nutrients in the Ambrosia artemisiifolia L. samples

Location	Investigated parameter (%)					
	Moisture	Crude protein	Crude fat	Crude fiber	Crude ash	NFE[1] *BEM*[1]
Zmajevo	5.36	13.95	1.44	23.66	12.38	43.21
Mali Iđoš	8.24	10.80	1.50	34.08	9.10	36.28
Novi Sad	5.13	11.65	1.26	31.81	10.60	29.92
Temerin	5.18	13.15	1.01	28.91	18.77	32.98
Odžaci	4.90	12.70	2.42	31.46	8.23	40.17
Palić	7.18	10.80	1.41	33.70	8.97	37.94
Šabac	5.40	11.10	1.49	28.55	11.59	41.87
X±Sd	5.91±1.27	12.02±1.25	1.50±0.44	30.30±3.62	11.38±3.59	37.48±4.80
$C_{V\%}$	21.49	10.40	29.33	11.95	31.55	12.81
Min-Max	4.90-8,24	10.80-13.95	1.01-2.42	23.66-34.08	8.23-18.77	29.92-43.21

NFE[1] - nitrogen free extract; BEM – extractive substances without nitrogen

Our chemical studies have shown that *A. artemisiifolia* has high protein content. The average value in the analyzed samples was 12.02±1.25, what is higher than the protein content in unshelled oats, but less than in dehydrated alfalfa hay: 11.6% and 15.2% respectively (Sinovec and Ševković, 1995). The values of protein content in the samples at some sites are quite consistent, with a low coefficient of variability. Slightly higher content was measured in the samples from Zmajevo and Temerin area, which can be attributed to soil type and agro technical measures, i.e. to the content of nitrogen in the soil. Hubbard and Boe, during 1984 and 1985, analyzed 27 plant species characteristic for swamp area that are located in the eastern parts of South Dakota. Among these plants were three samples of ragweed (*Ambrosia spp*.). The protein content in samples of ragweed was 15.7% in relation to dry matter at 100^{o}C. If this is applied on our dry weight, the protein content would be 14.77%, what is slightly higher from our value. This difference can be explained by another kind of ragweed, as well as the features of the soil from which the samples were taken.

The tests show a high content of cellulose fibers in common ragweed. The average value in our sample was 30.30±3.62. The values in the samples from different sites were quite close, what was indicated also by the coefficient of variation (11.95%). Crude fiber content of this weed plant was higher than in dehydrated alfalfa hay (with 15% prot.) and unshelled oats: 26.4 and 11.0% respectively (Sinovec and Ševković, 1995).

High level of ash in the samples (11.38±3.59%) indicates the presence of minerals (macro and microelements). The values of ash content in samples from some sites were quite different ($C_{V\%}$ 31.55%). The variability in the crude ash content was expected because the soils on different sites differ in their mineral status. Hubbard and Boe (1988), in the above mentioned study, examined the samples of ragweed and the crude ash value was 12.7% of dry matter at 100^{o}C, which, when calculated on our dry matter was 11.95% and agrees with our data of 11.38%, although we tested another type of weed plants.

Table 2 compares juvenile rats' uterus dose dependent weight changes in control and experimental groups after 17-β estradiol treatment.

Based on the results in the control group (without the 17-β estradiol) and experimental groups (s.c. application of 0.3 mg and 1.0 mg 17 β-estradiol) a dose response by increasing of the uterus weight was determined and equation for calculating the equivalent doses of 17-β estradiol to the uterus growth in experiments with extract of ragweed ($y = 0,5 \times X – 0,5667$) was established (Figure1).

Table 2 Standardized curve of dose depend increase of juvenile rats' uterus (sacrificed at 24 days old) after treatment with various doses of 17-β estradiol

Groups of animals	Average body weight of the animal (g)	Average weight of the uterus (g)	The relative weight of uterus (g / 100g BW)	The dose of 17-β estradiol
I - Control group	27.2	0.019	0.068	-
II – exp. group	28.8	0.053	0.185	0.3 µg
III – exp. group	28.6	0.075	0.264	1 µg

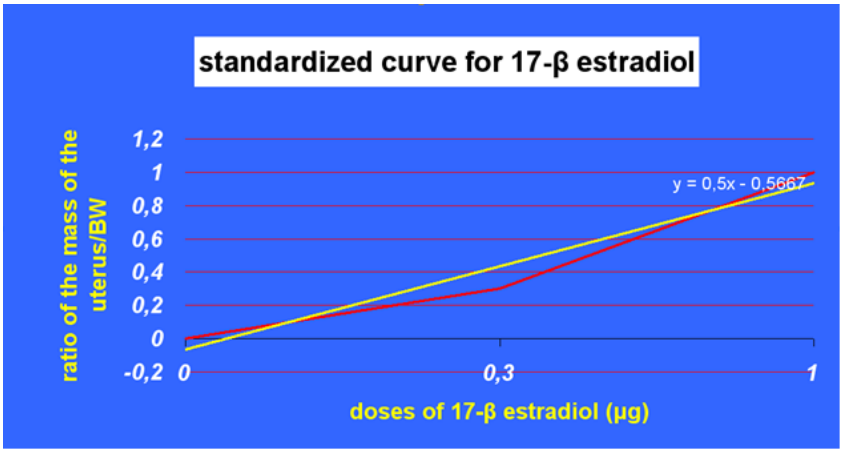

Figure 1. Standardized curve of increase uterine of juvenile rats (sacrificed 24 days of age) with various doses of 17-β estradiol

In Table 3 results of estrogen activity of ragweed samples from different location were presented. The results indicate that ragweed has low estrogenic activity, which is not within the limits of statistical significance. Up to now there are not similar research of phytoestrogens activity of ragweed in the available literature. During treatment there were no clinical signs of disease in rats and evident post-mortem changes on the internal organs.

Table 3. Results of estrogenic activity ragweed samples (uterotrophic assay)

Location	Average body weight of the animal (g)	Average weight of the uterus (g)	The relative weight of uterus (g / 100g BW)	Equivalent doses of estradiol, significance
Zmajevo	58.254 ± 5.934	0.036 ± 0,006	0.061 ± 0.007	0.138 n.s.
Mali Iđoš	52.200 ± 4.999	0.026 ± 0,002	0.050 ± 0.004	0.099 n.s.
Novi Sad	33.364 ± 3.066	0.029 ± 0,006	0.086 ± 0.016	0.132 n.s.
Temerin	49.928±10.472	0.032 ± 0,009	0.063 ± 0.007	0.143 n.s.
Odžaci	48.180 ± 2.117	0.029 ± 0,004	0.061 ± 0.008	0.137 n.s.
Palić	26.211 ± 3.854	0.017 ± 0,002	0.065 ± 0.013	0.147 n.s.
Šabac	28.666 ± 1.601	0.032 ± 0,002	0.111 ± 0.008	0.0136 n.s.
Average, ± SD	42.400±12.714	0.029 ± 0.006	0.071 ± 0.021	0.116 ± 0.048 n.s.

Two week feeding period of sheep with ambrosia did not led to the appearance of adverse effects on health and behavior. Animals ate ragweed with pleasure, especially at the stage before flowering. In case of harvesting ambrosia after flowering, palatability was different between animals. Several factors affect the palatability of a plant, including texture, leafiness, fertilization, moisture content, pests, and compounds in the plant. Many studies do not include palatability trials to observe if the plants will actually be consumed. Marten *et al.* (1987) did include a palatability study and found that most herbaceous weeds were less palatable than alfalfa or smooth bromegrass. The lambs used in their study basically rejected Jerusalem artichoke, curly dock, hoary asylum and Canada thistle, which may be due to physical characteristics such as spines and hairs on most of these species.
Giant ragweed (*Ambrosia trifida* L.) was less palatable than oats (*Avena sativa* L.) when tested by Marten and Anderson (1975), with a very low percentage (0.0 percent) being consumed after 12 days. The species, common ragweed (*Ambrosia artemisiifolia* L.) was classed as interacters; i. e. some sheep found them palatable, whereas other sheep refused to graze them. Palatability was not associated with nutritive value, indicating that sheep lacked "nutritional wisdom." Palatability is a key factor in determining the quality of weeds because there is no nutritive value for animals if they will not eat the species.

CONCLUSIONS

Our study shows that common ragweed has good nutritive values regarding to the content of basic nutrients. According to the chemical composition, *Ambrosia artemisiifolia* L. is categorized like grass of middle class quality. The results indicate that ragweed has low estrogenic activity, which is not within the limits of statistical significance, and it is not expected to disrupt the status of sex hormones of animals.
Based on the results of plant chemical analysis, uterotrophic assay for (phyto)estrogen presence and short time feeding experiment on sheep it can be concluded that common ragweed have certain nutritive value, have no phytoestrogens activity, good palatability in early phases of vegetation and could be used for the production of proteins of animal origin.
There is a need for further investigation of this weed, especially in the terms of its impact on the health of small ruminants (sheep and goats) when used in long-term animal nutrition. In rural areas, small ruminants may serve as a biological enemy in controlling the spread of ragweed, either grazing or after mowing. It is necessary to determine whether residue may be found in the products obtained from animals fed with *A. artemisiifolia* L., what can cause adverse affects on human health.

ACKNOWLEDGEMENTS

This study was financially supported by the Provincial Secretariat for Agriculture, Water Management and Forestry of Autonomous Province of Vojvodina, grants No 104-401-02180/2006-04.

REFERENCES

Baldwin, W. P. and C.O. Handley (1976). Winter food of bobwhite quail in Virginia. J. Wildl. Manage 10:142-149.

Bennetts HW, Underwood EJ, Shier FL. (1946). A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust Vet J. 22:2–12.

Bremness, L. (1998). Aromatic and Medical Plants. Egyetemi Nyomda, Budapest, Hungary.

Chollet, D., Y. Drieu, J. Molines and J. Pauget (1999). Comment lutter contre l'ambroisie á feuilles d'armoise. Persp. Agric. 250: 78-82.

Heneweer Marjoke, Houtman René, Poortman Jenneke, Groot Maria, Maliepaard Chris and Peijnenburg Ad (2007).Estrogenic Effects in the Immature Rat Uterus after Dietary Exposure to Ethinylestradiol and Zearalenone Using a Systems Biology Approach,Toxicological Sciences,Volume 99 (1), 303-314

Hubbard, E.D., Boe, A. (1988). IVDDM and Chemical Constituents in Selected Species of Wetland Plants. Proc. S.D. Acad. Sci., 67:44-58

Husveth, F., Szegleti, CS., Beres, I., Munkas, I., Magyar, L., and Szolloskei, G.(1999). Examination of Ambrosia artemisiifolia reticulo - ruminalis *in vivo* and *in vitro*. Állattenyésztés és Takarmányozás,48:707-708.

Jaraine, M. (2003). Pannon Encyclopedy. Flora of Hungary. (Pannon Enciklopédia Magyarország növényvilága). Budapest, Hungary.

Kazinczi, G., Béres, I., Novák, R., Bíró, K., Pathy, Z. (2008). Common ragweed Ambrosia artemisiifolia): a review with special regards to the results in Hungary. I. Taxonomy, origin and distribution, morphology, life cycle and reproduction strategy. Herbologia Vol. 9, No.1, 55-93

Kazinczi, G., Béres, I., Pathy, Z., Novák R. (2008b). Common ragweed (Ambrosia artemisiifolia L.): a review with special regards to the results in hungary: II. Importance and harmful effect, allergy, habitat, allelopathy and beneficial characteristics. Herbologia Vol. 9, No.1, 93-119

Konstantinović, B., Meseldžija, M., Konstatinović B. and Dakić Z. (2004). Control of allergic weed species *Ambrosia artemisiifolia* L. in the region of the city of Novi Sad. Herbologia 5:73-78.

Liu J, Burdette Joanna, Xu H, Gu C, van Breemen R, Bhat Christina, Booth Nancy, Constantinou A, Pezzuto J, Fong H, Farnsworth N, Bolton Judy. (2001), Evaluation of Estrogenic Activity of Plant Extracts for the Potential Treatment of Menopausal Symptoms, Journal of Agricultural and Food Chemistry, Volume 49, Number 5, 2472-2479.

Marten, G. C., and R. N. Andersen (1975). Forage nutritive value and palatability of 12 common annual weeds. *Crop Science* 15: 821-827.

Marten, G. C., C. C. Sheaffer, and D. L. Wyse. (1987). Forage nutritive value and palatability of perennial weeds. *Agronomy Journal* 79: 980-986.

Official method of analysis of AOAC 990.03 Protein – Crude in Animal Feed Combustion Method

Sinovec, Z., Ševković, N. (1995). Praktikum iz ishrane, Veterinarski fakultet, Beograd

Szentey, L, Toth Á. and Dancza I. (2004). Ambrosia-free Hungary is our common interest. Plant and Soil Protection Central Service, Budapest, Hungary.

Toth, Á., Benecsne B. G. and Beres I. (2001). The role of allelopathy in the distribution of Ambrosia artemisiifolia and Cirsum arvense in Hungary. Weeds, Weed Control 2:21-29.

ULTRA HIGH TEMPERATURE (UHT) TREATMENT EFFECT ON IODINE FORTIFIED MILK THROUGH COW FEED

Fernando Vicente, José Ángel Suárez Medina, Amelia González-Arrojo, Ana Soldado, Begoña de la Roza-Delgado*

Department of Nutrition, Grasslands and Forages. Regional Institute for Research and Agro-Food Development (SERIDA), PO Box 13, 33300 Villaviciosa, Spain

*Corresponding author:
E-mail address: broza@serida.org

ABSTRACT

Iodine is an essential trace element for humans, because it is necessary for the synthesis of thyroid hormones. Its metabolic action influences many physiological functions, facilitating the expense of fat excess, and is also present in the growth of teeth, hair and nails. The daily Dietary Reference Intake (DRI) recommended for iodine is 150 µg/d. Natural sources of dietary iodine include seafood and vegetables growing on iodine-rich soils. However, the milk, being a universal food, could be used as a rich source of iodine, because iodine concentration in milk depends of iodine intake by the animal. The aim of this work was to study the relationship between the intake of KI in dairy cow and the iodine content in raw milk and after the heat treatment applied, in order to reach more than 15% of the DRI. The cows were supplemented with 1 g of KI at 10% daily and were compared to a control group. Individual dry matter intake was monitored by a computerized system and the production of milk was recorded in a robotic milking system. Milk samples were collected weekly for analysis of fat, protein, solids-not-fat, lactose by mid-infrared and iodine by elemental mass spectrometry. There were no significant intake, production and milk composition differences between groups. After supplementation, the average level of iodine was 60 mg $I_2$100 ml^{-1} of milk, reaching the goal after 2-3 weeks of supplementation starting. The UHT industrial processing did not affect the milk iodine concentration, losing less than 15% of total I_2 content.
***Keywords**: Iodine supplementation, Milk, Dairy cows, Heat-treatment of milk*

INTRODUCTION

Iodine is an essential trace element for humans, being necessary for the synthesis of thyroid hormones. Iodine deficiency and related risks of medical and /or developmental disorders in humans are a worldwide problem (Hejtmánková *et al.,* 2006). Its physiological function as a constituent of thyroxine requires the control of intake levels, as deficiency or excessive exposure both have a detrimental effect on thyroid function control of metabolism. Its metabolic action influences many physiological functions, facilitating the expenditure of excess fat, and is also present in the growth of teeth, hair and nails. According to the World Health Organization, the optimal daily intake of iodine is 150–300 µg in adults, and less than 100 µg per day is thought to be insufficient. Therefore, the daily Dietary Reference Intake (DRI) recommended for iodine is 150 µg * d^{-1}. Natural sources of dietary iodine include seafood and vegetables growing on iodine-rich soils.

Cow milk, as universal food, is an excellent source of protein, calcium, vitamins and minerals. Its composition is not static, and varies depending on many factors. Many studies have investigated non genetic sources of variation in milk mineral content, demonstrating that it is influenced by stage of lactation, nutritional status and climate (Cashman, 2006; Gaucheron, 2005). The milk could be used as a rich source of iodine, because iodine concentration in milk depends of iodine intake by the animal (Knowles *et al.,* 2006). The normal concentration of iodine in cow's milk is around 10 µg L^{-1}. Underwood and Shuttle (1999) showed that with an optimal concentration of iodine in the ration, the concentration of iodine in milk is 44 µg kg^{-1}.

The aim of this work was to examine the relationship between the ingestion of KI as a supplemental source of iodine in dairy cow rations and levels of iodine in natural milk and the

subsequent industrial treatment in a pilot plant, in order to increase the iodine concentration of cow's milk through dietary supplementation with KI, to supply at least 15% of the DRI.

MATERIAL AND METHODS

Experimental design

This work was undertaken in the spring of 2010 (from 9th April to 22th June), in compliance to the standards of the European Union Animal Welfare Directive Number 86/609/EEC. The experiment was carried out with four Holstein-Friesian cows in the second third of lactation and daily milk production between 23 and 38L. These cows were randomly allocated in two groups: control group and supplemented group with KI to 10% dosage of 1 gram per day. Cows were milked twice daily at 07:30 and 19:30 and individual milk yields were recorded at each milking in an automatic milking system (DeLaval).To post-milking bath was used a commercial solution of accelerated hydrogen peroxide (DeLaval) free iodine. After morning milking, the KI supplementation mixed with the concentrate was offered to cows. Once they ate it, they had access to the total mixed ration (TMR) offered *ad libitum.* The TMR intake of individual animals was recorded with a computerized system of control of intake based on the described by Bach *et al*., (2004), following by grazing time till next milking. The TMR was formulated according to NRC (2001) requirements for dairy cattle and composed by corn silage, grass silage, straw of cereal and concentrates, being the relation of forage: concentrate 75:25 on dry matter basis.

A sample aliquot of milk for macronutrients analysis and total iodine content determination, consisting of morning and afternoon milking aliquots combination for each cow, were taken once a week during the experimental period. Samples were stored at -40ºC before analysis. TMR samples and orts were taken daily during the measurement period. Concentrates and pasture were sampled at the beginning of experimental period.

Sample analysis

TMR and forage samples were dried (60 ºC, 24 h) and ground (0.75 mm); concentrate samples were ground through a 1 mm screen. Dry matter (DM), ash, crude protein (CP), ether extract, starch, neutral and acid detergent fibres (NDF, ADF) were determined by near infrared spectroscopy (NIRS; FOSS NIRSystem 5000).

Milk samples were analysed to determine fat, protein, solids-not-fat and lactose contents by mid-infrared (MilkoScan FT 6000).

Iodine content in milk and feed samples was analysed using the method of analysis described by González Arrojo *et al*., (2014) by ICP-MS (Agilent 7500c Octopole Reaction System (ORS); Agilent Technologies, Tokyo, Japan) and previous microwave mineralization (Ethos One, Milestone, Sorisole, Italy). Calibration standards were made up in the range of 1 - 25 ng g^{-1} using KI 99.5%.

Statistical analysis

Milk production, milk composition and concentration of iodine in milk data were analysed using the MIXED procedure (SAS, 1999), according to the model: Yijk = μ + Ti + Eijk, where Yijk=the dependent variable, μ =the overall mean, Ti = the effect of treatment, and Eijk = the residual error.

RESULTS AND DISCUSSION

The composition of the TMR was expected according to the ingredients used (Table 1) and the concentration of iodine found in forage and feed were also within the range of previous studies (Borucki *et al*., 2011).The average dry matter intake of TMR (14.68 kg d^{-1}), concentrate (0.53 kg d^{-1}) and grass (between 5 to 8 kg d^{-1}) were within the values reported in previous studies in Asturias (Morales Almaráz *et al*., 2010, 2011). It is possible to estimate that the iodine intake from the diet was 9.28 mg of I_2 * day^{-1} from the TMR, of 3 mg I_2 * day^{-1}

from concentrate and approximately 2.5 mg I_2 * day^{-1} from grass. All of this assumes a baseline intake of 14.78 mg of I_2 daily.

Table 1. Chemical composition (% on DM) and energy value (Mcal kg^{-1}DM) of the Total Mixed Ration and grass

Parameters	**Total Mixed Ration**	**Grass**
Dry matter	54.16	16.49
Organic matter	93.24	90.76
Crudep rotein	13.82	17.57
Ether extract	3.98	N.D.[1]
Neutral detergent fibre	35.82	41.39
Acid detergent fibre	22.71	22.71
Starch	28.16	N.D.
Iodine	0.665	0.385
Net energy	1.66	1.63

[1]*Not determined*

No differences were found between control and supplemented group respect to milk production (30.15 *vs* 29.16). So, it could be concluded that supplementation with KI does not affect the production of milk, according other authors (Norouzian *et al*., 2009). No significant differences were observed between treatments with respect to macronutrients composition of milk. The obtained results are summarized in Table 2.

Table 2. Average of milk composition in macronutrients for the treatments

Components	**TREATMENT**			
	CONTROL	**IODINE**	**Rsd(1)**	**P**
Fat (%)	3.10	3.37	0.074	0.0823
Protein (%)	3.21	3.19	0.017	0.5676
Lactose (%)	4.85	4.80	0.019	0.2350
SNF(2) (%)	8.81	8.77	0.017	0.2712

(1) *Residual standard deviation*
(2)*Solids-not-fat*

Milk is an important source of iodine, and its excretion increases with the increase in the iodine intake. This study presents an increase of values in the concentration of iodine from the second week, already above the target value (22.5 µg I_2*100 ml^{-1}). Although there is a great variation in the concentrations of iodine throughout the weeks of trial (Figure 1), the recovery of iodine in milk already exceed the limit established from the second week of treatment. KI supplementation increases highly significant iodine concentration in milk from a 9.94 in the control group *vs*. 52.12 in the animals subjected to trial (µg I_2*100 ml^{-1}; $p<0.001$) (Table 3). This value is increased up to 60.97 ±20.04 µgI_2*100 ml^{-1}, when the two first weeks of treatment were not considered. However, the recovery of iodine in milk in the control group was higher than in supplemented group, reaching 19% in the former *vs*. 13% in the latter.

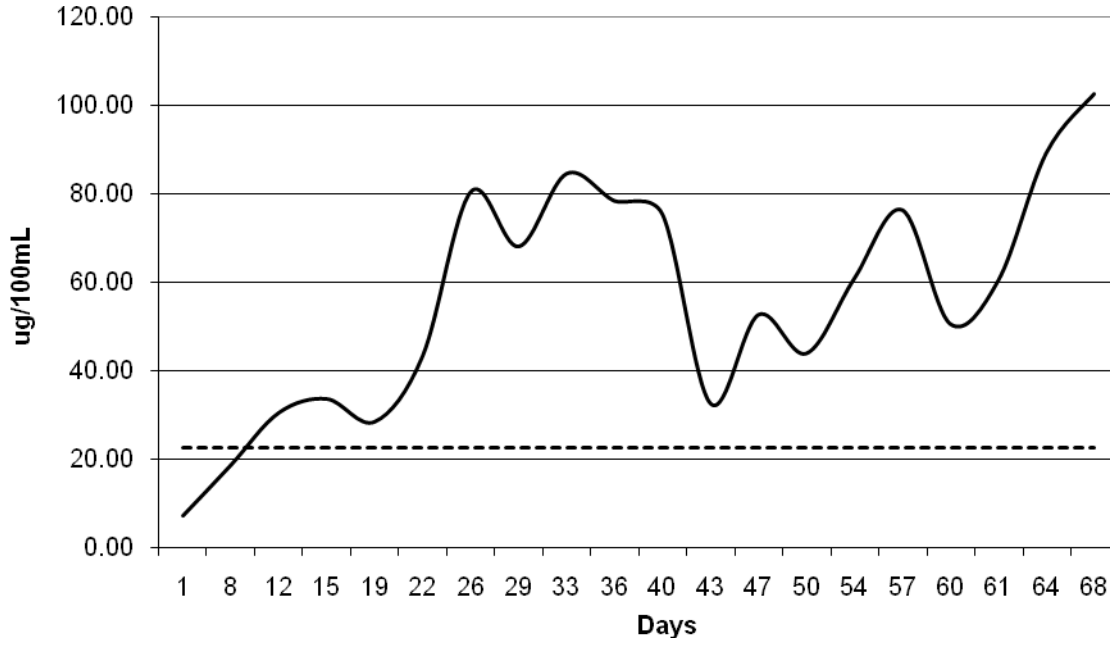

Figure1. Excretion of iodine in milk from supplemented cows throughout the experiment

*Table 3. Excretion of iodine in milk (µg * 100 ml^{-1}) from cows of the experiment*

Component	TREATMENT			
	CONTROL	IODINE	Rsd(1)	P
Iodine	9.94	52.12	2.668	0.0001

(1) Residual standard deviation

Several studies with radio isotope of iodine demonstrated that there is an excretion of iodine via milk between 8 to 12 percent of the ingested iodine (Swanson *et al*., 1990). Nevertheless, these values slightly exceeded those obtained in some studies, and they are lower than others, as the study of Franke *et al*., (2009), in which the transfer of iodine in milk was from 30 to 56 percent if the protein source were distiller dried grains and from 11 to 25 when was used flour rapeseed.

Analysing the results, it was observed that iodine milk concentration, once the plateau of maximum concentration was reached, approximately three weeks from the start of supplementation with KI, is above to 60.97 µg I_2 100 ml^{-1}.This means approximately 3 times the target value (22.5 µg I_2*100 ml^{-1}), then, theoretically it may be possible to reduce the dosage significantly of supplement with KI from 1 g to 0.31 g per day. Nevertheless, due to the animal variability, it is recommended not supplemented less than 0.50 g KI to 10%.

Effects of industrial sterilization of milk on the final concentration of iodine

As a final stage of the trial, the milk production in the last two days of assay was collected in order to carry out the study of milk iodine recovery after the usual industrial sterilization of UHT (Ultra High Temperature) process. The average concentration of iodine in milk before UHT process was 175 µg *100 ml^{-1}. After the UHT process, it presented a concentration of iodine of 155 µg 100 ml^{-1}. So, it seems that the thermal process that increases temperature in short period of time, reduces slightly the concentration of iodine, as had been observed by other authors (Aumont *et al*., 1987). Norouzian *et al*. (2009) showed a great and significant decreasing of iodine concentration after pasteurization treatment. It could be explain by the

property of iodine to be volatile. When milk is subject to processes that need heat for long periods of time, such as pasteurization, it sublimated because the 83 percent of the iodine in milk is free and unbound to protein, that do not occur in UHT treatment, with temperatures of 150 °C for 6 seconds.

CONCLUSIONS

In conclusion, the intake of feed for the cows is not altered by supplementation with 1 g of KI to 10% by animal and day, as well as the milk yield and chemical composition.
After supplementation of iodine with 1 g of KI to 10% to cow diets, it can reach levels of iodine in milk around 60 µg I_2 *100 ml^{-1}. These data placed the excretion of iodine in milk three times above the marked target, due to 15% of the DRI is 22.5 µg I_2 *100 ml^{-1}
After industrial treatment in the UHT pilot plant, the concentration of the iodine is not practically altered, losing less than 15% of the initial concentration of iodine obtained in raw milk.

ACKNOWLEDGEMENTS

This study was financially supported by the Spanish Project RTA2012-00063-C02-00 from the INIA and PC10-54 from the Asturias Regional Project and European Regional Development Fund (ERDF). Authors are also grateful to Nutrition, Grassland and Forages Staff of SERIDA and Scientific – Technical Services (SCTS) from Oviedo University for their technical assistance.

REFERENCES

Aumont G, LeQuerrec L, Lamand M, Tressol J. C. (1987). Iodine content of dairy milk in France in 1983 and 1984.Journal of Food Protection, 50,490–497.

Bach, A., Iglesias, C., Busto, I. (2004). Technical note: A computerized system for monitoring feeding behavior and individual feed intake of dairy cattle. Journal Nutrition, 130, 1548-1554.

Borucki, C.S.I., Lacasse, P., Fouquet, A., Beraldin, F., Robichaud, A., Berthiaume. (2011). Short communication: Feed iodine concentrations on farms with contrasting levels of iodine in milk. American Dairy Science Association, 94,4684-4689.

Cashman, K.D. (2006). Milk minerals (including trace elements) and bone health. International Dairy Journal, 16, 1389-1398.

Franke K., Meyer U., Wagner H., Flachowsky G. (2009). Influence of various iodine supplementation levels and two different iodine species on the content of the milk of cows fed rapeseed meal or disteller dried grains with solubles as the protein source. Journal of Dairy Science, 92, 4514-4523.

Gaucheron, F (2005). The minerals of milk. Reproduction Nutrition Development, 45, 473–483.

González-Arrojo, A., Soldado, A., Vicente, F., Fernández-Sánchez, M.L., Sanz-Medel, A., de la Roza-Delgado, B. (2014). On-farm organic Se supplementation to dairy cows: effect over Co, Cu, I and Zn milk levels. Animal (Under revision).

Hejtmánková, A., Kuklík, L., Trnková, E., Dragounová, H. (2006). Iodine concentrations in cow's milk in Central and Northern Bohemia. Czech Journal of Animal Science, 51, (5), 189–195.

Knowles S.O., Grace N.D., Knight T.W., Mcnabba W.C., Lee J. (2006) Reasons and means for manipulating the micronutrient composition of milk from grazing dairy cattle. Animal Feed Science and Technology 131, 154-167.

Morales-Almaráz, E., Soldado, A., González, A., Martínez-Fernández, A., Domínguez-Vara, I., De La Roza-Delgado, B., Vicente, F. (2010). Improving the fatty acid profile of dairy cow milk by combining grazing with feeding of total mixed ration.Journal of DairyResearch, 77 (2), 225 – 230.

Morales-Almaráz, E., De La Roza-Delgado, B., González, A., Soldado, A., Rodríguez, M.L., Peláez, M., Vicente, F. (2011). Effect of feeding system on unsaturated fatty acid level in milk of dairy cows. Renewable Agriculture and Food Systems, 26 (3), 224 – 229.

National Research Council (2001).Nutrient Requirements of Dairy Cattle.421 7th rev. ed. National Academy Press, Washington, DC.

Norouzian M.A., Valizadeh R., Azizi F., Hedayati M., Naserian A.A., Eftekhari S.F. (2009). The effect of feeding different levels of potassium iodine on performance, T3 and T4 concentrations and iodine excretion in Holstein dairy cows. Journal of Animal and Veterinary Advances, 8 (1), 111-114

SAS (1999) SAS/STATTM. User's Guide. Statistical Analysis System Inst, 428 Cary, NC.

Swanson E.W., Miller I.K., Mueller F.J., Patton C.S., Bacon J.A., Ranesy N. (1990). Iodine in milk and meat of dairy cows fed different amounts of potassium iodine or ethylene diamindihydro iodine. Journal of Dairy Science, 73, 398-405.

World Health Organization (WHO).(1996). Trace elements in human nutrition and health.WHO Publication, Geneva: 49-71.

BACTERIOLOGICAL QUALITY OF DRINKING WATER AND IMPACT ON ANIMALS HEALTH

Igor Stojanov, Jasna Prodanov Radulović, Miloš Kapetanov, Milica Živkov-Baloš, Jelena Petrović, Radomir Ratajac

Scientific veterinary institute Novi Sad, Rumenački put 20, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: igor@niv.ns.ac.rs

ABSTRACT

Food Safety Law prescribes the duties and responsibilities of participants who are taking part in quality testing of the food and feed. In terms of the existing law the food is any substance or product that is used or can be expected to be used for human consumption. Drinking water used for public supply is also regarded as food. Water that is used by farm animals can be a source of bacterial contaminants that can affect the health of animals and indirectly people. The subject of this paper is microbiological control of water collected from different parts of the supply systems used in pig farms. The goal is to determine whether there are differences in the composition of the bacterial flora at different critical points in the water systems. Analyses have shown that water collected before entering premises differ in terms of number and types of bacteria comparing to water that animals consume from drinkers. The most important finding was that *Pseudomonas aeruginosa* and coliform bacteria *Escherichia coli*, including the presence of coliform bacteria of faecal origin could be found in drinking water at the farm. The results indicate the possibility of a negative impact of microbiologically contaminated water on animal health.

***Keywords**: water, bacteria, animal health*

INTRODUCTION

Food is one of the strategic choices of each society. Today's requirements for safe food production are increasingly improving and practical food safety is imperative for each producer. Monitoring the quality of food, especially foods of animal origin, begins with the control of hygiene and quality of food for the animals and subsequent follow the health status of the animals. In animal nutrition, different mixtures and feed additives are used whose quality and hygiene are controlled in laboratory. That ensures that the feed does not have a negative impact on the health and production characteristics of the animal.

According to the Law on Food Safety (Official Gazette 41/2009) Food is any substance or product, whether processed, partially processed or unprocessed, intended for human consumption, or can reasonably be expected to be used for human consumption. In the same way define the feed, ie, the feed is any substance or product, whether processed, partially processed or unprocessed, intended for animals used for food production. Under this law, and drinking water is food, including water in the original packaging (table water, mineral water and spring water), and water used, or added during preparation, processing and food production. Drinking water is the water in the original packaging and water for public water supply.

In compliance with the existing legislation (Official SFRY 33/1987) microbiological characteristics of drinking water which belong to the group of treated, disinfected and bottled water at the source must not contain bacteria *salmonella*, *shigella*, *vibrio cholera* and other pathogenic species, faecal streptococci, *Proteus species* and *Pseudomonas aeruginosa*. Water also must not contain in 100ml of water coliform bacteria and sulphate-reducing bacteria. As under the existing regulations, water quality does not have special provisions for animals and humans, it is considered that microbiological properties of water for animals have to satisfy these characteristics.

Natural water may contain a number of different microorganisms. The characteristics of these bacteria are often related to the ambient conditions in which they are located, and the

fact that they are in a medium which does not contain a nutritious substrate for these bodily functions which often are not culturable (Fricker 2003). On the other hand, the number and type of bacteria that can be found in the water depends on the water, whether it is a natural open or closed waters, capped waters and springs or groundwater (Official SFRJ 33/1987). Contamination of wastewater changing microbial composition of water and significantly increases the number of *Enterobacteriacae*.
Monitoring the microbiological safety of water, which is fed animals in our animal production, it is not given enough attention. For these reasons, the subject of our study was Microbiological control water for pigs in some parts of the water system facilities in pig production, our goal is to determine whether there are differences in the composition of the bacterial flora of the critical points in the system of water supply and if there are differences, we try to determine the extent of importance for the health of the animals.

MATERIAL AND METHODS

Water samples for testing were taken from the pig farm. Water was sampled from three facilities. For each object were taken by two water samples. The first sample was a water taken out of the building with a the tap of water works belonging to one of the object. Another water sample was taken from the drinkers inside the building at the place where the animals are supplied with water. Samples were taken from the supply system were collected in sterile glass bottles. Before collecting samples, the water is released to flow out of the tap and the drinkers in order to avoid potentially contaminated water sample, and in order to provide a valid sample.Take water samples were shipped to the laboratory on the same day in the fridge bag when they seeded. For the isolation and identification of bacteria from water samples passed, we used methods for bacteriological, virological, biological and parasitological examination of drinking water, Annex III, present Regulation on the method of sampling and laboratory methods for the analysis of drinking water (Official Gazette of the SFRY, 1987).

RESULTS AND DISCUSSION

Our studies have comprised water samples from the farm that supplied water from wells located within farms. In Table 1 are the results of the bacteriological examination of samples of tap water from farm objects. Results are related to the water samples from three facilities on the farm, which were taken from the spaces that do not perform pigs production.

Table 1 Results of bacteriological testing of tap water

	Sampling location	**Finding**
1.	Object 1	*Bacillus sp., Corynebacterium spp., Micrococcus sp., Staphylococcus epidermidis, Staphylococcus (koagulaza neg.), Flavobacterium sp., Aeromonas spp.*
2.	Object 2.	*Bacillus sp., Corynebacterium spp., Micrococcus sp., Staphylococcus epidermidis, Aeromonas spp.*
3.	Object 3.	*Staphylococcus epidermidis, Bacillus sp., Micrococcus sp., Flavobacterium sp., Aeromonas spp.*

Bacterial species identified in the tested samples are bacteria that are not pathogenic or it belong to the opportunistic pathogens (Fricker 2003). Those represents the part of the flora, which is commonly found in unprocessed water or water in public distribution (LeChevallier et al., 1980). The presence of bacteria in the wells indicate that the water from which water is used to power farms include a different species of bacteria which belong to conditionally pathogenic microorganisms. Of the isolated bacteria in samples of tap water should be pointed *Aeromonas spp*. This bacterium is a ubiquitous microorganism widespread in water

and soil. The significance of these bacteria to human health is not well understood but they are isolated in humans with diarrhea (especially in infants) (Ashiru et al. 1993). With respect to animals, *Aeromonas spp.* are bacteria which have a limited health importance (Quinn et al 2011). Besides its importance for fish, amphibians, reptiles, some of these bacteria can be pathogenic for cattle (abortion) and the younger categories of dogs (septicemia). *Flavobacterium spp.* are also present in water and soil, but they do not pose a health risk for the animals except for some species of fish.

In addition to the above facts the presence of coliform bacteria (*E. coli*, *Proteus vulgaris*, *Enterobacter sp*.), faecal streptococci, *Pseudomonas aeruginosa* and bile-tolerant Gram negative bacteria *Aeromonas spp*. in samples of water from the drinkers, indicating addition to high contamination of water prohibited bacteria and the possibility of attachment of these microorganisms to specific receptors of enterocytes which begins infections of the body. Changing intestinale microflora as well as the synergistic effect of pathogen causes damage to the mucous membranes of the digestive tract, the appearance of hypersecretion, atrophy of the villi, damage intestinal mucosa and the occurrence of diarrhea and necrosis (Quinn et al 2011).

Table 2 presents data on the test results of water samples taken from drinkers in facilities where there are animals in the production. Samples were taken from several places, ie with a large number of drinkers in the house, after which he made pooled sample that was tested.

Table 2: Results of bacteriological examination of water from the drinkers

	Sampling location	**Finding**
1.	Object 1.	*Escherichia coli, Pseudomonas aeruginosa, Pseudomonas sp., Bacillus sp., Micrococcus sp., Staphylococcus epidermidis, Streptococcus faecalis, Aeromonas spp., Protus spp.*
2.	Object 2.	*Escherichia coli, Enterobacter spp., Pseudomonas aeruginosa, Aeromonas spp., Corynebacterium spp., Bacillus sp., Streptococcus faecalis, Micrococcus sp., Staphylococcus epidermidis*
3.	Object 3.	*Escherichia coli, Enterobacter spp., Pseudomonas sp., Staphylococcus epidermidis, Streptococcus faecalis Bacillus sp., Micrococcus sp., Aeromonas spp., Protus spp.*

In samples taken from drinkers in facilities, where are the animals, are isolated bacterial species that indicate water contamination with coliform bacteria, as well as species that are opportunistic pathogens (*Pseudomonas spp*.). The presence of *Ps. aeruginosa* in water samples taken from drinkers in poultry production objects was published in the article (Stojanov et al. 2013). The paper said that the application of antibiotics can lead to changing the bacterial flora of water and potentiate survival of resistant bacterial strains. A very common finding *Ps. aeruginosa* in sewage and surface waters was presented by the authors (Fuentefria et al. 2011) where they emphasize the importance of these isolates as vectors in the spread of resistance to certain antibiotics.

The presence of different types of bacteria in the water and water supply system may be the result of specific adaptations of organisms on living conditions with little nutrients. Many bacteria may be able to colonize the water supply system (Reasoner et al 1989). Such properties may has bacteria such as *Enterobacter*, and *Klebsiella*, but also other opportunistic organisms such as *Aeromonas*, *Pseudomonas*, *Flavobacterium*, and *Acinetobacter*. The presence of these of viable but nonculturable bacteria (VBNC - viable but nonculturable) may occur as a result of poor nutritional conditions in the water and used equipment as well as by changes in pH, salinity and other factors of water (Fricker 2003).

CONCLUSIONS

Water analysis showed that there are differences in the composition of bacterial species between the samples of tap water and drinkers from objects. Number of opportunistic pathogenic bacteria is less in samples taken from the tap water. Samples of water from the drinkers are contained in addition to *Pseudomonas* species and coliform bacteria (*E. coli, Enterobacter spp. Proteus spp*.). Finding these bacteria in water samples from drinkers indicates that drinkers may be place which bacteria can colonize and which may persist. Reasons to bacterial persistence in the drinkers may be the inability of high-quality disinfection of the water system after the completion of the production cycle, lagging feed on drinkers after it were used by animals which creates favorable conditions for bacteria or biofilm formation.
The obtained results demonstrate the need for quality maintenance of the water system of the farm. Recommended measures of hygiene which predict rinse water supply system after each production cycle will contribute to reducing the number and species of bacteria. Also it seems to be important during the bacteriological control of water samples to check which kind of isolated strains were biofilm producer, and if it's possible impact on their presence.

ACKNOWLEDGEMENTS

This investigation was financially supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Project No. TR 31071.

REFERENCES

Ashiru, J.O., Salau, T. and Rotilu, I. O.(1993). Incidence of Aeromonas species in diarrhoeic stool, Comparative Immunology, Microbiology and Infectious Diseases, 16, 51-54.

Fuentefria Bopp Daiane, Alessandra Einsfeld Ferreira, Gertrudes Corção (2011). *Antibiotic-resistant Pseudomonas aeruginosa from hospital wastewater and superficial water: Are they genetically related?.* Journal of Environmental Management 92, 250-255

Lechevallier W. Mark, Ramon J. Seidler, And T. M. Evans (1980). *Enumeration and Characterization of Standard Plate Count Bacteria in Chlorinated and Raw Water Suppliest.* Applied And Environmental Microbiology, p. 922-930

Official Gazette of the SFRY; Ordinance on the method of sampling and laboratory methods for the analysis of drinking water. 1987, (Službeni list SFRJ; *Pravilnik o načinu uzimanja uzoraka i metoda za laboratorijsku analizu vode za pić*e. 1987)

Official Gazette of the Republic of Serbia: Law on Food Safety, no. 41/2009, (Službeni glasnik Republike Srbije: Zakon o bezbednosti hrane, br. 41/2009)

Quinn J.P., Markey K.B., Leonard C.F., FitzPatrick S.E., Fanning S., Hartigan J.P. (2011). Veterinary Microbiology and Microbial Disease, Second Edition, Wiley-Blackwell

Reasoner D.J., Blannon J.C., Geldreich E.E. and Barnick J. (1989): Nonphotosynthetic pigmented bacteria in a potable water treatment and distribution system. Applied And Environmental Microbiology, 55, 912-92.

Stojanov I., Kapetanov M., Stojanović D., Ratajac R., Prodanov-Radulović J. (2003). The antibiotic therapy and their influence on bacterial flora of water on the farm = Uticaj antibiotske terapije na bakteriološku floru vode za napajanje na farmama.Environmental protection of urban and sububan settlements : Proceedings, XVII International Eco-Conference, 25-28th September 2013, Novi Sad, project editor Nikola Aleksić, Novi Sad, Ecological Movement of Novi Sad, Str.121-125, ISBN 978-86-83177-47-9 (eng)

World Health Organization. The Presence of bacteria in water after regrowth. (2003) Heterophilic plate Counts and Drinking-water Safety, Edited by J. Bartam, J. Cotuvo, M. Exner, C. Fricker, A. Glasmacher,Published by IWA Publishing London, UK.

UTILIZATION OF PROTEIN AND ENERGY FROM FEED MIXTURES CONTAINING DIFFERENT CONTENT OF PROTEINS IN CARP YEARLINGS

Marko Stanković[1]*, Zorka Dulić[1], Nada Lakić[1], Božidar Rašković[1], Ivana Živić[2], Vesna Poleksić[1], Zoran Marković[1]

[1]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia
[2]University of Belgrade, Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia

*Corresponding author:
E-mail address: markos@agrif.bg.ac.rs

ABSTRACT

Proteins present a necessary component in animal feed due to its important role in various physiological processes. The raising cost of fish meal (FM) on the world market initiated a lot of research among fish specialists to try and find possibilities for replacement of FM with more sustainable alternatives and optimize the protein level in diets for a variety of fish species and categories.
The aim of this study was to investigate the utilization of proteins and energy in feed mixtures with different content of proteins in diets for carp yearlings.
Fish were fed with concentrate mixtures having 38% (feed A), 41% (feed B) and 44% (feed C) of proteins.
Based on the results of Tukeys' test, fish fed with concentrate mixture containing 38% of proteins had significantly lower protein intake (1167.43 g) than fish fed with feed containing 41% (1457.73 g) and 44% of proteins (1569.83). Values of protein efficiency ratio (PER) were in the range from 0.96 (feed A) to 1.47 (feed C).
Average values of energy intake were 600.57 kJ (A), 695.16 kJ (B) and 718.13 kJ (C), providing significant difference between fish fed mixtures with the highest and the lowest content of proteins in diet and significant difference between fish fed mixtures with 41 and 44% of proteins.
The obtained values for energy efficiency ratio were 1.86 (A), 2.42 (B) and 3.21 (C), providing significant difference depending on the level of proteins in the diet. The highest feed utilization was obtained with feed C where FM is the prevailing protein.
***Keywords**: protein utilization, carp yearlings, fish feed*

INTRODUCTION

Proteins present a necessary component in feed for In fish, as in other domesticated animals, due to its important role in various physiological processes. Fish meal (FM), as the most commonly used component in diets for fish, is considered to be the best source of proteins in feed since it provides best production results (Barlow, 2003; Jackson, 2010). The raising cost of this component on the world market mostly due to the stagnating global capture fisheries production (Rana et al., 2009; Brinker and Reiter, 2011) initiated a lot of research among fish specialists to try and find possibilities for replacement of FM with more sustainable alternatives and optimize the protein level in diets for a variety of fish species and categories (Glencross et al., 2008; Marković et al., 2012).
From the economical and environmental point of view it is important to create aquafeed that will provide low conversion rate, high growth rate and good health of fish, high quality of fish meat, low organic and especially inorganic phosphorus and nitrogen load (Jahan et al., 2003). Nevertheless, fish producers are also trying to improve the technological process and lower the feed loss thereby enhancing the production profitability (Bailey and Alanärä, 2005). Therefore, the aim of this study was to investigate the utilization of proteins and energy if feed mixtures with different content of proteins in diets for carp yearlings.

MATERIAL AND METHODS

The experiment was carried out in the Laboratory for Fish Nutrition of the Faculty of Agriculture, University of Belgrade. Fish were fed with concentrate mixtures having 38% (feed A), 41% (feed B) and 44% (feed C) of proteins. The bigger share of the protein part in A were plant proteins (PP), in C fish meal (FM), while in B the share of FM and PP was approximately the same (Table 1).

Table 1: Components and chemical composition of experimental diets (% dry matter)

Feed	**A**	**B**	**C**
Fish meal	26.0	30.0	32.0
Soybean meal	29.0	30.0	31.0
Yeast	2.0	6.0	8.0
Wheat gluten	5.0	5.0	5.0
Wheat	11.5	11.5	11.5
Corn	24.0	15.0	10.0
DCP	1.2	1.2	1.2
Calcium	0.3	0.3	0.3
Min. Vit.premix	1.0	1.0	1.0
Total	100.00	100.00	100.00
DM gkg^{-1}	937	937	892
Protein	38.10	41.52	43.72
Lipid	8.54	9.07	9.64
Ash	9.50	9.61	10.76
Fiber	2.03	2.45	2.02
[1]NFE	41.83	37.35	33.86

[1]*NFE= 100 –proteins (g) –fat (g) –ash (g) –cellulose (g)*

In total, 12 independent tanks, with 120 L of usable water volume and flow rate of 0.34 $Lmin^{1}$ were used. Fish were acclimated to laboratory conditions during the period of 2 weeks. Each tank was stocked with 24 carp yearlings, average weight 95.6 g.

Water quality and environmental conditions (dissolved oxygen, water temperature, electroconductivity, and pH) were measured in each tank daily using MULTI 340i/SET (WTW, Weilheim, Germany). Air was supplied constantly by a blower to maintain the O_2 concentration around 6 mg/L. The water temperature (23 ± 1°C) was controlled by a thermostat. Experiment duration was 90 days.

Fish were fed with same percentage of feed depending on the total quantity of fish in each tank, i.e. 3.5% of the ichthyomass, using semiautomatic feeders with pendulum.

For every day measurements of feed quantity, as well as for control measurements each 30 days, a digital balance CASBEE, model MW 120; Casbee, Samsungm Korea, accuracy 0,01 g was used, while an ichthyometer was employed for length and height measurements.

Parameters for growth performances were calculated using following equations:

PI (Protein intake) = total feed intake × (crud protein % of diet / 100), Singh et al., 2011;
EI (Energy intake) = total feed intake × (gross energy % of diet / 100), Cho et al., 2001;
PER (Protein efficiency ratio) = weight gain / total protein intake, Singd et al., 2011;
EER (Energy efficiency ratio) = weight gain / total energy intake, Cho et al., 2001;
DEN (Digestible energy need) = (total feed intake × digestibility energy) / weight gain (Alanärä et al., 2001).

Statistical analysis was done using two-factorial analysis of variance with factors type of feed and period. Individual comparison of average values was carried out using Tukey test.

RESULTS AND DISCUSSION

During the experiment physical and chemical characteristics of water, according to Marković (2010), Flajšhans and Hulata (2007), Hover (1976), all the values monitored were within the optimal range for carp growth (temperature was 22.75 ± 0.02°C, electroconductivity was 524.35 ± 0.48 μS/cm, dissolved oxygen was 6.41 ± 0.04 mg/L, and pH value was 7.48 ± 0.01.

Table 2. Initial weight (IW), final weight (FW), feed intake (FI) in common carp fed the experimental diets

Parameter	A (mean±SE)	B (mean±SE)	C (mean±SE)	ANOVA	
				F	p
IW	95.59 ± 3.80^{NS}	95.33 ± 3.44^{NS}	95.23 ± 2.95^{NS}	0.016	≈1.000^{NS}
FW	149.79 ± 5.50^{a}	173.56 ± 6.78^{b}	200.18 ± 6.19^{c}	24.031	<0.001**
FI	1.55 ± 0.05^{NS}	1.57 ± 0.03^{NS}	1.65 ± 0.13^{NS}	0.812	0.497^{NS}
PI	1167.43 ± 86.26^{a}	1457.73 ± 58.84^{b}	1569.83 ± 94.71^{b}	15.962	<0.001**
PER	0.96 ± 0.06^{a}	1.15 ± 0.03^{b}	1.47 ± 0.08^{c}	19.728	<0.001**
EI	600.57 ± 44.37^{a}	695.16 ± 28.06^{b}	718.13 ± 43.33^{b}	5.782	0.003**
EER	1.86 ± 0.12^{a}	2.42 ± 0.07^{b}	3.21 ± 0.18^{c}	33.197	<0.001**
DEN	0.65 ± 0.04^{a}	0.50 ± 0.01^{b}	0.38 ± 0.02^{c}	14.963	<0.001**

Small letters indicate significant differences (p < 0.05) across rows

Based on the results from Tukeys' test, fish fed with concentrate mixture containing 38% of proteins had significantly lower (p<0,001) protein intake (1167.43 ± 86.26 g) than fish fed with feed containing 41% (1457.73 ± 58.84 g) and 44% of proteins (1569.83 ± 94.71 g). Values of protein efficiency ratio (PER) were in the range from 0.96 ± 0.06 (feed A) to 1.47 ± 0.08 (feed C). Significant difference for PER was noted between fish fed with feed A and C and between fish fed feed A and B (p<0.001 and p=0.028, respectively).

Average values of energy intake (EI) were 600.57 ± 44.37 kJ (feed A), 695.16 ± 28.06 kJ (feed B) and 718.13 ± 43.33 kJ (feed C), providing significant difference (p=0.003) between fish fed mixtures with the highest and the lowest content of proteins in diet and significant difference (p=0,017) between fish fed mixtures with 41 and 44% of proteins.

The obtained values for energy efficiency ratio (EER) were 1.86 ± 0.12 (feed A), 2.42 ± 0.07 (feed B) and 3.21 ± 0.18 (feed C), providing significant difference (p<0,001) depending on the level of proteins in the diet.

Values for digestible energy need (DEN) were ranging from 0.38 ± 0.02 (feed C) to 0.65 ± 0.04 (feed A). Tukey's test showed significant differences between fish fed mixtures with 38 and 44% (p<0.001), 38 and 41% (p=0.019) and 41 and 44% (p=0.017) of proteins, respectively.

Our results are in line with the study of Ahmad et al. (2012) that obtained the highest value for PER in fish fed with mixture containing 40% of proteins (2.66). They consider that the values of PER are in positive correlation with the optimal ≥40% of proteins in the mixture, as well as with the total content of fat in the mixture. Buyukcapar and Kamalak (2007) attained similar results for PER (from 1.7 to 2.2) but with no significant difference (p>0.05) when replacing fish meal up to 35% and soybean meal up to 60% with nut oil cake. However, with the further increase of nut oil cake level in the diet, the fiber content also raises directly affecting the digestibility of feed.

Cho et al. (2001) obtained significantly lower values of EER (0.20) by using feed mixtures with 35% of proteins compared to the mixtures with 40% (EER=0.23) and 45% (EER=0.24), justifying the results of the present investigation.

Significant difference was obtained for DEN between feed mixtures for carp with different protein content. Bailey and Alanara (2005) link DEN to the content of fat in the diet. Higher fat content in the fish diet lead to higher accumulation of body fat and therefore the increase of DEN values.

CONCLUSIONS

Based on the obtained results it can be concluded that the utilization of the proteins showed a statistically significant difference ($p<0.01$) in fish fed diets with different protein content. PER was positively correlated with the protein content in the diet. In addition, the protein efficiency ratio depended on the protein origin, so the highest values were observed in the group of fish fed with feed C (1.47 ± 0.08 g/g^{-1} protein), which had the highest proportion of fish meal.

The energy efficiency ratio was statistically significantly different ($p<0.01$) in fish fed diets with different amount of protein. Diet C had the best energy efficiency, 3.21 ± 0.18 g weight gain kJ^{-1}. The EER in fish fed diets A and B was 1.86 ± 0.12 g/kJ^{-1} and 2.42 ± 0.07 g/kJ^{-1}. It can be concluded that the significantly better utilization of protein and energy from diet C led to a significantly higher ($p<0.01$) final weight of fish fed this mixture.

ACKNOWLEDGEMENTS

The study was supported by the Ministry of Education, Science and Technological Development, the project Improvement of production capacities of carp (*Cyprinus carpio*) through nutrition and selective breeding programs (TR 31075) and FP7 project AREA (316004).

REFERENCES

Ahmad, M., Qureshi T. A., Singh A. B., Manohar, S., Borana, K., Chalko, S. R. 2012. Effect of dietary protein, lipid and carbohydrate contents on the growth, feed efficiency and carcass composition of *Cyprinus carpio* communis fingerlings. International Journal of Fisheries and Aquaculture 4, 30–40.

Alanärä, A., Kadri, S., Paspatis, M. 2001. Feeding management. In: Food Intake in Fish (Houlihan, D., Jobling, M., Boujard, T., eds.). Blackwell Science, Oxford, UK, pp. 332–353.

Bailey, J., Alanärä, A. 2005. Digestible energy need of selected farmed fish species. Aquaculture 251, 438–455.

Barlow, S. 2003. World market overview of fish meal and fish oil. In: Advances in Seafood Byproducts: 2002 Conference Proceedings (Bechtel, P.J., ed.). Alaska Sea Grant College Program, University of Alaska Fairbanks, Fairbanks, pp. 11–25.

Brinker, A., Reiter, R. 2011. Fish meal replacement by plant protein substitution and guar gum addition in trout feed, Part I: Effects on feed utilization and fish quality. Aquaculture 310, 350–360.

Buyukcapar H. M., Kamalak, A. 2007. Partial replacement of fish and soyabean meal protein in mirror carp (*Cyprinus carpio*) diets by protein in hazelnut meal. South African Journal of Animal Science 37, 35–44.

Cho, S. H., Jo, J. Y., Kim, D. S. 2001. Effects of variable feed allowance with constant energy and ratio of energy to protein in a diet for constant protein input on the growth of common carp *Cyprinus carpio L*. Aquaculture Research 32, 349–356.

Glencross, B., Hawkins, W., Evans, D., Rutherford, N., Dods, K., Mccafferty, P., Sipsas, S. 2008. Evaluation of the influence of Lupinus angustifolius kernel meal on dietary nutrient and energy utilization efficiency by rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition 14, 129–138.

Jackson, A. 2010. The global supply and demand for fishmeal and fish oil. Fiaap Asia, March 4, 2010, Bangkok Thailand.

Jahan, P., Watanabe, T., Kiron, V., Satoh, S. 2003. Improved carp diets based on plant protein sources reduce environmental phosphorus loading. Fisheries Science 69, 219–225.

Marković, Z., Poleksić, V., Lakić, N., Živić, I., Dulić, Z., Stanković, M., Spasić, M., Rašković, M., Sørensen, M. 2012. Evaluation of Growth and Histology of Liver and İntestine in Juvenile Carp (*Cyprinus carpio*, L.) Fed Extruded Diets with or without Fish Meal. Turkish Journal of Fisheries and Aquatic Sciences 12: 1–2.

Rana, K. J., Siriwardena S., Hasan M. R. (2009): Impact of rising feed ingredient prices on aquafeeds and aquaculture production. FAO Fisheries and aquaculture technical paper 541. 63p.

Singh, P., Maqsood, S., Samoon, M. H., Phulia, V., Danish, M., Chalal, R. S. 2011. Exogenous supplementation of papain as growth promoter in diet of fingerlings of *Cyprinus carpio.* International Aquatic Research 3, 1–9.

EXTRACT FROM MEDICINAL PLANTS MIXTURE AS ANTICOCCIDIAL AND ANTIOXIDANT IN BROILERS

Sanja Teodosin[1], Ljiljana Kostadinović[1]*, Ivana Čabarkapa[1], Jovanka Lević[1], Ljubiša Šarić[1], Vojislav Banjac[1], Ljiljana Suvajdžić[2]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Medicine, Departman of Pharmacy, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: ljiljana.kostadinovic@fins.uns.ac.rs

ABSTRACT

The mixture (MHE) of *Artemisia absinthium, Thymus vulgaris, Menthae piperitae, Thymus serpyllum* was evaluated upon blood and liver oxidative status (glutathione peroxidase-GSHPx, superoxide dismutase-SOD and concentration of malondialdehyde-MDA) and anticoccidial effects in broilers experimentally infected with mixture of oocysts of *Eimeria* spp. (20,000 oocysts/bird), in comparison to coccidiostatic salinomycine.

The *in vivo* investigation was carried out on 120 day-old Arbor acres broilers separated into 4 equal groups with 3 replicates each. Group A was uninfected and untreated. Group B was infected and untreated. Group C preventively received coccidiostatic salinomycine in quantity of 60 mg/kg and was inoculated with coccidia species at 21st day-of-age. Group D consumed a basal diet supplemented with extracts of herbs mixture in quantity of 2 g/kg and was infected with *Eimeria* oocysts at 21st day-of-age. Clinical signs, number of oocysts per bird (OPB) and mortality were monitored daily for 42 days. The anticoccidial activity of chosen medicinal plants extract caused a significant decrease in output number of oocysts per bird in broilers challenged with *Eimeria* spp.

The obtained results indicated a statistically significant ($P < 0.05$) increase of GSHPx activity in blood hemolysates. Moreover, the catalytic activity of SOD showed a statistically significant increase in group B comparing with the group A. The preventive doses of coccidiostatic indicated a statistically significant ($P < 0.05$) decrease of MDA concentration, reduction of SOD activity and decrease of GSHPx activity compared with group B. The activity of GSHPx in liver homogenates of broilers group B showed a statistically significant increase in comparison to the group A. Furthermore, the SOD activity increased the level of statistical significance.

Medical plants mixture can be used as prophylactic feed additive and source of antioxidant in dietary supplement since reduces the severity of coccidial infection induced by *Eimeria* spp. and exhibits a significant antioxidant activity in broilers fattening.

Keywords: *broilers, medicinal plant mixture, antioxidative status, anticoccidial*

INTRODUCTION

Coccidiosis is one of the most detrimental diseases in poultry due primarily to causing a decrease in daily gain, prolonge fattening, poorer skin pigmentation, slower and increase mortality (Christaki *et al.*, 2004). Coccidiosis is traditionally treated by chemotherapy, but the persistent appearance of drug – resistant strains of coccidia indicate the importance of developing alternative strategies. Many authors investigated alternatives to antibiotics (Mellor, 2000; Ocak et al., 2008). Medicinal plants have recently reported as alternatives to antibiotics in animal production and are claimed to be "digestive enhancers" (Williams and Losa, 2001). They are very complex mixtures of compounds, such as tannins, terpenoids, alkaloids and flavonoids. Many *in vitro* studies (Demirel *et al.*, 2011; Lević *et al.*, 2011) reported antimicrobial properties of medicinal plants. In addition to their antimicrobial activity, medicinal plants possess various biological activities, one of them is antioxidant activity (Aliyu *et al.*, 2012; Radivojević *et al.*, 2012). Introduction of essential oils in animal feed may have promising potential as a growth and health promoter without adverse effects.

Serbia has a wide range of medicinal herbs which posses a number of chemical substances for the use in poultry. *Artemisia absinthium* L. (Asteraceae) contains β-thujone, (Z)-6,7-epoxyocimene and sabinyl acetate (Juteau *et al.*, 2003). Thyme essential oil is characterized by the presence of thymol, carvacrol, p-cymene and γ-terpinene. Menthol and menthone are the main compounds of mint essential oil. In essential oil of *Thymus serpyllum* were 55 compounds identified. The main components are (E)-nerolidol, caryophyllene oxide, myrcene, (E)-β-caryophyllene and germacrene D (Daferera *et al.*, 2000).
The present study was designed to observe the antioxidative and anticoccidial efficacy of aqueous mixed extract mixture of *Artemisia absinthium, Thymus vulgaris, Menthae piperitae* and *Thymus serpyllum.*

MATERIAL AND METHODS

Collection and processing of plant materials

The plants used for this study were *Artemisia absinthium, Thymus vulgaris, Menthae piperitae* and *Thymus serpyllum.* The stem barks of the plants were collected in April and May 2014 from the Pančevo region (Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade). The stem barks of the plants were dried under shade for 10 days at 8h per day and then ground into powder. The powdered extracts were individually exhaustively soxlet extracted with water for 8h at 60°C (Onyeyili *et al.*, 2001*)*. The soluble extract was then concentrated in a conical flask placed in a water bath maintained overnight at 60°C, collected, weighed and stored at 4°C for later use in the study.

Experimental animals

Experiments under *in vivo* conditions were performed on 120 broilers of both sexes of the heavy Arbor acres line. The chickens were randomly divided into non-infected and infected groups. The second group of broilers were infected with mixture of sporulated oocysts of *E. tenella* (5000 oocysts), *E. mitis* (5000 oocysts), and *E. necatrix* genus (10000 oocysts) collected from infected chicken farms. Coccidial oocysts of *E. tenella, E. mitis* and *E. necatrix* were obtained from the guts of infected chickens and they were preserved in 2.5% potassium dichromate solution to induce sporulation and subsequently kept in a refrigerator at 2 - 5°C until use. The challenge infection of 21st day–old chickens was performed by oral administration of 1 cm^3 oocyst suspension. Faecal samples were taken daily in order to monitor the possibility of infection.

Experimental protocol

Experiments under *in vivo* conditions were performed on broilers of the heavy line Arbor acres, of both sexes. One-day-old broilers, randomly selected, were divided into four groups, each containing 30 individuals:

Group A**:** uninfected and unmedicated broilers – negative control group. Decapitation of 10 chickens was carried out at 30th day-of-age.
Group B**:** infected and unmedicated broilers – positive control group. Inoculation of 21 day-old broilers was performed by *p.o.* application of 1cm^3 of coccidial suspension mixture of sporulated oocysts. Nine days later (30th day-of-age), when first clinical signs of disease appeared, decapitation of 10 chickens were carried out.
Group C: broilers which received preventively coccidiostat salinomycin in quantity of 60 mg/kg of feed (Group C_1) and the remaining broilers inoculated with laboratory derived coccidia species at 21st day-of-age (Group C_2). Decapitation of 10 chickens was carried out at 30th day-of-age.
Group D: broilers which received aqueous extract from medicinal plants in quantity of 2 g/kg (Group D_1) and the remaining broilers infected with *Eimeria* oocysts at 21st day-of-age (Group D_2). Blood and liver were collected at 30th day-of-age.

The oocyst output was measured daily in each group, during the period from 6th to 9th day after the infection. Oocyst counts were determined using McMaster chambers and presented as the number of oocysts per bird (Hodgson, 1970).
Levels of hemoglobin, necessary for the expression of the enzymatic activities in hemolysed blood, were determined using commercial test ("Dialab", Vienna, Austria) on a spectrophotometer (Multiscan MCC 340, Finland). Protein content was determined by the method of Prakash *et al.* (2010). In hemolysed blood and homogenized liver, products of lipid peroxidation and the activities of antioxidant enzymes (glutathione peroxidase -GSHPx, superoxide dismutase -SOD) were determined.

Preparation of blood hemolysate
Blood was collected by heart puncture of broilers into heparinized test tubes. After centrifugation (10 min at 3500 rpm and 4°C) and plasma removal, the erythrocytes were rinsed 3 times in saline. The resulting erythrocyte pellet was suspended in an equal volume of double distilled water and vortexed. After incubation for 1 hour at room temperature, the hemolysate was centrifuged for 15 min at 3500 rpm and supernatant aliquoted for further analysis (Vossen *et al.*, 2010).

Preparation of liver homogenate
One gram of the excised liver was minced with scissors and homogenized in an ultraturax in 3 volumes of isotonic buffer (0.05 mol/dm^3 tris-HCl, 0.25 mol/dm^3 sucrose, pH=7.5). The homogenate was filtered through gauze into ice-cold tubes and aliquoted for further analysis (Chiu *et al*., 1976).

Determination of enzymatic activity
The SOD (EC 1.15.1.1) activity was determined by the spectrophotometric method based on the inhibition of adrenaline reduction to adrenochrome at pH= 10.2 (Kostadinović *et al.*, 2001). The GSHPx (EC 1.11.1.9) activity was determined by spectrophotometric measurement of absorbance at 412 nm with cumenhydroperoxide as the substrate (Chiu *et al.*, 1976).
Lipid peroxidation was determined by thiobarbituric acid (TBA–test). The oxidation of cellular membrane lipids was measured via reaction of lipid peroxides with thiobarbituric acid (Simmon *et al.*, 1974).

Statistical analysis
The results given in tables are reported as the mean ± standard deviations (*SD*) of a number (*n*) of independent determinations. The one way ANOVA analysis was performed to assess data differences between various groups using STATISTICA Version 12 (2013). The data means were considered different at $P < 0.05$.

RESULTS AND DISCUSSION

Bloody diarrhoea was observed from the fourth to sixth day after the infection with *Eimeria* spp. in all experimental groups except the uninfected control group (A).
Excreted oocysts count in the groups C_2 and D_2 were lower in comparison to infected control group (B) (Table 1). Administration of a mixture of herbal extracts (MHE) and coccidiostat salinomycin before infection with *Eimeria* spp. was shown to be associated with the reduction of oocyst output. The summary of statistical values obtained from 30 chickens in each test groups is shown in Table 1. However, the non-treated chickens infected with *Eimeria* spp. (B) showed significant excretion of oocysts in faeces (Table 1). The salinomycin treated broilers (C_2) showed complete reduction of oocyst in faeces at 30th day.

Table 1. Effectivenes of salinomycin and MHE on faecal oocyst counts and mortality rate in different treatment groups of broilers

Group	Oocysts excretion (x 10^6)·bird^{-1}				Mortality rate (%)
	Day of infection	After infection			
	21 day	24 day	27 day	30 day	
A	0	0	0	0	3
B	2.3 ± 0.3^b	34.5 ± 1.8^c	3.8 ± 0.4^c	0.4 ± 0.1^c	12
C_2	1.0 ± 0.1^a	2.2 ± 0.2^a	0.1 ± 0.02^a	0^a	5
D_2	1.7 ± 0.2^b	7.3 ± 0.1^b	0.4 ± 0.01^b	0.08 ± 0.01^b	7

MHE- mixture of herbal extracts; A - negative control; B-positive control; C_2 - salinomycin 60 mg/kg of feed and infected; D_2 - MHE 2g/kg of feed and infected.
Results are given as mean ± standard deviation (n = 3);
$^{a-c}$Means within a column with no common superscript differ significantly at P < 0.05

In MHE treatment group (D_2) the oocysts output and mortality rate were lower in comparison to positive control group (B). Therefore it can be concluded that MHE was effective in reducing the oocyst output of the preventive treated and infected broilers. Some herbal extracts have already been shown to possess a coccidiostat activity (Youn and Noh, 2001). This biological activity has been mainly attributed to phenolic components. Phenols interact with the cytoplasmic membrane by changing its permeability for cations, like H^+ and K^+. The dissipation of ion gradients leads to the impairment of essential processes in the cell, allows leakage of cellular constituents, resulting in water unbalance, collapse of the membrane potential and inhibition of ATP synthesis, and finally cell death (Ultee *et al.*, 1999).
Presented results (Figure 1) indicate a significant increase of LPx content and catalytic activity of GSHPx and SOD in blood hemolysates of infected broilers (group B) compared with the negative control group (group A). The most likely explanation for the observed phenomena is that the pathological alterations intensify free radical processes by stimulating catalytic activities of enzymes involved in the antioxidative protection. However, during the disease period's lipolysis from the lipid depots is increased due to smaller food intake and exhaustion of the organism by diarrhea which leads to intensification of free radical processes and formation of larger quantities of lipid peroxides in blood. Newly formed lipid peroxides and their degradation products are transported by blood stream to inactive organs and tissues having toxic effect on them and generating cellular membrane damages. In order to protect itself the organism activates its antioxidative protection system. Concomitantly with the increased risk of lipid peroxidation in blood, there is an increase in the enzymatic activity of GSHPx.
The preventive doses of coccidiostatic salinomycin indicated a significant decrease of activity of GSHPx and significant reduce of catalase-activity of SOD. Also, it was noted statistically significant decrease of LPx content compared with the group B.
Infection in group of broilers C_2, nine days later (30^{th} day–of–age) resulted in significant decrease of LPx content and activity of investigated enzymes were significantly (GSHPx and SOD) lower comparing to group B.
The activity of GSHPx in blood hemolysates of broilers group supplemented with MHE (Group D_1) were significantly higher compared to the group A and group C_1. MHE did not affect on the LPx content in hemolysates of broilers. Broilers of group supplemented with MHE had greater activity of SOD than broiler chickens in control and salinomycine treated group. If we compare the results of the effects of preventive doses of salinomycin or MHE on the activity of antioxidative enzymes in blood hemolysates, we get a good agreement.
The content of LPx and the catalytic activity of selected enzymes of the antioxidative defense system found in the liver homogenates of control and experimental groups are shown in Figure 2. The content of LPX and activity of GSHPx and SOD in liver homogenates of broilers group B showed a significant increase in comparison with the group A.

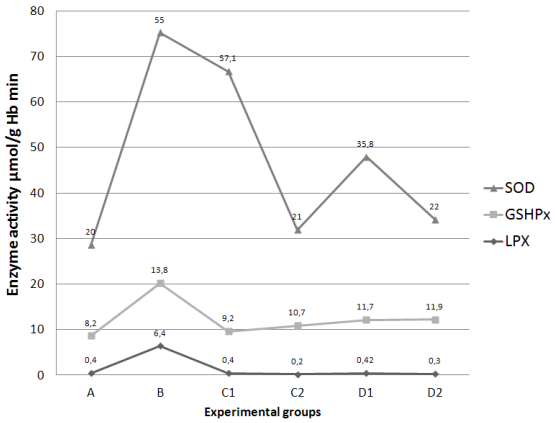

Figure 1. LPx content and the activity of GSHPx and SOD in blood hemolysates

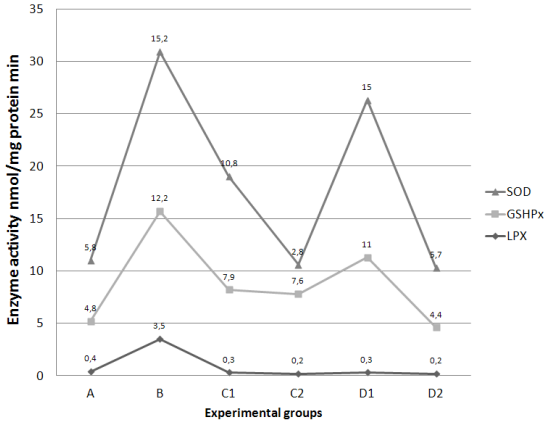

Figure 2. LPx content and the activity of GSHPx and SOD in liver homogenates

Infection with *Eimeria* spp. in group of broilers C_2 nine days later (30th day–of–age) resulted in lower activity of GSHPx, also content of LPx and activity of SOD were decreased compared to the group B and group of broilers C_1. On the basis of the observed changes of enzymes activity, we may conclude that induction and inhibition of their activity in liver homogenates of preventive treated with salinomycin and infected broilers continued until reaching the basic activity–level characteristic for the corresponding control group, i.e. having the tendency of eliminating the negative effects induced by the disease (Figure 2).

In liver homogenates of preventive MHE treated broilers and then infected was observed a highly significant decrease of LPx content, decrease of GSHPx activity and inhibition of SOD activity in comparison with the group A. The results in Figure 1 and 2 also indicate more pronounced changes in antioxidative protective system in blood hemolysates compared with those in the liver homogenates of infected chickens (Group B). This finding is in accordance with published data by Olanlokun (2008). When *Eimeria* enter the digestive system, different developmental stages secrete specific metabolities that may be absorbed and induce changes in the enzymatic activity of the antioxidative protective system in a variety of tissues. Observed changes show positive preventive effects of applied MHE. Its application leads to decreased number of coccidian and therefore, to the decrease of the intensity of the disease induced free-radical processes. Comparing the results of the present investigation on effects of MHE on the antioxidative system in blood hemolysates (Figure 1) and liver homogenates (Figure 2) of broilers from group D_2, it can be concluded that applied MHE in concentration of 2g/kg of feed, has a greater positive effect on the antioxidative system of erythrocytes. This may be caused by producing statistically more significant changes of these biochemical parameters in comparison to liver homogenates, which can be explained by larger antioxidative capacity of liver and larger exposure of erythrocytes to the oxidative stress (Hunter and Mohamed, 1986).

CONCLUSIONS

The results of this study indicate that MHE is an effective agent in reducing the oocyst output of the preventively treated and infected broilers and could be a potential source of protection agents against coccidiosis. Excreted oocysts in the groups treated with 2 g/kg of MHE were lower than in the infected control group, but higher than in the salinomycin group.

Pathological changes in blood hemolysates and liver homogenates of artificially infected broilers intensified free radical processes. The obtained results show that infection with *Eimeria* oocysts exhibit negative effects on the antioxidant defense system in the blood and liver of broilers and that MHE demonstrates protective role against *Eimeria* infection.

ACKNOWLEDGEMENTS

This paper is a part of the project III 46012 financed by Ministry of Education, Science and Technological Development of Republic of Serbia and project 114-451-4685/2013-03 financed by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina.

REFERENCES

Aliyu, A.B., Ibrahim, M.A., Ibrahim, H., Musa, A.M., Lawal, A.Y., Oshanimi, J.A., Usman, M., Abdul Kadir, I.E., Oyewale, A.O., Amupitan, J.O. (2012). Free radical scavenging and total antioxidant capacity of methanol extract of Ethulia conyoides groving in Nigeria. Romanian Biotechnological Letters, 17 (4), 7458-7465.

Chiu, D.T.Y., Stults, F.H., Tappel, A.L. (1976). Purification and properties of rat lung soluble glutathione peroxidase. Biochimica et Biophysica Acta, 445, 558-566.

Christaki, E., Florou-Paneri, P., Giannenas, I., Papazahariadou, M., Botsoglou, N.A., Spais, A.B. (2004). Effect of mixture of herbal extracts on broiler chickens infected with *Eimeria tenella.* Animal Research, 53, 137-144.

Daferera, D.J., Ziogas, B.N., Polissiou, M.G. (2000). GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum.* Journal of Agriculture Food Chemistry, 48, 2576-2581.

Demirel, Z., Yilmaz-Koz, F.F., Karabay-Yavasoglu, N.U., Ozdemir, G., Sukatar, A. (2011). Antimicrobial and antioxidant activities of solvent extracts and the essential oil composition of *Laurencia obtusa* and *Laurencia obtusa var. Pyramidata*. Romanian Biotechnological Letters, 16 (1), 5927-5936.

Hodgson, J.N. (1970). Coccidiosis: oocyst-counting technique for coccidiostat evaluation. Experimental Parasitology, 28, 99-102.

Hunter, M., Mohamed, J. (1986). Plasma antioxidant and lipid peroxidation products in Duchenne muscular dystrophy. Clinica Chimica Acta, 155, 123-132.

Juteau, F., Jerkovic, I., Masotti, V., Milos, M., Mastelic, J., Bessiere, J.M., Viano, J. (2003). Composition and anti-microbial activity of essential oil of *Artemisia absinthium* from Croatia and France. Planta Medica, 69, 158-161.

Kostadinović, Lj., Pavlovski, Z., Pavkov, S. (2001). Effect of amprolium on the lipid peroxidation and the activity of superoxide dismutase in broilers blood and liver. Archiv fur Geflugelkunde, 65 (3), 118-122.

Lević, J., Čabarkapa, I., Todorović, G., Pavkov, S., Coghill-Galonja, T., Kostadinović, Lj. (2011). In vitro evaluation of antibacterial activity of essential oils from family Lamiaceae. Romanian Biotechnological Letters, 16 (2), 6034-6041.

Mellor, S. (2000). Antibiotics are not the only growth promoters. World's Poultry Science Journal, 16 (1), 14-15.

Ocak, N., Erener, G., Burak Ak, F., Sungu, M., Ozmen, A. (2008). Performance of broilers fed diets supplemented with dry peppermint (Mentha piperita L.) or thyme (Thymus vulgaris L.) leaves as growth promoter source. Czech Journal of Animal Science, 53 (4), 169–175.

Olanlokun, J.O. (2008). Protective influence of vitamin E on the antioxidant defense system in the whole blood and liver of normal and alloxan-induced diabetic rats. Indian Journal of Clinical Biochemistry and Nutrition, 23, 62-66.

Onyeyili, P.A., Nwosu, C.O., Amin, J.D., Jibike, G.I. (2001). Anthelmintic activity of crude aqueous extract of *Nauclea latifolia* stem bark against ovine nematodes. Fitoterapia, 72, 12-21.

Prakash, B.D., Manjunath, S., Kadi, S., Chetana, K., Vanishree, J. (2010). Oxidative stress and enzymatic antioxidant status in rheumatoid arthritis: a case control study. European Review for Medical Pharmacological Sciences, 14 (11), 959-967.

Radivojević. I.D., Stanković, M.S., Stefanović, D.D., Topuzović, M.D., Čomić, J. (2012). Antioxidative and antimicrobial properties of different extracts from Sideritis montana L. Romanian Biotechnological Letters, 17 (2), 7160-7168.

Simmon, L.M., Fatrai, Z., Jonas, D.E., Matkovics, B. (1974). Study of metabolism enzymes during the development of *Phascoulus vulgaris*. Biochem Physiol Pflanz, 166, 389-393.

STATISTICA (Data Analysis Software System) (2013). v.12.0., Stat-Soft, Inc., USA (www. statsoft.com)

Ultee, A., Kets, E.P.W., Smid, E.J. (1999). Mechanisms of action of carvacrol on the food borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology, 65, 4606-4610.

Vossen, E., Ntawubizi, M., Raes, K., Smet, K., Huyghebaert, G., Arnouts, S., De Smet, S. (2010). Effect of dietary antioxidant supplementation on the oxidative status of plasma in broilers. Animal Physiology and Animal Nutrition, 95, 198-205.

Williams, P., Losa, R. (2001).The use of essential oils and their compounds in poultry nutrition. World's Poultry Science Journal, 17, 14-15.

Youn, H.J. & Noh, J.W. (2001). Screening of the anticoccidial effects of herb extracts against *Eimeria tenella.* Veterinary Parasitology*, 96,* 257-263.

IDENTIFICATION OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* ISOLATED FROM MILK SAMPLES FROM COW WITH MASITITIS

Ljiljana Suvajdžić[1], Jovanka Lević[2], Maja Velhner[3], Dubravka Milanov[3], Ivana Čabarkapa[2], Maja Bekut[4], Zoran Suvajdžić[5]

[1]Medical Faculty, Department for Pharmacy, University of Novi Sad,
[2]Institute of Food Technology in Novi Sad, University of Novi Sad,
[3]Scientific Veterinary Institute „Novi Sad", Novi Sad,
[4]Medical Faculty, University of Novi Sad
[5]Veterinary ambulance, STRIKS, Čerević

*Corresponding author:
E-mail address: giba57@eunet.rs

ABSTRACT

Corynebacterium pseudotuberculosis is the most frequent causative agent of the disease in sheep and horses, but also in other mammals and humans. It could induce mastitis in lactating animals. The organism can survive the phagosomal mechanisms, which can lead to subsequent formation of abscesses. It is often misidentified in routine work. The goal of this work is to suggest diagnostic algorithm that is cost-effective, available, applicable and reliable.
This research was performed at the farm of diary Holstein-Friesian cows during an outbreak of acute mastitis. Milk samples were collected from 560 lactating cows. The samples were taken after cleaning and disinfecting each quarter of the udder. Samples were collected into sterile sampling tubes. California mastitis test was applied in all samples by adding equal volumes of CMT reagent (provided with the test) and milk collected from each quarter. The changes in milk fluidity and viscosity were observed. Samples were than inoculated on to the 10% sheep blood agar, Endo agar, Sabouraud, thioglycolate medium and nutrient broth. The plates were incubated for 3 days at 37°C in aerobic conditions. Cultural, morphological and conventional biochemical testing was done. Double CAMP and plasma coagulation tube test were applied as well. Total 28 isolates were included in a synergistic haemolysis with *Rhodococcus equi* (ATCC 6939) and inverse CAMP phenomenon with *Staphylococcus aureus* and coagulated rabbit plasma. Additionally, *Corynebacterium pseudotuberculosis* was confirmed using API Coryne V 2.0 and relevant BioMerieux software program. The identity rate was 99.9%, accuracy rate was T = 1 and test count was 0. Based on the results we concluded that the *Corynebacterium pseudotuberculosis* is present in our country. It could be misdiagnosed since applicable diagnostic protocols are lacking. In this paper we are suggesting simple, inexpensive and reliable diagnostic method for identification of *Corynebacterium pseudotuberculosis*.
Keywords: *Corynebacterium pseudotuberculosis, mastitis, diagnostic protocol, double CAMP*

INTRODUCTION

Nokard was the first who isolated *C. pseudotuberculosis*, in 1888. from clinical samples originating from cattle. Preisz fully described this microorganism and noted its similarity to *Corynebacterium diphtheria* six years later (Merchant, 1935). Synonyms for this bacterium are *Bacillus pseudotuberculosis ovis, Bacillus pseudotuberculosis, Corynebacterium ovis* and *Preisz-Nocard bacillus*.
In 1933, Kelser considered it to be an important animal pathogen that can cause severe economic losses (Merchant 1935). It belongs to section 17 by Bergey, *Corynebacterium-Mycobacterium-Nocardia- Rhodococcus* (CMNR) group, genus *Corynebacterium* and has all the properties of this Genus (Holt et al., 1991). The high content of lipids in the cell wall provides intracellular survival (it is protected from phagolysosomal fusion), which leads to the formation of abscesses. Dominantly it causes caseous lymphadenitis (CLA), mainly in sheep (Merchant, 1935, Hawari et al., 2014, Queen et al., 1994, Brown et al., 1987) and ulcerative lymphangitis in horses (ULA) (Merchant, 1935, Aleman et al., 1996). The disease is

widespread throughout the world, but more in the regions with intensive breeding of sheep, horses and goats (Dorella et al., 2006).
The selectivity to mammalian species is not explicit. The microorganism is isolated from pigs (Suvajdžić et al., 2000, Biberstein et al., 1990), cattle (Suvajdžić et al., 2000, Yeruham et al., 1996, Shpigel et,al., 1993, Hommez et al., 1999), camels (Hawari, 2008, Afzal et,al., 2000), goats (Brown and Olander, 1987, Ameh and Tari, 2000, Ivanović et al., 2009) and humans (Dorella et al., 2006, Paviour et,al., 2002, Peel et, al., 1997). As an agent of mastitis, it is reported increasingly (Yeruham et al., 1996, Shpigel et al., 1993, Hommez et al., 1999, Adekeye et al., 1980, Yeruham et al., 2003, Yeruham et al., 2004). Its detection requires additional diagnostics compared to routine protocols. In our country, there is a report of *C. pseudotuberculosis* isolation from the lungs of pigs and calves (Suvajdžić, 2000) and from the lymph nodes of goats (Ivanović et al., 2009). In this paper we report the identification of 28 strains of *C. pseudotuberculosis* from dairy cows with mastitis and propose a simple, reliable and accessible diagnostic test.

MATERIALS AND METHODS

Cattle farm

The study was carried out in the summer of 2010, during an outbreak of acute mastitis on a large cattle farm situated in the central part of the Autonomous Province of Vojvodina, Republic of Serbia. The farm is characterized by closed housing system of diary Holstein-Friesian cows. Most of the year, the cows are held in corals, but during the winter animals are tied in a stall barn. The animals are fed silage, dry beet pulp, brewer's grain containing 16% protein and green crop. Milking is performed according to standard regimen, twice a day, with an average milk yield of 6,500 liters. Udder papillae are disinfected before and after milking using chlorine based solutions.

Milk samples

Milk samples from 450 lactating cows were collected in sterile sampling tubes. Before the collection of quarter milk samples, the udder was thoroughly cleaned with soap and water and rubbed to dry. The teats were disinfected with cotton wool moistened with 70% ethyl alcohol and allowed to be air-dried. The first few squirts of milk were discarded. The quarter milk samples were stored in ice container and transported as soon as possible to the microbiological laboratory.

California Mastitis Test (CMT)

All collected milk samples were examined for mastitis using California mastitis test, which was carried out by the method first described by Schalm and Noorlander (1957). Briefly, equal volumes (5 mL) of commercial CMT reagent and quarter milk were mixed and the changes in milk fluidity and viscosity were observed (Madut and Abdelgadir, 2011).

Microbiological examination

Some of the milk samples were watery and contained clots (Adekeye et, al., 1980). Primary processing of all these samples was performed according to methodology by Suvajdzic et all (2012).
The same methodology was used in the following diagnostic steps: all isolates were presumptively identified based on colonial morphology, tinctorial status (using Gram, Neisser and Ziehl-Nielsen methods), rabbit plasma coagulation tube test, the production of CAMP phenomenon in double CAMP test with *Rhodococcus equi* (ATCC 6939) and *Staphylococcus aureus*.
Double CAMP test was performed on a separate Petri-dish with blood agar using *Staphylococcus aureus* and *Rhodococcus equi* (ATCC 6939) as diagnostic strains inoculated as vertical and parallel lines with an aim of confirmation or exclusion of both CAMP phenomenons by the investigated isolate (horizontal streak): synergistic haemolysis with *R. equi* and inverse CAMP phenomenon with *S. aureus* (Clarridge, 1989). For controls at

double CAMP test *Streptococcus agalactiae*, *Listeria monocytogenes*, *Streptococcus* non A non B group, *Corynebacterium* sp., *Corynebacterium pseudotuberculosis* and *Arcanobacterium pyogenes* were used.
Catalase and oxidase tests on nutritive agar were performed, as well as biochemical tests: fermentation of lactose and xylose, liquefaction of gelatin and hydrolysis of urea. The definitive biochemical identity of the bacteria was confirmed using API Coryne V 2.0 and software program-BioMerieux1. For control in plasma coagulation and catalase test *Staphylococcus aureus* was used, whilst *Pseudomonas aeruginosa* was used as control in oxidase test. Human isolates were used as the controls in staining procedures, i.e.: *Streptococcus pyogenes*, *Mycobacterium tuberculosis* and *Corynebacterium diphteriae* type gravis for Gram-, Ziehl Nielsen- and Neisser-staining, respectively.

RESULTS AND DISCUSSION

Out of 450 examined milk samples from cows with clinical mastitis and positive California-mastitis test result, 28 isolates were suspected as *Corynebacterium ulcerans* / *pseudotuberculosis*.
All 28 suspect strains formed visible colonies on 10%-sheep blood agar after 18 h of incubation. The colonies were ivorysh, smooth but matte and dry. They were clearly margined, with β-haemolysis zone, resembeling to hemolytic *staphylococci*, approximately 1 mm in diameter after 24 h (Figure 1). The colonies were ingrowing in to the agar and taking off without disintegration. Dispersion of the colonies in liquid media was difficult.

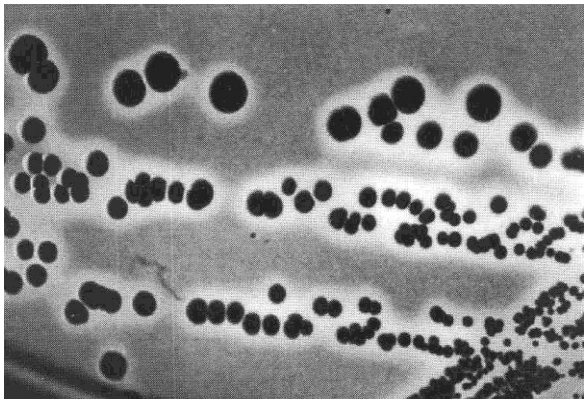

Figure 1. Corynebacterium pseudotuberculosis, 48h of incubation, complete lysis of erythrocytes

Subcultures on thyoglycolate medium revealed bacterial growth in all incubation conditions; however, the best growth was observed in microaerophylic conditions. The isolates survived at 4ºC, but the phenomenon of "cold enrichment" was not observed. The growth of colonies on a nutritive agar was observed after 24 h, but their size was significantly smaller then of those grown on blood agar, reaching a diameter up to 0.5 mm. Gram-staining revealed Gram-positive rods and coccoid forms. Existence of metachromatic granules and acid-resistance was excluded by Neisser-staining and Ziehl-Nielsen staining, respectively. All the investigated strains coagulated rabbit plasma. In a double CAMP test all examined strains developed both CAMP phenomenons: a synergistic haemolysis with *Rhodococcus equi* (ATCC 6939), and inverse CAMP phenomenon with *Staphylococcus aureus* (Figure 2).

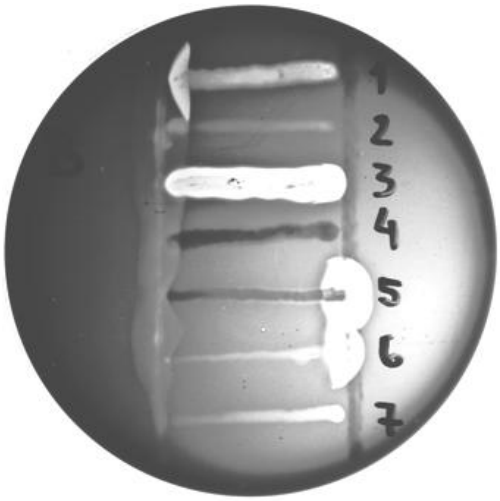

Figure 2- Double CAMP test, third day of incubation, Legend: 1. S. agalactiae; 2. S. non A non B; 3. L. ivanovi; 4. Corynebacterium sp.; 5. C. pseudotuberculosis; 6. A. haemolyticum; 7. A. pyogenes (Published in Acta Scientiae Veterinariae, 2012, 40(2): 1039. by Suvajdzic et al.)

Oxidase-test and catalase test with 3% H_2O_2 revealed a negative and a strongly positive result, respectively. The isolates resulted in neither lactose and xylose fermentation, nor gelatin liquefaction. On the other hand, all investigated isolates hydrolysed urea but not glycogen. All investigated strains were alkaline phosphatase (PAL) positive and fermented glucose, ribose and maltose. Bacteriological diagnosis was confirmed using API Coryne V 2.0 and software program, revealing an identity rate of 99.9%, accuracy rate T = 1, test count = 0. The identification rate was evaluated as excellent (Table 1).

Using this protocol, 28 *C. pseudotuberculosis* strains isolated from milk samples of cows with mastitis were identified. All identifications of *C. pseudotuberculosis* strains were confirmed applying the API Coryne V 2.0 - diagnostic kit and software.

This study demonstrated that the morphological, cultural and tinctorial traits of the isolates corresponded with the literature data [Merchant, 1935]. In this study, plasma coagulation caused by *C. pseudotuberculosis* was observed, which corresponds with our previous experience. However, there are no references on such experiences in the available literature. Gomes *et al*. (2009) reported that the coagulase tube test resulted in the formation of a thin layer of fibrin embedded in rabbit plasma by the non-toxigenic BR-CAT5003748 strain *C. diphtheriae*. All of our isolates protected erythrocytes from lysis (inverse CAMP phenomenon) and caused synergistic haemolysis with *Rhodococcus equi*. Soucek and Souckova (1972) reported that only phospholipase D produced by *Arcanobacterium haemolyticum*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* can protect erythrocytes from lysis by the staphylococcal β-toxin. Bernheimer *et al*. (Bernheimer et a., 1980) described gradual decomposition of erythrocyte membrane sphyngomyelyns influenced by phospholipase D excreted by *Corynebacteria*. Barksdale *et al*. (1981) defined the production of phospholipase D as a crucial marker in the genus *Corynebacterium*, because only *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* produce it. The gene encoding *Arcanobacterium haemolyticum* phospholipase D, which is responsible for the inverse CAMP-reaction, has been cloned and sequenced and showed some similarities to the corresponding genes of *C. pseudotuberculosis* and *C. ulcerans* (Cuevas and Songer, 1993). Synergism with *Rhodococcus equi* is corresponding with our previous experience and literature data (Clarridge, 1989, Clarridge and Spiegel, 1995). *C. pseudotuberculosis* always produced both, inverse CAMP phenomenon and synergistic hemolysis with *R. equi*.

Table 1- Biochemical characteristics of strains of Corynebacterium pseudotuberculosis isolated from milk samples of cows with clinical mastitis applying API Coryne V 2.0 and software program-BioMerieux1

Test	Reaction	Isolate reaction	
		Investigated strain	Identification table
		1	2
NIT	NITrate reduction	0/28	1
PYZ	PYraZinamidase	0/28	0
PyrA	Pyrolydonil Arylamidase	0/28	0
PAL	ALkaline Phosphatase	28/28	54
β-GUR	beta GlucURonidase	0/28	0
β -GAL	beta GALactosidase	0/28	0
α-GLU	alpha GLUscosidase	0/28	25
β-NAG	N-Acetyl-β-Glucosaminidase	0/28	0
ESC	ESCulin (β-glucosidase)	0/28	0
URE	UREase	28/28	100
GEL	GELatine (hydrolysis)	0/28	0
O	Oxidase	0/28	0
GLU	GLUcose (fermentation)	28/28	100
RIB	RIBose (fermentation)	28/28	100
XYL	XYLose (fermentation)	0/28	0
MAN	MANitol (fermentation)	0/28	0
MAL	MALtose (fermentation)	28/28	75
LAC	LACtose (fermentation)	0/28	0
SAC	SACharose (fermentation)	0/28	0
GLYG	GLYcoGen (fermentation)	0/28	0
CAT	CATalase	28/28	100

Biochemical features of examined strains confirmed by API Coryne V 2.0 and software program, were in accordance with the identification table. The obtained results confirmed the identity of *C. pseudotuberculosis* with an identity rate of 99.9% and an accuracy rate T = 1.

We are of the opinion that colonial resemblance of *C. pseudotuberculosis* with species of the genus *Staphylococcus* organisms is the main reason for "missing" these agents in routine diagnostics. Crucial explanation for such "missing" is the fact that they, same as some staphylococci, cause plasma coagulation in the test tube. As common diagnostic minimum in most bacteriology laboratories includes tube coagulation test and mannitol fermentation test, we are of the opinion that introduction of double CAMP test is necessary. This test proves presence of phospholipase D enzyme that prevents erythrocyte-lysis caused by hemolysin of *Staphylococcus aureus* (inverse CAMP test) and synergistic hemolysis with *R. equi*. This enzyme is produced by *C. pseudotuberculosis, C. ulcerans*, and *A. haemolyticum*, but it is not produced by any of *Staphylococcus* strains. Thus, positive double CAMP test, along with positive plasma tube test indicates presence of only two bacterial species - *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans*, whilst *A. haemolyticum* is plasma-negative and resembles streptococci. The species *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* may differ from one another by their ability of glycogen or starch degradation (always-positive *Corynebacterium ulcerans* and always-negative *Corynebacterium pseudotuberculosis*).

Appearance of colonies can deceive in the direction of *Nocardia*-like organisms, in which case smear and Gram staining usually solves the dilemma.

CONCLUSION

In this study, 28 isolates of *C. pseudotuberculosis,* from milk samples of cows with mastitis were identified. Since *C. pseudotuberculosis* was isolated in pure culture from milk samples, we can confirm that it is the causative agent of cows mastitis, what is in accordance with the work of other authors (Yeruham et al., 1996, Shpigel et, al., 1993, Hommez et al., 1999, Adekeye et al., 1980, Yeruham et al., 2003, Yeruham et al., 2004). We recommend introduction of double CAMP test in every atypical coagulase-positive *Staphylococcus* sp. Cost-benefit relation qualifies this test as the method of choice because it is available in every bacteriological laboratory, and provides reliable results.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technology of the Republic of Serbia (Grant Number 46012 and 41012).

REFERENCES

Adekeye, J.D., Shannon, D, Addo, P.B. (1980). Mastitis in a cow caused by *Corynebacterium pseudotuberculosis* (*C. ovis*). Veterinary Record, 106 (12), 270.

Aleman, M, Spier, S.J., Wilson, W.D., Doherr, M. (1996). *Corynebacterium pseudotuberculosis* infection in horses 538 cases (1982-1993). Journal of American Veterinary Medical Association, 209 (4), 804-9.

Afzal, M., Sakir, M., Hussain, M.M., (1996). *Corynebacterium pseudotuberculosis* infection and lymphadenitis (taloa or mala) in the camel. Tropical Animal Health and Production, 28 (2), 158-62.

Ameh, J.A., Tari, I.S., (2000). Observations on the prevalence of caprine mastitis in relation to predisposing factors in Maiduguri. Small Ruminant Research, 35 (1), 1-5.

Barksdale, L., Linder, R., Sulea, I.T., Pollice, M., Phospholipase. D. (1981). Activity of *Corynebacterium pseudotuberculosis* (*Corynebacterium ovis*) and *Corynebacterium ulcerans*, a Distinctive Marker Within the Genus *Corynebacterium*. Journal of Clinical Microbiology, 13 (2), 335-43.

Bernheimer, A.W., Linder, R., Avigad, L.S. (1980). Stepwise degradation of membrane sphingomyelin by corynebacterial phospholipases. Infection and Immunology, 29 (1), 123-31.

Biberstein, E.L., Zee, Y.C. (1990), editors. Review of Veterinary Microbiology. Cambridge Massachusets: Blackwell Science Inc.

Brown, C.C., Olander, H.J. (1987). Caseous Lymphadenitis of Goats and Sheep, a review. Veterinary Bulletin, 57, 1-11.

Clarridge, J.E. (1989). The recognition and significance of *Arcanobacterium haemolyticum*. Clinical Microbiology Newsletter, 11 (6), 41-5.

Claridge, J.E., Spiegel, C.A. (1995).*Corynebacterium* and Miscellaneous Irregular Gram- Positive Rods, *Erysipelothrix* and *Gardnerella*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of Clinical Micribiology. 6th edition. Washington D.C., AMS Press. p. 357–78.

Cuevas, W.A., Songer, J.G. (1993). *Arcanobacterium haemolyticum* phospholipase D is genetically and functionally similar to *Corynebacterium pseudotuberculosis* phospholipase D. Infection and Immunity, 61 (10), 4310-6.

Dorella, A., Pacheco, L.G., Oliveira, S.C., Miyoshi, A., Azevedo, V. (2006). *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Veterinary Research, 37 (2), 201-18.

Gomes, D.L., Martins, C.A., Faria, L.M., Santos, L.S., Santos, C.S., Sabbadini, P.S., et al (2009). *Corynebacterium diphtheriae* as an emerging pathogen in nephrostomy catheter-related infection: evaluation of traits associated with bacterial virulence. Journal of Medical Microbiology, 58 (Pt 11), 1419-27.

Hawari, A.D. (2008). *Corynebacterium pseudotuberculosis* Infection (Caseous Lymphadenitis) in Camels (Camelus dromedarius) in Jordan. American Journal of Animal and Veterinary Sciences, 3 (2), 68-72.

Hawari, A.D., Obeidat, M., Awaisheh, S.S., Al-Daghistani, H.I., Al-Abbadi, A.A., Omar, S.S., et al (2014). Prevalence of mastitis pathogens and their resistance against antimicrobial agents in Awassi sheep in Al-balqa province of Jordan. American Journal of Animal and Veterinary Sciences, 9 (2), 116-21.

Holt, J., Krieg, N., Sneath, P., Staley, J., Williams, S. (1991). Bergey's Manual of Determinative bacteriology. 9th ed. Washington: Williams & Wilkins.

Hommez, J, Devriese, L.A., Vaneechoutte, M., Riegel, P., Butaye, P., Haesebrouck, F., (1999). Identification of Nonlipophilic *Corynebacteria* Isolated from Dairy Cows with Mastitis. Journal of Clinical Microbiology, 37 (4), 954-7.

Ivanović, S., Žutić, M., Pavlović, I., Žujović, M., (2009). Caseous lymphadenitis in goats. Biotechnology in Animal Husbandry, 25 (5-6), 999-1007.

Madut, N, A., Abdelgadir, A.E. (2011). Susceptibility of *Corynebacterum* spp. responsible for bovine mastits against commonly used antibiotics in Kuku dairy farms, Sudan. Journal of Cell and Animal Biology, 5 (1), 6-10.

Merchant, I.A. (1935). A study of the Corynebacteria associated with diseases of domestic animals. Journal of Bacteriology, 30 (1), 95–117.

Paviour, S., Musaad, S., Roberts, S., Taylor, G., Taylor, S., Shore, K., et al (2002). *Corynebacterium* Species Isolated from Patients with Mastitis. Clinical Infectious Diseases, 35 (11), 1434-40.

Peel, M.M., Palmer, G.G., Stacpoole, A.M., Kerr, T.G. (1997). Human Lymphadenitis Due to *Corynebacterium pseudotuberculosis:* Report of Ten Cases from Australia and Review. Clinical Infectious Diseases, 24 (2), 185-91.

Queen.C., Ward, A.C., Hunter, D.L. (1994). Bacteria isolated from nasal and tonsillar samples of clinically healthy Rocky Mountain bighorn and domestic sheep. Journal of Wildlife Diseases, 30 (1), 1-7.

Schalm, O.W., Noorlander, D.O. (1957). Experiments and observations leading to development of the California mastitis test. Journal of the American Veterinary Medical Association, 130 (5), 199-204.

Shpigel, N.Y., Elad, D., Yeruham, I., Winkler, M., Saran, A. (1993).An outbreak of *Corynebacterium pseudotuberculosis* infection in an Israeli dairy herd. Veterinary Record, 133 (4), 89-94.

Součkova, A., Souček, A. (1972). Inhibition of the hemolytic action of and beta-lysins of *Staphylococcus pyogenes* by *Corynebacterium haemolyticum*, *C.ovis* and *C.ulcerans*. Toxicon, 10 (5), 501-9.

Suvajdžić, Lj. (2000): Izučavanje karakteristika *Actinomyces pyogenesa* i sličnih mikroorganizama izolovanih iz pluća teladi i prasadi sa pneumonijom [dissertation]. Beograd: Fakultet veterinarske medicine.

Suvajdžić, Lj., Potkonjak, A., Milanov, D., Lako, B., Kocić, B., Milić, N**.**, Čabarkapa I**. (**2012)**.** A Proposal of a Diagnostic Protocol for Isolation of *Corynebacterium ulcerans* from Cow's Milk. Acta Scientiae Veterinariae, 40 (2), 1-7.

Yeruham, I., Braverman, Y., Shpigel, N.Y., Chizov-Ginzburg, A., Saran, A., Winkler, M. (1996). Mastitis in dairy cattle caused by *Corynebacterium psudotuberculosis* and the feasibility of transmission by houseflies I. Veterinary Quaterly, 18 (3), 87-9.

Yeruham, I., Elad, D., Friedman, S., Perl, S. (2003). *Corynebacterium pseudotuberculosis* infection in Israeli dairy cattle. Epidemiology and Infection, 131 (2), 947-55.

Yeruham, I., Friedman, S., Perl, S., Elad, D., Berkovich, Y., Kalgard, Y. (2004). A herd level analysis of a *Corynebacterium pseudotuberculosis* outbreak in a dairy cattle herd. Veterinary Dermatology, 15 (5), 315-20.

PATHOGENS OF ANIMALS AND HUMANS – PHOSPHOLIPASE D PRODUCERS AND THEIR DIAGNOSTIC AND THERAPEUTIC FAILURES

Ljiljana Suvajdzic[1], Zoran Suvajdzic[2]

[1]Faculty of Medicine, Department of Pharmacy, University of Novi Sad,
[2]Veterinary infirmary, STRIKS, Čerević

*Corresponding author:
E-mail address: giba57@eunet.rs

ABSTRACT

Arcanobacterium haemolyticum, Corynebacterium ulcerans and Corynebacterium pseudotuberculosis produce phospholipase D that significantly facilitates their laboratory diagnosis. Phospholipase D is easily and reliably identified in every bacteriological laboratory which will be shown in this paper.
The test was performed as well as conventional CAMP test. Instead of synergistic hemolysis, the absence of hemolysis caused by *Staphylococcus aureus* on blood agar was observed. Phospholipase D protects erythrocytes from lyse by staphylococcal haemolysin, resulting in inversa CAMP phenomenon. It is possible to perform *Rhodococcus* CAMP test in the same petri plates. In that case, synergistic hemolysis is observed between phospholipase D and *equi* factors of *Rhodococus equi*. Thus, with high level of certainty, the identity of *Arcanobacterium haemoiticum*, and *Corynebacterium ulcerans/pseudotuberculosis* is proven. These agents are often misidentified in routine work, either in human or veterinary bacteriology. These zoonotic species can cause not only mild opportunistic infections, but also serious clinical conditions and often require treatment different from usual. Diagnostic and therapeutic failures prolong hospitalisation and sick leave period in medicine and lead to unnecessary economic losses in veterinary medicine.
Without etiological diagnosis there can be no rational antimicrobial therapy. Non rational antibiotic therapy contributes to drug resistance, which is considered to be the plague of twenty-first century. This paper points out the most common reason for diagnostic and therapeutic failures of diseases caused by these bacteria. We also propose a simple, reliable and accessible test for sufficient bacteriological diagnosis of these three bacteria, available in any laboratory.
***Keywords**: phospholipase D producers, double CAMP test*

INTRODUCTION

Phospholipase D is an enzyme that destroys the membrane of mammalian cells. Thus, it is an important virulence factor of the microbes that produce it. Phospholipase D production is associated with only three bacterial species. Confirmation of this enzyme is a crucial diagnostic parameter (Součkova and Souček 1972; Barksdale et al., 1981). All three species were classified into the Genus *Corynebacterium* until early eighties: *Corynebacterium haemolyticum*, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans*. In 1982, Collins et al. proposed a separate Genus, *Arcanobacterium* for *Corynebacterium haemolyticum* (Collins et al., 1982), thus the organism was renamed to *Arcanobacterium haemolyticum*. The other two species, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans*, are still members of the Genus, *Corynebacterium*.

ARCANOBACTERIUM HAEMOLYTICUM (*A. HAEMOLYTICUM*)

Arcanobacterium haemolyticum predominantly causes diseases of the upper respiratory tract of human population. In the tropics, skin ulcers caused by these bacteria can appear. It is not a frequent agent, so it is of minor epidemiological significance. However, it can produce erythrogenic toxin, in which case it can clinically mimic the scarlatina, exanthema toxialergicum and rash fever. Therefore, it is of great importance in differential diagnosis for clinical practice and epidemiological assessment of public health (Banck and Nyman 1986).

Its misidentification leads to diagnostic and therapeutic failures, increasing the number of hospital days and time spent on sick leave (Clarrindge 1989; Kovatch et al., 1983).
It is primarily associated with pharyngitis, especially in teenagers and young adults (Carlson et al., 1983; Gaston and Zurowski 1996; Mackenzie et al., 1995). Pharyngitis is associated with rash in 30% of cases. Peritonsillar abscess can be the only clinical manifestation (Miller and Brancato 1984). The organism rarely causes severe health problems and complications such as sepsis (Ford et al., 1995), endocarditis (Worthington et al., 1985), mixed wound infections (Barker et al., 1992), neurological complications (Chandrasekar and Molinari 1987) and cavitary pneumonia (Waller et al., 1991).
In our country, a case of seventeen year old girl was reported. The patient had mild symptoms of pharyngitis, marked urticarial rash and heavy desquamation of palms and soles. According to the antibiogram and bacteriological diagnosis of *Streptococcus* non A non B group, the patient was treated with penicillin; however, ineffectively. Escalation of urticaria and failure of the initial penicillin therapy shifted the diagnosis towards exanthema toxialergicum and thus to the treatment with corticosteroids and antihistaminics, yet with no improvement. Repeated bacteriological examination of throat swabs applying more complex diagnostic procedures confirmed the identity of *Arcanobacterium haemolyticum*. Erythromycin 500 mg, twice a day for seven days, resulted in complete eradication of the causative agent. The patient fully recovered (Suvajdžić et al., 2006).
A. haemolyticum rarely causes disease in animals, or at least it is rarely isolated. There are only few cases reported from animal samples. It is a commensal of the respiratory tract of domestic animals (Holt et al., 1991). The lungs are the most commonly infected organs (Roberts 1969; Suvajdžić 2000; Suvajdžić et al., 2002; Suvajdžić et al., 2012a), but it was also reported from bull semen (Richardson and Smith 1968) and the central nervous system of goat (Richardson and Smith 1968). Its rare identification is most likely a consequence of diagnostic failure rather than its absence in animal samples. Since it is a commensal, it is present in the respiratory mucosa. Such misidentification is most probably due to the "mimicry" of its colonial appearance to another species, *Arcanobacterium pyogenes* that is frequently isolated agent in animal samples. Better understanding of the causes and appropriate routine diagnostic tool could be useful in veterinary medicine in order to apply rational antibiotic therapy.

Treatment and control

The course of untreated pharyngitis has been rarely described so far, although MacLean points out that patients, who received only symptomatic treatment, recovered spontaneously within two weeks (MacLean et al., 1946). Despite numerous reports of symptoms withdrawal three days after the introduction of penicillin therapy, many clinical failures were noted in the per oral and parenteral administration. Banck described 18 patients treated with penicillin V per os 25 mg per kg a day in two daily doses during seven to ten days. Patients had *A. haemolyticum* in the throat 2 to 4 weeks after therapy. (Banck and Nyman, 1986) Based on the high level of penicillin tolerance in 40 isolates, Nyman found that penicillin V is ineffective in the treatment of *A. haemolyticum*. (Nyman et al., 1990) Osterlund is of the same opinion, interpreting that with the intracellular survival of microorganisms (Osterlund 1995). Long-term carrier status was observed in patients regardless of whether they were or were not treated with penicillin.
Uniform in vitro sensitivity to erythromycin (Carlson et al., 1994) and an excellent effect in clinical practice qualifies erythromycin as antibiotic of choice for treatment of *A. haemolyticum* infections. Erythromycin has proven to be effective in oral administration of 250 mg four times a day during ten days and at a dose of 500 mg twice a day during seven days.

CORYNEBACTERIUM PSEUDOTUBERCULOSIS AND *CORYNEBACTERIUM ULCERANS*

The diseases caused by these pathogens are widespread throughout the world. *C. pseudotuberculosis* is a well-known animal pathogen, with rare reports of the isolation in humans. Epidemiological data for *C. ulcerans* are inverted.
C. pseudotuberculosis is an ubiquitous microorganism that survives well in organic detritus and humid environment. The sources of infections are secreta and/or excreta of infected animals and humans. Sick individuals (humans and animals) and soil, especially stables, corrals and pens are well established reservoirs of infection. Although sheep (Queen et al., 1994; LeaMaster et al., 1987; Brown and Olander 1987; Pepin et al., 1997) and horses (Merchant 1935; Aleman et al., 1982-1983) are most frequently infected species, the other species than mammals are prone to infection as well. The organism was isolated from goats (Brown and Olander 1987; Gezon et al., 1991), pigs (Suvajdžić 2000; Biberstenin and Zee, 1990), cattle (Suvajdžić 2000; Yeruham et al., 1996; Songer et al., 1988), camels (Songer et al., 1988) and humans (Jones and Collins 1986). Skin wounds are the most common entry portal of infection (Aleman et al., 1982-1983) while the skin and lymphoid tissue is usually its target (Biberstein and Zee, 1990). The organism can be transmitted by insects (Aleman et al., 1982-1983) *Hematobia irritans, Musca domestica, Stomaxys calcitrans* and *Culicidae,* in whose feces and sputum they can survive several days (Yeruham et al., 1996), what is important for epidemiological prognosis (Yeruham et al., 1997). High lipid content in the cell wall explains intraphagocytic survival and leukotoxicity (Aleman et al., 1982-1983). Survival of phagolysosomal mechanisms enables formation of abscesses, which remain localized if the toxin is absent or neutralized (Biberstein and Zee, 1990). Distribution and spectrum of changes vary depending of the entrance portal of infection and paths of spreading. Lymphogenic dissemination is always included (Biberstein and Zee, 1990), but hematogenic and *per continuitatem* just occasionally (Aleman et al., 1982-1983). The exudate produced during the infection demonstrates greenish opalescence. Lesions are caseous to dry and crumbly. Neutrophilic infiltration and endothelial damage are always present. Macrophages, giant cells and fibrous tissue are present only in old lesions (Biberstein and Zee, 1990).

Pathogenicity for humans

In the late seventies and early eighties (Barksdale et al., 1981; Jones and Collins 1986), it became clear that within the Genus *Corynebacterium* there is a group of related microorganisms able to produce real "diphtheria" toxin. To produce this toxin, they have to be lysogenized by beta phages. Besides the three varieties of *Corynebacterium diphtheriae*, this group includes *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*. This group differs from the other members of the Genus by the type of main non-hydroxylated amino acids in the cell wall, and the fact that they are pyrazinamidase negative and neuraminidase positive (Jones and Collins 1986).
Beta phage is the carrier of Tox gene for diphtheria toxin. In this case, they produce real diphteric toxin (Jones and Collins, 1986), and cause a clinical condition similar to signs and symptoms of diphtheria (Hust et al., 1992; de Carpentier et al., 1992; Gubler et al., 1990). Pseudomembranes can be present (Gubler et al., 1990) and the disease can escalate to clinical picture of malignant diphtheria (de Carpentier et al. 1992).
Cases of skin manifestations (in the form of gangrenous dermatitis) (Olson et al., 1988) and granulomatous necrotizing pneumonia, where *C. ulcerans* was isolated as a monoculture, are described. A case of one patient treated with penicillin during seven days was reported. The recurrence wasn't recorded even after two years.
If not lysogenized by beta phage, the organisms produce only their own toxin, i.e., the ovis toxin. Its main component is phospholipase D. Determination of ovis toxin structure has ended a half-century long speculation about the nature of this toxin, which is produced independently of the presence of beta phage (Barksdale et al., 1981).

From an epidemiological point of view, it is important to emphasize that C ulcerans could be transferred to humans by milk and dairy products. This microorganism was the only causative agent of food borne outbreaks associated with milk consumption in two of the 27 cases in England and Wales (1983; 1984).
This indicates the importance of mandatory ruling out the presence of *C ulcerans* in milk samples in cases of mastitis, as well as in consumable milk and milk products.

MICROBIOLOGICAL DIAGNOSIS

Zaharova and Kubelka (1960) found that some bacteria produce substances that protect erythrocytes contained in blood agar, against lysis by staphylococcal toxin. They named this phenomenon "the inverse CAMP phenomenon", because the hemolysis of erythrocytes was prevented rather than enhanced as in the classical CAMP test.
In the following years and decades, this was confirmed by numerous researchers: Souckova and Soucek (1972), Lamler and Blobel (1988), Comman (1996), and JE Claridge (1989; 1995)**.** Based on this phenomenon and synergistic hemolysis with equi factors of *Rhodococcus equi* produced by these bacteria, Jill E. Claridge developed in 1989 and in 1995(Claridge and Spiegel, 1995) a simple and reliable test that is performed on a single blood agar plate. This test is a supreme tool for proving the identity of all three phospholipase D producers**.**
In our country, Suvajdzic et al. (1998a; 1998b; 2000; 2002; 2012a; 2012b) described the performances of this test and its importance in the diagnosis of *Actinomyces pyogenes, Arcanobacterium haemolyticum, Corynebacterium pseudotuberculosis, Corynebacterium ulcerans, Listeria monocytogenes* and *Listeria Iwanovi* through serial reports during the period 1998-2012
Despite persuasive arguments that this test replaces expensive commercial kits and even more expensive molecular diagnostics, double CAMP test did not find wider application in routine microbiological laboratory work. It is probably one of the reasons why *Arcanobacterium haemolyticum* and *Corynebacterium ulcerans* are rarely found in clinical specimens of animal origin, although both bacteria are described as commensals in domestic and wild animals. Also, *Corynebacterium pseudotuberculosis* is found only in more detailed and extensive studies that go beyond everyday routine work.

TREATMENT AND CONTROL

In sheep and goats, the treatment of infections caused by the *Corynebacteria* is not efficient. Prevention of disease spreading is limited to the separation of sick animals, limiting the exposure of infection, sanitary care and hygiene measures. Bacterin-toxoid combination could be useful in infection limiting. Abscesses are treated surgically. Prolonged treatment with penicillin can be applied in the prevention of agents' dissemination, or in the treatment of the disseminated form of the disease. According to our experience, local administration of gentamicin gives better results than penicillin, which is consistent with the experience in human medicine.
Erythromycin is an effective drug for pneumonia treatment in humans (Carlson et al., 1994) caused by this microorganism.

REASONS FOR DIAGNOSTIC WANDERING AND HOW TO AVOID THEM

What is this all about? Colonies of *A. haemolyticum* resemble beta hemolytic species of the Genus *Streptococcus* and *Trueperella pyogenes* colonies*.* Beta hemolytic *Streptococcus* is a frequent "guest" in human bacteriological laboratory, while *A. pyogenes* is common in materials of animal origin.
In bacteriological jargon, all of the mentioned genera and species are known as "beta small". Thus, a veterinary bacteriologist will "see" in the smear the gram positive pleomorphic rods

that correspond to expected agent, *A. pyogenes.* Medical microbiologist will pursue, through normal routine procedure, this isolate to the "bacitracin, CAMP test". The next day, during result interpretation, he will conclude that CAMP "today falls short", and interpreate the result as *Streptococcus* none A none B group. In this way, the usual routine work and diagnostic protocol successfully allows missing of these rare or "rare" pathogens.
As for the diagnosis of *Corynebacterium ulcerans,* there is an even bigger trap: colonies can mimic species of the Genus *Staphylococcus*. Usually, they are creamy, yellowish or ivory, buttery in consistency, easy to remove from the surface and just as easily dispersed. Colonies cause beta hemolysis on blood agar, more often narrow than wide, or their hemolysis occurs after removing the colony from agar surface. Each microbiologist, medical or veterinarian, will subject such colonies to plasma coagulation in a test tube, before they make smears. Since plasma in a tube test will be positive (detection of free coagulase), neither human nor veterinarian microbiologist will have any reason to doubt that he proved "coagulase positive staphylococcus". Depending on the work style of the institution, laboratories or individuals it can be a definitive diagnosis of *Staphylococcus aureus*, and the testing will be supplemented by mannitol fermentation and Cadnes-Graves test. In case of negative mannitol test, the diagnosis will be *Staphylococcus intermedius*, because this is a common algorithm in most routine laboratories.
C. pseudotuberculosis could be confused with the species of the Genus *Staphylococcus*, although its colonies are usually hard, ingrown into the substrate, and it is difficult to remove and disperse them in liquid medium. However, if the diagnostician is not specialized and advised about this entity, colony of this agent could "pass" as species of Genus *Staphylococcus, Nocardia*-like organism or diphtheroids (which it is, but a significant one). Sometimes we declare unrecognized colonies as "Luft bacteria" (air bacteria), which implies insignificant contamination.
Most of the reports, statements and papers are precise about *C. pseudotuberculosis*, probably due to the best knowledge of this microorganism, its biology and pathology. The clinicians are usually the first to suspect of *Corynebacterium pseudotuberculosis* infection. Therefore, clinicians commonly alert microbiologists on the delivery of the material or samples for examination. However, it would be more rational to control each "beta small" colony in a double instead of plain CAMP test, which would otherwise be routinely done (there is not increase in the costs, one only needs to draw two, instead of one vertical line on the blood agar). Each atypical staphylococci and nocardia-like colonies, especially if the smear is misleading to diphtheroid appearance, needs to be examined also in a double-CAMP test. At the price of one blood agar plate, we can confirm diagnosis for all three phospholipase D producers, as well as few other species that are rarely found in clinical specimens. We should not forget that we can find only what we know to look for.
What a field veterinarian may and should do? When the field veterinarian faces (noted) a sample that is watery or even lymph-like, containing clots, associated with apparent swelling and soreness usually in one quarter, and learns from anamnesis about failed local penicillin therapy, further "blind" therapy is not recomended . According to a protocol, the veterinarian has to take a sample, sent it to the laboratory examination and rinse affected quarter with saline. Until obtaining of the antibiogram, gentamicin therapy should be initiated , i.e., gentamicin added in a saline for rinsing along with symptomatic treatment including analgesics and antipyretics. The veterinarian has to wait for the microbiological diagnosis and antibiogram and then, if it is necessary, to adjust the antibiotics until accomplishing the successful treatment.

CONCLUSION

Phospholipase D producers are present in our environment. They are often missed by diagnostic filter, which leads to diagnostic wandering and therapeutic failure. In human medicine, this leads to an increase in the number of hospital days and days spent on sick leave, while in breeding animals it can cause unnecessary economic losses. In most

instances, some pathogens should not be treated with antibiotics, whereas in some cases, penicillin, which is generally used, is not the drug of choice.
All three microorganisms are ubiquitous, surviving well in the external environment, especially humid one, the detritus and soil of pens and stables. Animal feed, especially if it is not properly stored, could be a source of infection.
We are witnessing the increase of antimicrobial resistance, for which we are greatly responsible because of uncritical use of antibiotics. For a bacterium, it is not relevant whether its resistance is developed in the animal or human body, in animal or human food: it will transfer its "knowledge" to its descendants and contemporaries, wherever bacterium received plasmid of resistance or gene that supports it. Accurate etiologic diagnosis is the first prerequisite for rational antibiotic therapy. Thus, a diagnostician plays an important role in the fight against bacterial resistance, whose findings direct therapy. For the diagnosis of phospholipase D producers, double CAMP test should be the method of choice.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technology of the Republic of Serbia (Grant Number 46012 and 41012).

REFERENCES

Afzal, M, Sakir, M, Hussain, MM. (1996) Corynebacterium pseudotuberculosis infection and lymphadenitis (taloa or mala) in the camel. Trop Anim Health Prod, 28(2), 158-62. A2.
Aleman, M, Spier, SJ, Wilson, WD, Doherr, M. (1982-1993) Corynebacterium pseudotuberculosis infection in horses 538 cases, J Am Vet Med Assoc. 1996;209(4), 804-9.
Banck, G, Nyman, M. (1986). Tonsillitis and rash associated with Corynebacterium haemolyticum. J Infect Dis, 154 (6), 1037-40.
Barker, KF, Renton, NE, Lee, PY, James, DH. (1992). Arcanobacterium haemolyticum wound infection. J Infect, 24(2), 214-5.
Barksdale, L, Linder, R, Sulea, IT, Pollice, M. (1981) Phospholipase D: Activity of Corynebacterium pseudotuberculosis (Corynebacterium ovis) and Corynebacterium ulcerans, a Distinctive Marker Within the Genus Corynebacterium. J Clin Microbiol, 13(2), 335-43.
Barrett, NJ. (1986) Communicable disease associated with milk and dairy products in England and Wales: 1983-1984. J Infect, 12(3): 265-72.
Biberstein, E.L., Zee, Y.C. (1990). Review of Veterinary Microbiology, Blackwell Science Inc.,Cambridge Massachusets.
Brown, CC, Olander, HJ. (1987) Caseous Lymphadenitis of Goats and Sheeps: a review. Vet Bull, 57, 1-11.
Carlson, P, Kontianen, S, Renkonen, OV. (1994) Antimicrobial susceptibility of Arcanobacterium haemolyticum. Antimicrob Agents Chemother, 38(1), 142-3.
Carlson, P, Renkonen, OV, Kontiainen, S. (1994) Arcanobacterium haemolyticum and streptococcal pharyngitis. Scand J Infect Dis, 26(3), 283-7.
Chandrasekar, PH, Molinari, JA. (1987) Corynebacterium haemolyticum bacteremia with fatal neurological complication in an intravenous drug addict. Am J Med, 82 (3 Pt 2), 638-40.
Claridge, J.E., Spiegel, C.A. (1995) Corynebacterium and Miscellaneous Irregular Gram- Positive Rods, Erysipelothrix and Gardnerella. In Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H (Eds.), Manual of Clinical Micribiology,vol. 29 (pp. 357-378), Washington D.C.: AMS Press.
Clarridge, J.E. (1989). The recognition and significance of Arcanobacterium haemolyticum. Clinical Microbiology Newsletter, 11(6), 41-45.
Collins, MD, Jones, D, Schofield, GM. (1982) Reclassification of "Corynebacterium haemolyticum" (McLean, LeLiebow & Rosemberg) in the genus Arcanobacterium gen. nov. as Arcanobacterium haemolyticum nom.rev., comb.nov.. J Gen Microbiol, 128(6), 1279-81.
Coman, G, Panzaru, C, Dahorea, C. (1996) The isolation of Arcanobacterium haemolyticum from the pharyngeal exudate of children. Bacteriol Virusol Parazitol Epidemiol, 41(3-4),141-4.
de Carpentier, JP, Flanagan, PM, Singh, IP, Timms, MS, Nassar, WY. (1992) Nasopharyngeal Corynebacterium ulcerans: a different diphtheria. J Laryngol Otol, 106(9), 824-6.
Ford, JG, Yeatts, RP, Givner, LB. (1995) Orbital cellulitis, subperiosteal abscess, sinusitis, and septicemia caused by Arcanobacterium haemolyticum. Am J Ophthalmol, 120(2), 261-2.
Gaston, DA, Zurowski, SM.(1996) Arcanobacterium haemolyticum pharyingitis and exanthem. Three case reports and literature review. Arch Dermathol, 132 (1), 61-4.
Gezon, HM, Bither, HD, Hanson, LA, Thomson, JK. (1991) Epizootic of external and internal abscesses in a large goat herd over a 16-years period. J Am Med Assoc, 198(2), 257-63.

Gubler, JG, Wust, J, Krech, T, Hany, A. (1990) Classical pseudomembranous diphtheria caused by Corynebacterium ulcerans. Schweitz Med Wochenschr, 1; 120(48), 1812-6.

Holt, J, Krieg, N, Sneath, P, Staley, J, Williams, S. (1991) Bergey's Manual of Determinative bacteriology. 9th ed. Washington: Williams&Wilkins.

Hust, MH, Metzler, B, Schubert, U, Weidhase, A, Seuffer, RH. (1994) Toxic diphtheria caused by Corynebacterium ulcerans. Dtsch Med Wochenschr, 119(15), 548-52.

Jones, D, Collins, MD. (1986) Irregular, nonsporing Gram-positive rods. In: Smeath PHA, Mair NS, Sharpe M.E, Holt JG, editors. Bergey's manual of systematic bacteriology. Baltimore: Williams and Wilkins, p. 1261–1434.

Kovatch, AL, Scuit, KE, Michaels, RH. (1983) Corynebacterium haemolyticum peritonsillar abscess mimicking diphtheria. JAMA, 249(13), 1757-8.

Lämmler, C, Blobel, H. (1988) Serological recognition of Actinomyces pyogenes and Arcanobacterium haemolyticum in serum soft agar. Zentralbl Bakteriol Mikrobiol Hyg A.267(4),528-30.

LeaMaster, MR, Shen, DT, Gorham, JR, Leathers, CW, Wells, HD. (1987) Efficacy of Corynebacterium pseudotuberculosis bacterin for the immunologic protection of sheep against development of caseous lymphadenitis. Am J Vet Res, 48(5), 869-72.

Mackenzie, A, Fuite, LA, Chan, F, King, J, Allen, U, MacDonald, N. (1995) et al. Incidence and pathogenicity of Arcanobacterium haemolyticum during a 2-year study in Ottawa. Clin Infect Dis, 21(1), 177-81.

MacLean, PD, Liebow, AA, Rosenberg, AA.(1946) A haemolytic Corynebacterium resembling Corynebacterium ovis and Corynebacterium pyogenes in man. J Infect Dis,79,69-90.

Merchant, IA. (1935) A study of the Corynebacteria associated with diseases of domestic animals. J Bacteriol, 30(1), 95–117.

Miller, RA, Brancato, F. (1984) Peritonsillar abscess associated with Corynebacterium haemolyticum. West J Med, 140(3), 449-51.

Nyman, M, Banck, G, Thore, M. (1990) Penicillin tolerance in Arcanobacterium haemolyticum. J Infect Dis, 161(2), 261-5.

Olson, ME, Goemans, I, Bolingbroke, D, Lundberg, S. (1988) Gangrenous dermatitis caused by Corynebacterium ulcerans in Richardson ground squirrels. J Am Vet Med Assoc, 193(3), 367-8.

Osterlund, A. (1995) Are penicillin treatment failures in Arcanobacterium haemolyticum pharyngotonsillitis caused by intracellularly residing bacteria? Scand J Infect Dis, 27(2), 131-4.

Pepin, M, Seow, HF, Corner, L, Rothel, JS, Hodgson, AL, Wood, PR. (1997) Cytokine gene expression in sheep following experimental infection with various strains of Corynebacterium pseudotuberculosis differing in virulence. Vet Res, 28 (2), 149-63.

Queen,.C, Ward, AC, Hunter, DL. (1994) Bacteria isolated from nasal and tonsillar samples of clinically healthy Rocky Mountain bighorn and domestic sheep. J Wild Dis, 30(1), 1-7.

Richardson, A, Smith, PJ. (1968) .Herd fertility and Corynebacterium haemolyticum in bovine semen. Vet Rec, 83(6), 156-7.

Roberts, RJ. (1969). Isolation of Corynebacterium haemolyticum from a case of ovine pneumonia. Vet Rec, 84. 490.

Songer, JG, Beckenbach, K, Marshall, MM, Olson, GB, Kelley, L. (1988) Biochemical and genetic characterization of Corynebacterium pseudotuberculosis . Am J Vet Res, 49(2), 223-6.

Součkova, A, Souček, A. (1972) Inhibition of the hemolytic action of and beta-lysins of Staphylococcus pyogenes by Corynebacterium haemolyticum, C.ovis and C.ulcerans. Toxicon, 10 (5), 501-9.

Suvajdžić, Lj, Ašanin, J, Lako, B, Potkonjak, A, Sakač, V, Čabarkapa, I. (2012a) et al. Isolation of Arcanobacterium haemolyticum from calves with pneumonia and proposal of a diagnostic protocol. Vet Glasnik, 66 (3-4), 165-174.

Suvajdžić, Lj, Ašanin, R, Jovičin, M, Kovačević, M, Lalić, M, Boboš, S. (1998a) Applicacation of double CAMP test for differential diagnosis of pathogenic bacteria important for the aetiology of bovine genital tract diseases. Proceedings, International Symposium on Animal Reproduction, Veterinary Institut, Department of reproduction, Skopje.

Suvajdžić, Lj, Ašanin, R, Knežević, N, Košarčić, S. (2002) Caracterisation of Arcanobacterium haemolyticum isolates originating from pneumonic piglets (the first isolation from piglet lungs in Yugoslavia). Acta veterinaria, 52(4), 223-34.

Suvajdžić, Lj, Mrđa, E, Bogavac, M, DŽambas, Lj. (2006) Urtica caused by Arcanobacterium haemolyticum. Diagnostic and therapeutic failures. Proc Nat Sci. Matica Srpska, 110, 35-43.

Suvajdžić, Lj. (1998b) Arcanobacterium haemolyticum – prepoznavanje uzročnika i klinički značaj. Profilaksa, 6, 1-11.

Suvajdžić, Lj. (2000). The study of characteristics of Actinomyces pyogenes and related organisms isolated from the lungs of calves and pigs with pneumonia, PhD thesis, Faculty of Veterinary Medicine, Univesity of Belgrade, Serbia.

Suvajdžić, Lj., Potkonjak, A., Milanov, D., Lako, B., Kocić, B., Milić, N., Čabarkapa, I. (2012b). A Proposal of a Diagnostic Protocol for Isolation of Corynebacterium ulcerans from Cow's Milk. Acta Scientiae Veterinariae, 40(2), 1039.

Waller, KS, Johnson, J, Wood, BP. (1991) Cavitary pneumonia due to Arcanobacterium haemolyticum. Am J Dis Child, 145(2), 209-10.

Worthington, MG, Daly, BDT, Smith, FE. (1985) Corynebacterium haemolyticum endocarditis on a native valve. South Med J, 78(10), 1261-2.

Yeruham, I, Elad, D, Van-Ham, M, Shpigel, NY, Perl, S. (1997) Corynebacterium pseudotuberculosis infection in Israeli cattle: clinical and epidemiological studies. Vet Rec, 140(16), 423-7.

Yeruham, I., Braverman, Y., Shpigel, N.Y., Chizov-Ginzburg, A., Saran, A., Winkler, M. (1996). Mastitis in dairy cattle caused by Corynebacterium pseudotuberculosis and the feasibility of transmission by houseflies I. The Veterinary Quarterly, 18(3), 87-89.

Younan, M, Drescher, B. (1996) Zeckenbefall Als ausloeser einer eitrigen meningitis bei einer burenziegen. Tieraertzl Umschau, 51, 356-61.

Zahorova, L, Kubelka, V. (1960) A reverse CAMP diagnostic test with Corynebacterium pyogenes varietas hominis. Folia Microbiol, 5(1), 57-9.

ANTIMICROBIAL RESISTANCE OF *SALMONELLA* SPP ISOLATED FROM POULTRY FARMS IN SOUTHERN BAČKA AND SREM REGION

Maja Velhner*, Dalibor Todorović, Marko Pajić, Igor Stojanov

Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

*Corresponding author:
E-mail address: maja@niv.ns.ac.rs

ABSTRACT

The goal of our work was to detect salmonella on poultry farms situated in Southern Bačka and Srem County and to test their antimicrobial resistance.
During the year 2013, we isolated salmonellas from 69 farms. *Salmonella* Enteritidis, *Salmonella* Infantis resistant to nalidixic acid and tetracycline and *Salmonella* Typhimurium were detected in broilers. The most frequently isolated serotypes found in layer chickens were *S*. Enteritidis as well as *Salmonella* Infantis resistant to nalidixic acid and tetracycline. Broiler breeders (three farms were included) were infected with serovar Enteritidis and Infantis as well. These results correlate well with previous research on the clonal spread of *S*. Infantis in Serbia. *Salmonella* Newport was found in breeder eggs and one broiler farm. These isolates were multiresistant, harboring resistance to ampicillin, nalidixic acid and tetracycline.
Improved farm management is important in order to minimize the rate of salmonella infection in poultry flocks. Prudent use of antibiotics in the livestock industry is necessary to prevent the spread of resistant bacteria along the food chain.
***Keywords**: Salmonella, resistance, monitoring, poultry*

INTRODUCTION

Poultry products and meat can be contaminated with salmonella and such foods may be implicated in salmonella outbreaks in humans. Salmonella could develop different resistance mechanisms to antibiotics (Velhner et al., 2012). If humans become infected with resistant strains the therapy is compromised in young patients, elderly people and immunodeficient individuals. Monitoring antimicrobial resistance (AMR) in Serbia is obligated under regulations provided in the Official Gazette numbers 21/2012, 91/2013 and 24/2014. These regulations require monitoring the AMR in 170 salmonellas isolated from breeder flocks, broilers, layers and turkeys. Subsequently, the risks posed to human health, if they become infected with resistant strains, can be evaluated.
Good management practice is the most important way to minimize infection with salmonella in poultry. Therapy with antimicrobial agents does not have long lasting effects and salmonella dissemination is therefore not prevented. Immunization of poultry flocks is recommended but only to reduce salmonella shedding. In such circumstances farmers are constantly investing in maintaining good health for each flock and maintaining good management practice on daily basis (Velhner et al., 2013).
To obtain resistotyping of salmonella, we collected single isolates from a single poultry farm in Southern Bačka and Srem region, during the year 2013. If different serotypes of salmonella were found at the same locality, these isolates were included in the research as well. The goal was to find out what serotypes are dominant on our farms and how these bacteria respond to antibiotics.

MATERIAL AND METHODS

Salmonella was isolated from poultry feces during a routine monitoring system. The samples of feces was placed in buffer peptone water (Biokar, Beauvais Cedex, France) and incubated at 37 °C for 24 hours. Next day modified semi-solid Rappaport Vassiliadis (Biokar, Beauvais

Cedex, France) plates were inoculated by placing 100 µl of peptone to three or four distant individual spots. Plates were incubated at 42°C for 24 to 48 hours and the full loop taken from the edge of the growth resembling opaque halos, was transferred to Salmonella differential agar (HIMEDIA, Mumbai, India). The suspect colonies were inoculated on triple sugar agar and serotyping was done using antiserum for somatic and flagellar antigens (OIE 2012). Salmonella was collected from 62 farms and single isolates were included in the study. If more than 1 serotype was found on the same locality, they were also tested on antimicrobial resistance and those isolates are recorded in the table 1.
Resistotyping was done according to recommendation provided in the CLSI documents M07-A9 and M100-S22 (CLSI 2012). First, the overnight culture in trypto-casein-soy broth (Biokar, Beauvais Cedex France) was obtained and the dilution of McFarland 0.5 was prepared. Mueller Hinton agar (Oxoid, Basingstoke, UK) was used for plating salmonella. Discs with the following concentration of antibiotics were used: Ampicillin 10 µg (AMP), Amoxycillin/clavulanic acid 20 µg + 10 µg (AMC), Chloramphenicol 30 µg (CAP), Ciprofloxacin 5 µg (CIP), Gentamycin 10 µg (GEN), Nalidixic acid 30 µg (NAL), Streptomycin 10 µg (STR), Sulphonamides 300 µg (SSS), Tetracycline 30 µg (TET), Trimethoprim+sulfamethoxazole 1.25 µg + 23.75 µg (SXT), Trimethoprim 5 µg (TMP), Cefpodoxime 10 µg. (CPD), Cefotaxime 30 µg (CTX), Ceftazidime 30 µg (CAZ), BioRad (Marnes –la-Coquette, France). The inhibition zone was measured the next day and recorded for each isolate.

RESULTS AND DISCUSSION

The most frequent samples delivered to the laboratory for salmonella control were from broiler farms. Thus *S*. Enteritidis (SE) and *S*. Infantis (SI) were often found in broilers. Surprisingly, SI was detected in layers on three farms out of 16 examined. S. Enteritidis and SI have been isolated in broiler breeders as well. Therefore, at least three serotypes of salmonellas are most frequently circulating among poultry flocks in both Counties. Recent findings of the clonal spread of NAL TET^r SI in Serbia, in poultry and humans, supports the results from this work and implicates significant contamination of poultry farms with the resistant clone (Velhner et. al., 2014). The presence of SE and SI on poultry farms clearly shows that management on farms is not satisfactory. Recently we performed molecular typing of SE by random amplified polymorphic DNA analysis (RAPD) and we have found that genetically related salmonella are shared in poultry and humans. In Serbia SE presents a serious contaminant in the food chain (Kozoderović et al., 2011). According to the regulation provided in the Official Gazette No 7/2010, self monitoring protocols have to be developed for each farm and all flocks infected with SE, ST, SI, *S*. Hadar and S. Virchow must be recorded. This regulation allows treatment of poultry infected with salmonella, as an optional strategy, but also slaughter of infected poultry is requested. The EU regulation (EC 1003/2005, 2160/2003) proposes that the prevalence of SE, ST, SI, S. Hadar and *S*. Virchow has to be 1% or less in all commercial breeder poultry flocks. The comparison of data obtained in this work with the occurrence of Salmonella in EU countries shows that the percent of SE and SI in broilers and layers is higher in our counties. ST is found in 6.1 % of broiler farms examined, while in the EU, ST is found in 3.0% of broiler farms (Miller et. al., 2010). Correspondingly, the National Salmonella Control Program in Serbia is urgently needed, or the financial consequences to the poultry industry will be significant and all three serotypes of Salmonella (SE, ST and SI) will continue to be dominant on our farms.

Table 1: Prevalence of Salmonella spp in single poultry farms from Southern Bačka and Srem region

Chicken flock	Number of farms tested*	*Salmonella* serotype**			
		S. Enteritidis no/%	*S*. Infantis no/%	*S*. typhimurium no/%	*S. Newport no/%*
Broilers	44	25 (56.82)	18(40.90)	3 (6.81)	1 (23.00)
Layers	15	13 (86.66)	3 (20.00)	-	
Broiler breeders	3	2 (66.66)	1 (33.33)		
Breeder eggs					1

**Salmonella isolates from single farms are recorded*
***If there were different serotypes found on the same farms those isolates were also included*

S. infantis was resistant to NAL and TET while SE serotype was susceptible to 14 antibiotics recommended by CLSI (2012, document M100-S22) for Enterobacteriaceae. It is interesting to note that ST, having usually a multiple resistant phenotype, was susceptible to all classes of antibiotics that have been included in resistotyping. The ST was found in broilers only, suggesting that broiler farms in Southern Bačka and Srem County are contaminated with three, otherwise most frequent, serotypes. The discovery of SE in layers is of the highest burden because of their vertical transfer through eggs, which enhances the possibility of food contamination. The resistance mechanism in SI NAL-TET^r was studied recently in detail and it was shown that point mutation on *gyrA* (Ser83→Tyr) and *parC* genes (Ser80→Arg) are principally responsible for the resistance to quinolones. It is important to note that MICs to CIP in some SI isolates have increased to 2 μg/mL, (Velhner et al., 2014). Multiple resistant *S*. Newport was found in broilers. This serotype harbors resistance to: ampicillin, tetracycline and nalidixic acid. Intermediate resistance to ciprofloxacin is also noted. Monitoring the antimicrobial resistance must be evaluated as a continuous process in the future (EC/2003/99/EC, article 7 and EC407/2007) while prudent use of antimicrobial agents is urgently needed.

Table 2: Resistotype of Salmonellas isolated in Souther Bačka and Srem region and their mechanism of resistance

Salmonella serotype	Resistotype	Mechanism of resistance
S. Enteritidis	Sensitive	-
S. Infantis	NAL, TET	Mutations on topoisomerase genes, efflux pump
S. typhimuirum	Sensitive	-

CONCLUSIONS

Poultry farms in two Counties, Southern Bačka and Srem are contaminated with salmonella. The most prevalent serovars are SE and SI. Surprisingly, SI was also found in layer chickens, implicating that contamination with NAL TET^r SI has spread to layers. Resistance to NAL and TET was found only in SI. This finding supports our recent data of clonal spread of SI in Serbia. Other serotypes were susceptible to different classes of antibiotics. The National Control Program for Salmonella is urgently needed in Serbia.

ACKNOWLEDGEMENTS

This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project number TR 31071.

REFERENCES

Clinical and Laboratory Standards Institute (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard-ninth edition. CLSI document M07-A9. CLSI, Wayne, Pa, USA.

Clinical and Laboratory Standards Institute (2012). Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. Clinical and Laboratory Standards Institute document M100-S22. CLSI, Wayne, Pa, USA.

Kozoderović G., Velhner M., Jelesić Z., Stojanov I., Petrović T., Stojanović D., Golić N. (2011): Molecular typing and antimicrobial resistance of Salmonella Enteritidis isolated from poultry, food, and humans in Serbia. Folia Microbiologica, 56 (1), 66-71.

Miller T., Prager R., Rabsch W., Fehlhaber K., Voss M. (2010): Epidemiological relationship between *Salmonella* Infantis isolates of human and broiler origin. Lohmann Information, 45 (2), 27-31.

OIE Manual of Diagnostic tests and vaccines for terrestrial animals, Seventh Edition, 2012, Salmonellosis, Chapter 2.9.9.

DIRECTIVE EC/2160/2003 of the EUROPEAN PARLAMENT AAND OF THE COUNCIL of Novemer 2003, on the control of salmonella and other specified food-borne zoonotic agents.

DIRECTIVE EC/2003/99 OF THE EUROPEAN PARLAMENT AND THE COUNCIL of November 2003, on monitoring of zoonoses and zoonotic agents, amending Council Decision 90/4242/EEC and repealing Council Directive 92/117/EEC.

COMMISSION REGULATION (EC) No 1003/2005 of 30 June 2005, implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of *Gallus gallus* and amending Regulation (EC) No 2160/2003

DIRECTIVE EC407/2007, decisions commission of 12 June 2007 on a harmonised monitoring of antimicorbial resistance in Salmonella in poultry and pigs, .

Velhner M., Kozoderović G., Jelesić Z., Stojanov I., Potkonjak D., Petrović J. (2012). Plasmid mediated resistance to quinolones in Salmonella. Arhiv veterinarske Medicine, 5 (1), 19-29.

Velhner M., Potkonjak D., Stojanović D., Mitevski D., Stojanov I.,Petrović J. (2013) Rezistencija salmonela na antibakterjske lekove I mere kontrole u živinarskoj proizvodnji. Veterinarski glasnik, 67(1-2), 87-96.

Velhner M., Kozoderović G., Grego I., Galić N., Stojanov I., Jelesić Z., Kehrenberg C. (2014). Clonal Spread of Salmonella enterica Serovar Infantis in Serbia: Acquisition of mutations in the topoisomerase genes *gyrA* and *parC* leads to Increased resistance to fluoroquinolones. Zoonoses and Public Health, 60 (5), 364-370.

INFLUENCE OF GRINDING METHOD AND GRINDING INTENSITY OF CORN ON MILL ENERGY CONSUMPTION AND PELLET QUALITY

Đuro Vukmirović[1]*, Jovanka Lević[1], Aleksandar Fišteš[2], Radmilo Čolović[1], Tea Brlek[1], Dušica Čolović[1], Olivera Đuragić[1]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: djuro.vukmirovic@fins.uns.ac.rs

ABSTRACT

The aim of this research was to investigate influence of grinding method (hammer mill vs. roller mill, i.e. wide vs. narrow distribution of particle size) and grinding intensity of corn (coarseness of grinding) on mill specific energy consumption (SEC) and on pellet quality.
Grinding on roller mill resulted in more uniform particle size distribution (PSD) compared to hammer mill. As it was expected, increasing of grinding intensity significantly increased SEC of both hammer mill and roller mill ($p < 0.05$), while increase was more pronounced for the hammer mill. When comparing SEC for similar grinding intensity on hammer mill and roller mill (similar geometric mean diameter), SEC was higher for the hammer mill. Pellet quality decreased with coarser grinding on hammer mill but, surprisingly, this effect was not observed for the roller mill. Generally, pellet quality was better when roller mill was used compared to hammer mill and this was attributed to more uniform PSD of corn ground using roller mill.
From the obtained results it can be concluded that high energy savings of grinding process could be achieved by coarser grinding of corn before pelleting. But, from the aspect of pellet quality, if coarser grinding is applied it is better to use roller mill, concerning that more uniform PSD of corn ground on roller mill results in more uniform PSD in pellets and this provides better pellet quality
***Keywords**: grinding, energy consumption, pellet quality, poultry, corn*

INTRODUCTION

Dominating principle in poultry breeding is to use complete mixtures (diets) in pelleted form (Svihus, 2011) because it has been shown that pelleting (compared to using diets in mash form) increases feed intake (Engberg et al., 2002), reduces feed wastage (Jensen, 2000), prevents birds from selecting larger particles (Abdollahi et al., 2013), prevents segregation of diet components (Greenwood and Beyer, 2003), etc. The first step in production of pelleted poultry feed is grinding of diet ingredients. Grinding is most commonly done by hammer mills while roller mills are not widely used in animal feed production even though they work with lower energy consumption than hammer mills. Hammer mills produce some large and many small particles, while roller mills produce more uniform particle size distribution (PSD) (Nir et al., 1990; Koch, 1996).
Although fine grinding was considered as a key for achieving good pellet quality and good utilization in poultry digestive system (Svihus et al., 2004), there is a lot of literature that emphasizes importance of coarse particles presence in poultry diets due to their positive effect on digestive system (Svihus, 2011). Thus, coarser grinding of cereals, as the main component of poultry diets, should be applied. Even though it is well known that pelleting reduces size of micro-particles that constitute the pellets (Abdollahi et al., 2013), it is expected that coarser grinding before pelleting will increase the share of coarse particles in pellets.
Problem with coarse ground cereals is their influence on pellet quality because dominating belief is that pellet quality decreases with coarser grinding (Amerah et al., 2007b). But Reece et al. (1986) determined that coarseness of grinding has no effect on pellet quality. As it can be seen, results about influence of grinding intensity on pellet quality are contradictory.

The aim of this research was to determine the influence of mill type (hammer mill vs. roller mill, i.e. wide vs. narrow distribution of particle size) and coarseness of grinding on mill energy consumption and pellet quality.

MATERIAL AND METHODS

Experiments were conducted at pilot-plant facility of Institute of Food Technology (University of Novi Sad, Serbia). Dent corn obtained from local company (Agrobacka a.d., Backa Topola, Serbia) was ground using hammer mill (ABC Engineering, Pančevo, Serbia) and roller mill (ROSKAMP TP650-9, California pellet mill, USA) equipped with three pairs of rollers with 1.8 – 5.5 corrugations per cm and differential speed of 1 : 1.5 for each pair of rollers.
By using hammer mill equipped with sieve openings diameter of 3, 6 and 9 mm, three different coarseness of corn were obtained: fine (treatment HM-F), medium (HM-M) and coarse (HM-C), respectively. For the roller mill, gap between two higher pairs of rollers was fixed at 4.4 and 2.6 mm for all grinding treatments while gap between lower pair of rollers was set to 1.4, 2.0 and 2.6 mm for obtaining three different coarseness, i.e. medium (RM-M), coarse (RM-C) and very coarse (RM-VC).
Specific energy consumption (kWh/t) of hammer mill and roller mill was measured according to equation described by Payne et al. (1994):

$$Specific\ energy\ consumption = \frac{(I - I_0) * U * cos\varphi * \sqrt{3}}{1000} * \frac{1}{Q}$$

where I (A) and I0 (A) are average hammer mill or roller mill motor amperage with and without material, respectively, U (V) is the voltage, cosφ is the power factor (ratio between the actual load power and the apparent load power drawn by an electrical load) and Q (kg/h) is the throughput of material.
Moisture content of ground corn was adjusted to 16% by the addition of water in the double shaft pedal mixer (SLHSJ0.2 Muyang, China) and corn was pelleted using flat pellet press (14-175, Amandus Kahl, Germany) with 6 mm diameter of die openings and 24 mm thickness.
PSD of ground corn was determined according to ISO 1591-1 1988 (E) using sieve shaker (Endecotts, UK) with the following size of sieve openings: 5600, 4000, 3150, 2000, 1600, 1000, 630, 250 and 125 µm. Geometric mean diameter (GMD) and geometric standard deviation (GSD) were determined according to A.S.A.E. standard (A.S.A.E., 2003) using the equations:

$$GMD = log^{-1}\left[\frac{\sum_{i=1}^{n}(W_i * log d_i')}{\sum_{i=1}^{n} W_i}\right]$$

$$d_i' = \sqrt{d_i + d_{i+1}}$$

$$GSD = log^{-1}\left[\frac{\sum_{i=1}^{n}(\log d_i - log\ GMD)}{\sum_{i=1}^{n} W_i}\right]$$

where di (µm) is the size of sieve openings of i^{th} sieve and W_i (g) is the mass on i^{th} sieve.

Pellet quality was measured using Holmen Pellet Tester (NHP 100, Norfolk, UK) and expressed as pellet durability index (PDI) which is calculated as the ratio of mass of pellets after the test and the mass of pellets before the analyses. Duration of treatment was 30 s and pellets were sieved before and after the treatment using a sieve with 4.8 mm size of sieve openings.
One-way analysis of variance (ANOVA) and Tukey HSD test were used for comparison of sample means to analyze variations of the results (statistical software Statistica 12,

Oklahoma, USA). Differences between the means with probability $p<0.05$ were accepted as statistically significant.

RESULTS AND DISCUSSION

Obtained GMDs and GSDs for different grinding treatments of corn are presented in Table 1. As it can be seen, wide range of GMDs was achieved, and GSDs were different for different mill type (hammer vs. roller mill), while for grinding treatments within the same mill, values were similar. According to A.S.A.E. (2003) when GSD is equal to 1 all particles are exactly the same, while when GSD is about 3 or more there is a lot of variation in particle size. This implies that for roller mill PSDs were more uniform.

Table 1. Grinding treatment and obtained GMDs and GSDs

Grinding treatment	GMD (µm)	GSD (µm)
HM-F	643	2.97
HM-M	1108	3.01
RM-M	1099	2.34
HM-C	1550	2.91
RM-C	1518	2.42
RM-VC	2072	2.27

In production of pelleted poultry diets cereals are usually finely ground using 3 to 4.5 mm hammer mill SOD (Svihus et al., 2004b). Thus, treatment HM-F presented grinding that is usually performed in practice. In other treatments of this study coarseness of grinding was increased, with extreme coarseness for treatment RM-VC. PSDs of the finest (HM-F) and the coarsest ground corn (RM-VC) are presented in Figure 1.

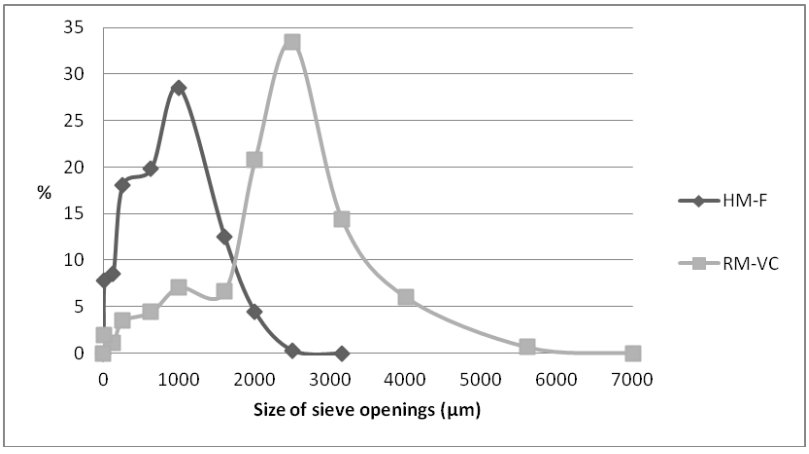

Figure 1. PSD of corn ground at hammer mill with 3 mm SOD (HM-F) and at roller mill with 2.6 mm gap between lower pair of rolls (RM-VC)

For treatments RM-M and RM-C the distance between lower pair of rollers was selected to reflect 6 mm and 9 mm hammer mill grinding, i.e. to obtain similar GMD between HM-M and RM-M, and between HM-C and RM-C (Table 1). Even though GMDs of these pair of treatments were similar, differences in PSD were very pronounced, which can be seen in the Figure 2 and 3.

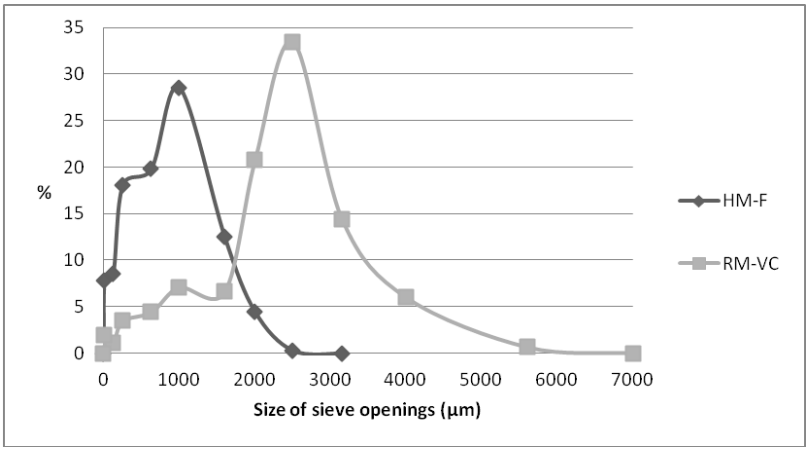

Figure 2. PSD of corn ground at hammer mill with 6 mm SOD (HM-M) and at roller mill with 1.4 mm gap between lower pair of rolls (RM-M)

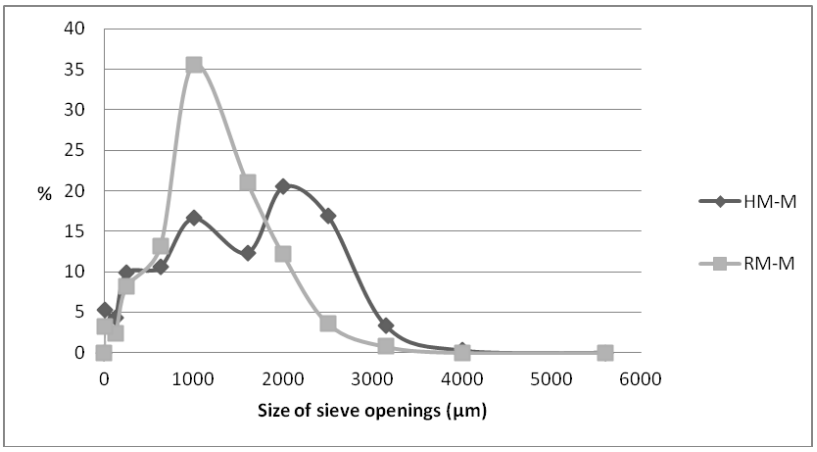

Figure 3. PSD of corn ground at hammer mill with 9 mm SOD (HM-C) and at roller mill with 2.0 mm gap between lower pair of rolls (RM-C)

Markedly higher specific energy consumption for grinding was obtained for the treatment HM-F comparing to other treatments (Figure 4). When comparing pairs of treatments with similar GMD, obtained with hammer mill and roller mill (HM-M vs. RM-M and HM-C vs. RM-C), it can be seen that specific energy consumption was higher when hammer mill was used. Additionally, there was no significant difference between treatments RM-M and HM-C, which further indicates possibilities for energy savings when using roller mill instead of hammer mill. Expected decrease of pellet quality (expressed as PDI) with coarser grinding was observed for corn ground using hammer mill (Figure 5). Surprisingly, this was not observed for corn ground using roller mill where obtained PDI values for different grinding intensities were not significantly different within each other. Generally, pellet quality was better (higher PDI) when roller mill was used compared to hammer mill. Only for the treatment HM-F, obtained pellet quality was not significantly different from RM treatments, yet it was slightly lower.

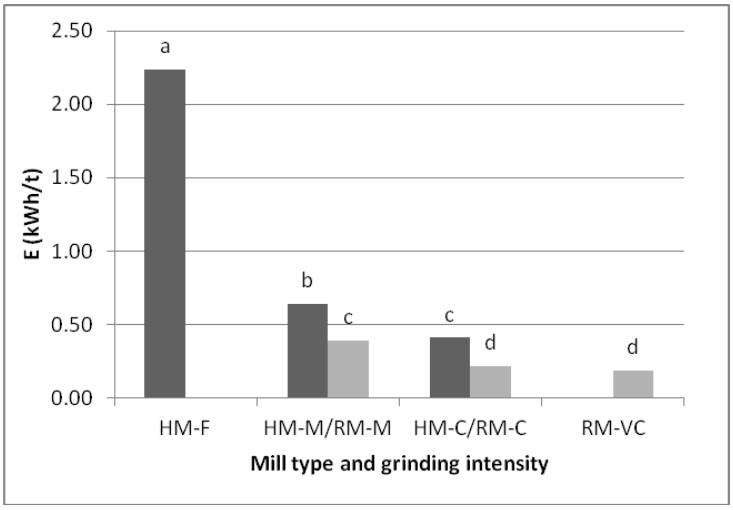

Figure 4. Influence of mill type (hammer mill (HM) or roller mill (RM)) and grinding intensity (fine (F), medium (M), coarse (C) and very coarse (VC)) on specific energy consumption of mill. Values with different letters are significantly different ($p < 0.05$)

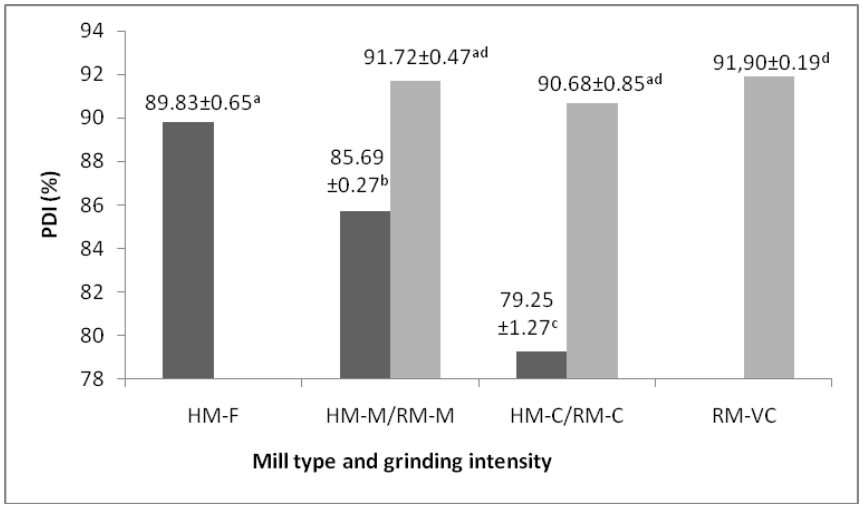

Figure 5. Influence of mill type (hammer mill (HM) or roller mill (RM)) and grinding intensity (fine (F), medium (M), coarse (C) and very coarse (VC)) on PDI. Values with different letters are significantly different ($p < 0.05$)

Possible reason for lower PDI values of pellets produced with corn ground on hammer mill could be wider distribution of particle size which results in more inhomogeneities in pellet structure. Pellets are particularly sensitive near the points of inhomogeneities in their structure because local stresses and strains are highest near such imperfections (Thomas and van der Poel, 1996). Grinding with roller mill results in more uniform PSD and in can be assumed that this resulted in more uniform PSD in pellets, compared to pellets made of hammer milled corn. As the result of narrow PSD and more homogeneous structure, quality of pellets produced from material ground on roller mill was better.

CONCLUSIONS

From the obtained results it can be concluded that high energy savings could be achieved by coarser grinding of corn before pelleting. But, from the aspect of pellet quality, if coarser grinding is applied, it is better to use roller mill, concerning that more uniform PSD after roller mill result in more uniform PSD in pellets and this provides better pellet quality.

ACKNOWLEDGEMENTS

Authors would like to express their gratitude to Serbian Ministry of Education, Science and Technological Development who supported this work as a part of Project No III 46012. Authors also would like to thank to Province Secretariat of Science and Technological Development of Autonomous Province of Vojvodina on financial support.

REFERENCES

Abdollahi, M.R., Ravindran, V., Svihus B. (2013). Pelleting of broiler diets: An overview with emphasis on pellet quality and nutritional value. Animal Feed Science and Technology 179: 1-23.

Amerah, A.M., Ravindran, V., Lentle, R.G., ThomaS, D.G. (2007). Influence of feed particle size and feed form on the performance, energy utilisation, digestive tract development, and digesta parameters of broiler starters. Poultry Science 86, 2615–2623.

American Society of Agricultural and Biological Engineers (A.S.A.E.) (2003). Methods of Determining and Expressing Fineness of Feed Materials by Sieving. Standard no. S319.3., pp. 202–205.

Engberg, R.M., Hedemann, M.S., Jensen, B.B. (2002). The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. British Poultry Science 43, 569–579.

Greenwood, M.W., Beyer, R.S. (2003). Effect of feed manufacturing practices on nutrient availability and feed quality, Proceedings of the 30th Annual Carolina Poultry Nutrition Conference, Raleigh, NC, USA, 7–16.

Jensen, L.S. (2000). Influence of pelleting on the nutritional needs of poultry. Asian-Australian Journal of Animal Science 13, 35–46.

Koch, K. (1996). Hammer mills and roller mills. In: MF-2048 Feed Manufacturing (pp. 1–4). Department of Grain Science and Industry Kansas State University, Manhattan, KS, USA.

Payne, J., Rattink, W., Smith, T., Winowiski, T. (1994). Objectives in pelleting. In: Payne, J., Rattink, W., Smith, T., Winowiski, T. (Eds), The Pelleting Handbook, pp. 11–12, Norway, Borregaard Lignotech.

Reece, F.N., Lott, B.D., Deaton, J.W. (1986). The effects of hammer mill screen size on ground corn particle size, pellet durability, and broiler performance. Poultry Science 65: 1257-1261.

Svihus, B. (2011). The gizzard: function, influence of diet structure and effects on nutrient availability. Worlds Poultry Science Journal 67, 207–223.

Svihus, B, Kløvstad, K.H., Perez, V., Zimonja, O., Sahlström, S., Schüller, R.B., Jeksrud, W.K. & Prestløkken, E. (2004). Physical and nutritional effects of pelleting of broiler chicken diets made from wheat ground to different coarsenesses by the use of roller mill and hammer mill. Animal Feed Science and Technology, 117, 281–293.

Thomas, M. and van der Poel, A.F.B. (1996) Physical quality of pelleted animal feed: 1.Criteria for pellet quality. Animal Feed Science and Technology 61, 89–112.

EFFECTS OF Cr (III) SUPPLEMENTS IN GROWING PIG DIETS ON NUTRITIONAL QUALITY OF LOIN (*Longissimus Dorsi*)

Arabela Elena Untea, Tatiana Dumitra Panaite, Iulia Varzaru*, Margareta Olteanu, Gabriela Maria Cornescu, Mariana Ropota

National Institute for Research Development in Animal Biology and Nutrition (IBNA- Balotesti), Romania

*Corresponding author:
E-mail address: iulia_maros@yahoo.com

ABSTRACT

Trivalent chromium it was recognized to play a role in regulating glucose and lipid metabolism in humans and laboratory animals. There are relatively few papers on the effect of Cr supplementation on pork quality.
The objective of this study was to highlight the positive effects of the chromium picolinate on nutritional quality of loin.
A 6-week study on growing pigs evaluated the effect of the dietary chrome picolinate (CrPic) on the growth performances and nutritional quality of loin (Longissimus dorsi). The experiment was conducted on 8 castrated Landrace × Large White males with an initial bodyweight of 17.16 ± 0.62 kg, assigned to 2 groups (C, E), housed in individual metabolic cages and fed on corn-soybean meal-based diets (18.75% CP; 3063 kcal/kg ME). The diets of E group was supplemented with 200 µg/kg CrPic. Blood samples were collected at the end of the experiment, following which all animals have been slaughtered and samples of loin were collected. The nutritional quality of the collected samples was evaluated for: proximate analysis, amino acids profile, fatty acids profile, mineral content.
No significant differences of productive parameters were noticed. In loin samples, the fat / protein ratio was lower in group E (22.18% fat; 63.4% protein), than in group C (22.27% fat; 57.57% protein). There were no significant differences between groups for fatty acids analysis but it was noticed a significant increase of methionine concentrations (0.73±0.07% for C and 0.88±0.06% for E). Chromium supplements decreased, but not significant (P>0.05) Fe and Zn deposition in loin (32.16±2.57 ppm (C); 28.15±0.56 ppm (E) for Fe and 40.22±2.50 ppm (C); 39.12±1.67 ppm (E) for Zn). The antagonist relation between Cr and Fe was expected due to the same minerals transporter.
***Keywords**: trivalent chromium, pigs, nutritional quality, loin*

INTRODUCTION

Chromium is the 21th most abundant mineral in the crust of the earth and its essentiality for human nutrition was demonstrated for over 35 years (Jeejebhoy et al., 1977). At the late of 1990s, chromium started to be studied as an essential mineral in livestock animals (cattle, sheep, pigs and poultry) (Pechova, 2007). The main role of trivalent chromium it was recognized to be the regulating glucose and lipid metabolism in humans and animals. A lack of chromium in the body results in tissues becoming less responsive or insulin resistant. Insulin is a key factor in the regulation of skeletal muscle protein synthesis. Postprandial changes in protein synthesis are positively correlated with changes in circulating insulin concentrations in pigs (O'Conner, 2003). Also, chromium (III) seems to influence the lipogenic activity of the organism, modifying the amount of fat deposits in monogastric animals (Lambertini, 2004).
There are relatively few papers on the effect of Cr supplementation on pork quality. Page et al., (1993) found that chromium picolinate supplementation increased gain in growing-finishing pigs experiments, reduced backfat and increased percentage of muscle. Similar results were reported by Jackson et al., (2009), using chromium propionate supplements. Lindemann et al., (2004) reported that chromium supplementation increased the number of pigs born alive.

Muscle and fat contents are critical attributes of the quality of pig carcasses. The objective of the present research was to investigate the effects of chromium picolinate supplements in growing pigs, on nutritional quality of loin.

MATERIAL AND METHODE

Experimental design

The experiment was conducted on 8 growing castrated TOPIGS male pigs, under balance experiment conditions and it ran for 45 days. Throughout the experimental period, the piglets were randomly assigned to 2 groups (4 animals per group), kept in individual metabolic cages (AGRICO, RYBARSKA, Czech Republic) with an area of 0.87 m^2, placed in an experimental hall under controlled environmental conditions (temperature of 24^0C, humidity 50-60 %). The piglets were fed the respective diets daily, at 8.00 a.m., *ad libitum*. Water was supplied *ad libitum* via drinking nipples. The pigs had an average initial body weight of 17.16 ± 0.6 kg. They received a commercial diet designed for this category of animals differed between groups by the level of Cr^{3+} supplement. The source of Cr^{3+} was Chromium picolinate (Sigma Aldrich, Germany) and it brought the chromium level to 200 µg Cr/kg feed in E group. The productive parameters were calculated from the records of the body weights and feed intake. At the end of experiment, after blood samples collection, all pigs were slaughtered and meat samples (loin) were collected.

Chemical analysis

The crude protein of the diet and of the muscles was determined using a semiautomatic classical Kjeldahl method using a Kjeltek auto 1030 – Tecator (SR EN ISO 5983-2, 2009 and SR ISO 973, 2007, respectively). The fat was extracted using an improved version of the classical method by continuous extraction in solvent, followed by fat measurement with Soxhlet after solvent removal (SR ISO 6492, 2001).

Gas chromatograph Perkin-Elmer Clarus 500 (Massachusetts, United States), fitted with Flame Ionization Detector (FID) and capillary separation column with high polar stationary phase (TRACE TR-Fame, (Thermo Electron, Massachusetts, United States), 60m X 0.25 mm X 0.25 µm. thickness film) was used in order to determine fatty acids composition of meat samples. Each sample was prepared as described previously (Habeanu et al., 2011).

HPLC Surveyor Plus Thermo Electron, (Massachusetts, United States) and HyperSil BDS C18 column (Thermo Electron, Massachusetts, United States), dimensions 250mm X 4.6 mm X 5 µm were used in order to determine the amino acids profile of meat samples. Each sample was prepared as described previously (Varzaru et al., 2013).

The meat samples were analysed for iron and zinc concentrations applying flame atomic absorption spectrometry (FAAS) as described by Untea et al., (2012) after the microwave digestion. The used Equipment was as follows: Atomic absorption spectrometer Thermo Electron – SOLAAR M6 Dual Zeeman Comfort (Cambridge, UK), with deuterium lamp for background correction and air-acetylene flame and microwave digestion system with remote temperature measurement, BERGHOF, Speedwave MWS-2 Comfort (Eningen, Germany).

Statistical analysis

The analytical data were compared performing analysis of variance (ANOVA), using STATVIEW for Windows (SAS, version 6.0). The differences between mean values in the groups were considered significant at $P<0.05$.

RESULTS AND DISCUSIONS

No significant differences of productive parameters were noticed. At the end of experiment, blood samples were collected and the results of haematological parameters were similar for both of studied groups proving a normal state of health.

Crude protein and crude fat concentrations were determined in loin samples. The results obtained are presented in figure 1.

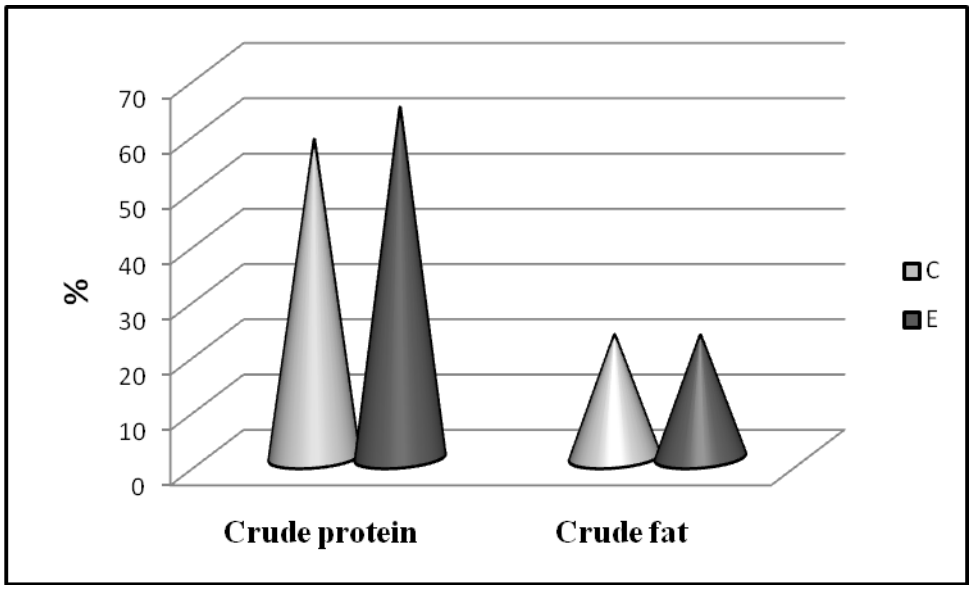

Figure 1. Crude protein and crude fat concentrations in loin

No significant differences were noticed for crude protein and crude fat concentrations between groups. In loin samples, the fat / protein ratio was lower in group E (22.18% fat; 63.4% protein), than in group C (22.27% fat; 57.57% protein). Mooney et al. (1997), observed that chromium supplementation increased the rate of protein deposition and reduced the rate of fat deposition in pigs carcass.
A significant increase (*P≤ 0.05*) of methionine concentrations, an essential amino acid for pigs it was noticed. No significant differences were observed for fourteen amino acids analized. The results are presented in table 1.

Table 1. Amino acids concentrations in loin

Amino acid	**C %**	**E %**
Aspartic acid	5.373 ± 0.13	5.409 ± 1.02
Glutamic acid	10.327 ± 0.18	10.225 ± 1.95
Serine	3.146 ± 0.05	3.138 ± 0.57
Glycine	2.702 ± 0.22	2.699 ± 0.30
Threonine	2.955 ± 0.02	2.892 ± 0.49
Arginine	4.975 ± 0.16	4.937 ± 0.81
Alanine	3.537 ± 0.17	3.436 ± 0.55
Tyrosine	1.923 ± 0.06	1.951 ± 0.39
Valine	1.764 ± 0.12	1.827 ± 0.30
Phenylalanine	1.464 ± 0.11	1.576 ± 0.23
Isoleucine	2.645 ± 0.14	2.737 ± 0.53
Leucine	4.364 ± 0.13	4.283 ± 0.87
Lysine	5.278 ± 0.35	4.947 ± 0.82
Cystine	0.532 ± 0.04	0.566 ± 0.03
Methionine	0727 ± 0.07[a]	0.882 ± 0.06[b]

Note: a – significantly (*P≤ 0.05*) different of E; b – significantly (*P≤ 0.05*) different of C

The fatty acids profile was split into three main categories: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). There were no significant differences between groups for the three fatty acids categories (fig 2). In human trials, Cr didn't have effects on lipid metabolism (Anderson, 1983; Rabinowitz, 1983). These authors suggests that the lipid metabolism is a function of nutritional status of Cr in organism and depends of bioavailability of Cr from food and supplements.

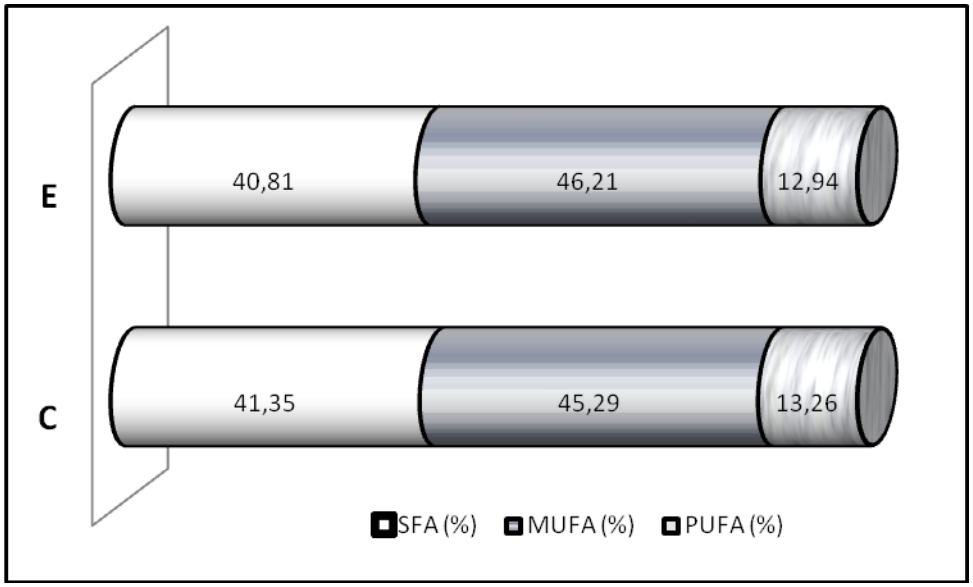

Figure 2. Fatty acids (SFA; MUFA and PUFA) concentrations in loin

Chromium supplements decreased, but not significant (P>0.05) Fe and Zn deposition in loin (Fig. 3). The antagonist relation between Cr and Fe was expected due to the same minerals transporter (transferrin). When the concentration of Fe is higher (conventional diets for pigs include Fe above requierements of animal category) the two minerals (Cr and Fe) compete for the same binding sites (Pechova, 2007). Ani and Mostaghie, (1992), noticed that chromium may impair Fe metabolism.

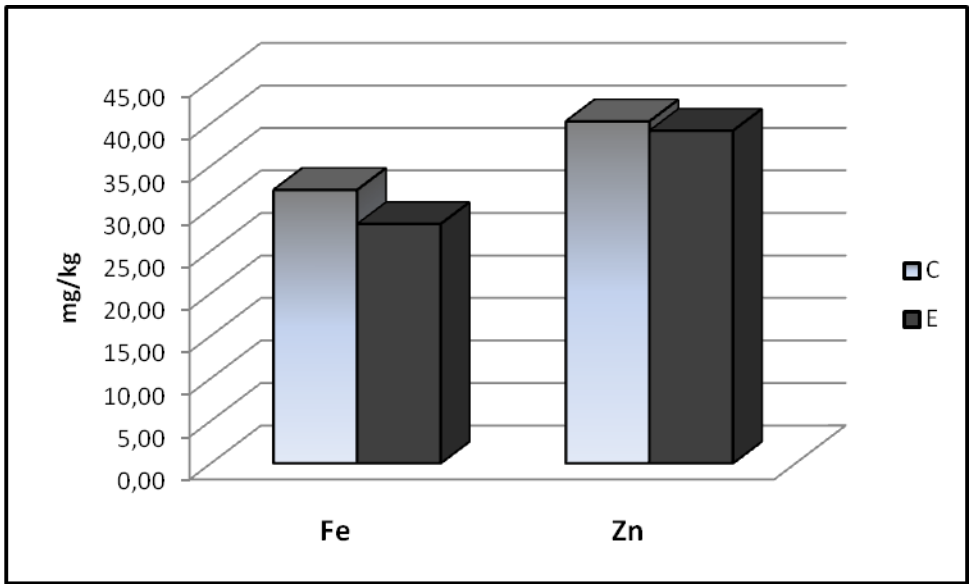

Figure 3. Minerals (Fe and Zn) concentrations in loin

CONCLUSIONS

The use of chromium supplements as chromium picolinate in growing pigs diet (200 ppb) didn't affect significantly the nutritional quality of lean meat such as loin. We consider that more research is necessary to evaluate the effect of chromium on other anatomical parts of pigs valuable for human nutrition.

REFERENCES

Anderson, R.A., Polansky, M.M., Bryden, N.A., Patterson, K.Y., Veillon, C., Glinsmann, W.H. (1983). Effects of chromium supplementation on urinary Cr excretion of human subjects and correlation of Cr excretion with selected clinical parameters. Journal of Nutrition, 113, 276–281.

Ani, M., Mostaghie, A.A. (1992). The effect of chromium on parameters related to iron metabolism. Biological Trace Element Research, 32, 57-64.

Habeanu, M., Hebean, V., Taranu, I., Ropota, M., Lefter, N., Marin, D. (2011). Dietary ecologic camelina oil – a beneficial source of n-3 PUFA in muscle tissue and health status in finishing pigs, Romanian Biotechnological Letters. 16 (5), 6564-6571.

Jackson, A. R., Powell, S., Johnston, S. L., Matthews, J. O., Bidner, T. D., Valdez, F. R., & Southern, L. L. (2009). The effect of chromium as chromium propionate on growth performance, carcass traits, meat quality, and the fatty acid profile of fat from pigs fed no supplemented dietary fat, choice white grease, or tallow. Journal of animal science, 87(12), 4032-4041.

Jeejebhoy, K.N., Chu, R.C., Marliss, E.B., Greenberg, G.R., Bruce-Robertson, A. (1977). Chromium deficiency glucose intolerance and neuropathy reversed by chromium supplementation in a patient receiving long-term total parenteral nutrition. American Journal of Clinical Nutrition, 30, 531-538.

Lindemann, M. D., Carter, S. D., Chiba, L. I., Dove, C. R., LeMieux, F. M., & Southern, L. L. (2004). A regional evaluation of chromium tripicolinate supplementation of diets fed to reproducing sows. Journal of animal science, 82(10), 2972-2977.

Lambertini, L., Vignola, G., Beone, G. M., Zaghini, G., & Formigoni, A. (2004). Effects of chromium yeast supplementation on growth performances and meat quality in rabbits. World Rabbit Sci. 12, 33 – 47.

Mooney, K.W., Cromwell, G.L. (1997). Efficacy of chromium picolinate and chromium chloride as potential carcass modifiers in swine. Journal of Animal Science, 75, 2661–2671.

O'Connor, P. M., Bush, J. A., Suryawan, A., Nguyen, H. V., & Davis, T. A. (2003). Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. American Journal of Physiology-Endocrinology and Metabolism, 284(1), E110-E119.

Page, T.G., Southern, L.L., Ward, T.L., Thompson, D.L. (1993). Effect of chromium picolinate on growth and serum and carcass traits of growing finishing pigs. J.Anim.Sci., 71, 656-662.

Pechova, A., Pavlata, L. (2007). Chromium as an essential nutrient: a review. Veterinarni Medicina, 52 (1), 1–18.

Rabinowitz, M.B., Gonick, H.C., Levine, S.R., Davidson, M.B. (1983). Clinical trial of chromium and yeast supplements on carbohydrate and lipid metabolism in diabetic men, Biological Trace Element Research, 1983, 5, 449–466.

Untea, A. R. A. B. E. L. A., Criste, R. C., & Vladescu, L. (2012). Development and validation of a microwave digestion–FAAS procedure for Cu, Mn and Zn determination in liver. Revista de Chimie, 63(4), 341-346.

Varzaru, I., Untea, A. E., Martura, T., Olteanu, M., Panaite, T. D., Schitea, M., & Van, I. (2013). Development and Validation of an RP-HPLC Method for Methionine, Cystine and Lysine Separation and Determination in Corn Samples. Revista de Chimie, 64(7), 673-679.

NUTRITIVE VALUE OF VITAMINIZED SILAGES

Milica Živkov-Baloš[1], Sandra Jakšić[1], Željko Mihaljev[1], Saša Obradović[2], Dragana Ljubojević[1], Igor Stojanov[1], Milovan Jovičin[1]

[1]Scientific Veterinary Institute "Novi Sad", Rumenački put 20, 21 000 Novi Sad, Republic of Serbia
[2]State University of Novi Pazar, Department of Chemical-Technological Sciences, Vuka Karadžica bb, 36300 Novi Pazar, Republic of Serbia

*Corresponding author:
E-mail address: milica@niv.ns..ac.rs

ABSTRACT

Plant species that are suitable for silage have a higher dry matter yield in the field and higher digestibility, low buffer capacity and a higher amount of water soluble carbohydrates. Whole plant maize silage is used worldwide in rations for cattle. However, β-carotene content in corn silage, which is a popular main feed for dairy cows, is very low. In our region, pumpkin and carrot may be considered as alternative crops and an option for production of vitaminized silage. Ensiling can be considered as the method for using wet raw materials more efficiently. The aim of this work was to evaluate the quality of silage based on the whole corn plant and carrots as well as pumpkin for possible use in dairy cows nutrition. For ensilaging, the following combinations of raw materials were used: whole corn plant and carrot, oat grits and carrot and corn grits and pumpkin. Silage samples were taken from the top, centre and bottom parts of the trench silo, i.e. the barrel. Lactic and volatile organic acids (butyric and acetic acid) were determined by distillation. Determination of moisture, fat, crude fiber, ash, calcium and phosphorus in the samples was performed by standard methods, while the protein was determined by measuring total nitrogen using total combustion. The fermentation process parameters and nutritional composition indicated that investigated vitaminized silages can be successfully prepared, preserved and utilized as a feed for dairy cows.
***Keywords**: vitaminized silage, carrot, pumpkin, dairy cows*

INTRODUCTION

Organic milk production is based on organic principles and objectives including naturalness and reciclyng of nutrients. According to current regulations, the feed used in organic milk production must be 100% organic grown (EC, 1999). However, the integrity of the organic production could be further enhanced if the frequent use of vitamin supplementation with artificial fat-soluble vitamins could be substituted by vitamins from natural sources (Mogensen et al., 2012).

During recent decades, milk production per cow has increased dramatically as a cause of improved management, nutrition, and genetic selection, however, the reproductive performance of high-producing dairy cows has been declining. The positive relationship between supplemental β-carotene and reproductive performance has been demonstrated and reviewed in many studies (Hurley and Done, 1989), and a recent study has reported that β-carotene supply improved embryo production and quality in super ovulated cows (Kawashima et al., 2010). In ruminants, as in other animals, carotenes (mainly β-carotene) are precursors of retinol (i.e. Vitamin A) through cleavage (Yang and Tume, 1993). Among the roles of retinol in ruminants, as in other mammals, its influence on reproduction has been extensively studied. A deficiency in retinol may reduce reproductive efficiency in dairy cows, especially through impaired ovarian function and increased incidence of abortion (Hurley and Done, 1989). Retinol is also involved in various functions, such as vision, growth and male fertility. In addition, the conversion rate of β-carotene to vitamin A in granulosa cells has been enhanced by follicular growth, and intrafollicular concentrations of vitamin A correlated positively with those of estradiol and follicle diameter (Schweigert et al., 1989).

The β-carotene content in corn silage, which is a popular main feed for dairy cows, is very low. The amount of β-carotene in grasses varies with plant species and stage of growth. The

level of β-carotene in pasture grass, corn, alfalfa, etc. is highest in a young plant but decreases as the plant becomes older (Nozière et al., 2006). In Serbia, pumpkins and carrots can be considered as alternative crops and an option to replace the synthetic vitamin A and β-carotene in cattle diets.

Plant species that are suitable for ensiling have a higher dry matter yield in the field and higher digestibility, low buffer capacity, and a higher amount of water soluble carbohydrates. Maize silage is characterized by high concentration of soluble carbohydrates that allow a good fermentation, good energy supply, and high rumen degradability. Root crops, alternative crops and agricultural residuals are highly moisture materials. Although these materials have nutritive value, they are difficult to incorporate into a commercial feeding program because of preservation problems. Instability of pumpkin fruit on storage rules out its wide application in industrial processing. In this situation, the cost of collection and transportation is likely to be high. Another problem is that many crops are seasonal and all become available at the same time of the year. This means that these crops need to be treated for storage if they are to be made available as a feed resource during lean season. Ensiling can be considered as a method to use wet raw materials more efficiently. Prior to silage making, drying and chopping are also required, which makes the method a bit expensive and cumbersome for farmers. The basic principles are the same as those for fresh forages and maize silage, so attention must be paid to ensuring anaerobic conditions and there should be sufficient acid in the silage to restrict the activities of undesirable bacteria.

Carrots can be ensiled, typically with a dry forage (hay or straw) chopped at 7-8 cm length. They can be ensiled in layers with grass and alfalfa (Morel d'Arleux, 1990). Ensiling carrot roots and whole carrot plants was shown to decrease the β-carotene content by 34% during storage. Moisture and butyric acid increased, although the pH value was kept below 4.2 (Nonaka et al., 1994). Regarding different mixtures of carrot silages with grain and straw, the best quality silage was obtained with carrot and oat grain. Carrots were also able to control the moisture content of low-moisture late-harvested maize (Nonaka et al., 1994).

Having in mind the specificities of climate conditions and the type of agricultural farms in Serbia, carrot and pumpkin can be considered as potential raw materials for production of combined vitaminized silage. Thus, the aim of this study was to investigate the possibility to successfully ensiling pumpkin and carrot, and to determine nutritive value of produced silages.

MATERIAL AND METHODS

*Material*s

For ensilaging, the following combinations of raw materials were used:

1. Vitaminized silage of whole corn plant and carrot

Silage corn mass was 72 000 kg and carrot mass (whole root) was 3500 kg. The ensilaging was performed in a trench silo using an inoculant (Start supplement for silage, Natura point, Novi Sad).

2. Vitaminized silage of carrot and oat grits

Carrots (14 kg) were chopped in small particles (2 cm), and then 12.5 kg of oat-grits were added along with 1.5 l water. The ensiling was performed in a PVC barrel.

3. Vitaminized silage of corn grits and pumpkin, variety *Ishicu Kuru* (used for seed production) was prepared in a trench silo. Pumpkin's pulp (720 kg) was pulverized using a tractor chips and mixed with 480 kg of corn groats. Silage was carried out in six plastic barrels of 200 L.

Sampling procedure

Silage samples were taken from the top, centre and bottom parts of the trench silo, i.e. the barrel. The samples were collected into sterilized bags, kept in iceboxes and transported to the laboratory for analysis.
Methods of chemical analysis: Content of lactic and volatile organic acids (butyric and acetic acid) was determined by the method of Flieg (Balzer, 1961). Moisture content was determined by drying at 105°C and pH value by direct potentiometry method (Pravilnik,1987). Determination of moisture, crude fat, crude fiber, crude ash, calcium and phosphorus in the samples was performed by standard methods (ISO 6496; 6492; 6865; 5984; 6490-2; 6491), while crude protein was determined by measuring total nitrogen using total combustion according to Dumas (EN ISO 16634-1) and applying Elementar Rapid N Analyzer.

RESULTS AND DISCUSSION

Data on fatty acids content and pH values of the examined silage samples are presented in Table 1. There is limited literature about the chemical composition of combined silages made from pumpkin's pulp and from carrots.

Table 1. The parameters of fermentation changes in vitaminized silages

Fatty acids	Type of vitaminized silage		
	whole corn plant and carrot	oat grits and carrot	corn grits and pumpkin
pH value	3.85-4.08	3.97-4.28	4.04-4.07
Content of total acids (% of fresh matter)			
Acetic acid	0.27-0.63	0.19-0.24	0.21-0.31
Butyric acid	0-0.02	N.D.	N.D.
Lactic acid	1.37-1.77	0.54-0.55	0.56-0.98

N.D. – not detected

pH value and dry matter content are the most important factors affecting the intensity of proteolysis. By producing the drop in pH, lactic acid preserves the whole corn plant. Naturally fermented silages are characterized by low pH value, mostly ranging between 3.7 and 4.2, as well as high concentration of lactic acid [8]. In the investigated silage samples, we established pH values ranging from 3.85 to 4.28 (Table 1), which is considered optimal value for well-fermented silages.
The content and ratio of lactic, acetic and butyric acids in the examined silage samples strongly indicate a successful preservation process. Barnett (1954), classified the fermentation process of silage and reported that, at the first stage, carbon dioxides discharge by respiration of the plant ensiled in the silage; at the second stage, coli-type bacteria and other fungi produce acetic acid; at the third stage lactic acid bacteria produce lactic acid and the lactic fermentation begins (3 days); at the fourth stage lactic acid content reaches a peak and pH maintains at 4.2 or less (17-21 days). When water-soluble carbohydrates are insufficient and pH does not fall sufficiently, the butyric acid bacteria produce butyric acid, which is the fifth stage. Butyric acid was detected in traces only in the silage containing whole corn plant and carrot (Table 1).
Data on average chemical composition of the examined silage samples are displayed in Table 2. According to the research of Horrocks and Vallentine (1999), the desirable content of dry matter (DM) of corn plant for ensiling is 35%. This percentage provides an optimal proportion of starch, which is the „carrier" of energy value and soluble sugars essential in production of adequate amounts of lactic acid. Moisture content in the examined samples ranged from 45.33% to 76.92%. Reduced fermentation in carrot/oat grit silage is most

probably due to the high content of dry matter, which inhibits the microbial activity (Živkov-Baloš et al., 2013).
Crude protein (CP) content was 2.29-2.84% on fresh matter basis (average 9.32% in DM) in silage of whole corn plant and carrot, 7.13-7.52% (average 13.45% in DM) in silage of carrot and oat grits and 4.19-4.75% (average 8.11% in DM) in silage of corn grits and pumpkin (Table 1). Our results of silage CP were in between the results of other authors. Demirel et al. (2011) noted that silages of 12 maize hybrids have CP content from 4.07-6.91% in DM . Kamalak et al. (2002) findings revealed the content of CP from 8.5 to 9.6% for control and transgenic maize varieties. The effect of stage of maturity on the ensiling properties of whole crop maize was studied under laboratory conditions. The CP content decreased from 80 to 58 g/kg DM (Filya, 2004). Vranic et al. (2008) reported that the CP content in samples of corn silage for dairy cows nutrition, was 61.6 g CP / kg DM. Similar findings (68.72 g/kg DM) were reported by Đorđević et al. (2009) Carrots have 85-90% moisture content and about 10% crude protein in dry matter (Rust and Buskirk, 2008). Content of proteins in pumpkins (carving and pie pumpkins) is 14.3-14.4% in DM (Jenkins, 2010).

Table 2. Chemical composition of vitaminized silages (on dry matter basis, %)

Nutritional value	Type of vitaminized silage		
	Whole corn plant and carrot	Oat grits and carrot	Corn grits and pumpkin
Dry matter	27.58 ± 4.50	53.02 ± 5.59	55.13 ± 1.79
Organic matter	95.29 ± 4.44	96.72 ± 5.12	89.70 ± 3.60
Crude Protein	9.32 ± 0.36	13.45 ± 0.59	8.11 ± 0.28
Crude fat	1.20 ± 0.23	0.79 ± 0.30	0.54 ± 0.02
Crude fiber	23.86 ± 1.73	10.49 ± 1.02	8.98 ± 0.13
Crude ash	4.71 ± 0.06	3.28 ± 0.65	10.30 ± 1.82
Calcium	0.43 ± 0.02	0.09 ± 0.02	0.14 ± 0.01
Phosphorus	0.22 ± 0.01	0.21 ± 0.01	0.16 ± 0.01

Results are presented as means±SD

Crude fiber (CF) content in silages changed from 4.82 to 8.45% on fesh matter basis (8.98-23.86% in DM). The highest content of CF has been established in vitaminized silage of whole corn plant and carrot, and the lowest in vitaminized silage of corn grits and pumpkin. Our results are in compliance with the results of other autors. Demirel et al. (2011) estabilised that CF content in maize silages is between 17.96 and 27.28% DM. Đorđević et al. (2009) reported that the content of CF in DM of whole plant maize silage is 17.65%, and Galila et al. (2012) reported that the content of CF in corn stalks, after the process of fermentation (that lasted 28 day) was 24.54-27.20%. Carrot root contains 8.1-12.1% crude fibre in DM (Nonaka et al., 1994). Fresh pumpkin *(Cucurbita sp.*) contains on average 13.2% CF in DM (Enishi et al., 2004).
Crude fat/Ether extract (EE) ranged between 0.54% and 1.20% DM. Our silage results were lower than the results of other authors. The maize silages EE content changed from 2.68% to 4.40% in DM (Demirel et al., 2011). Djordjević et al. (2009) reported that EE content in dry matter of maize silages is 8.76%. EE content in carrot is on average 1.0% (0.2-1.9%) in DM (Nonaka et al., 1994). Fresh pumpkin contains about 2.8% of crude fat in DM (Enishi et al., 2004).
Crude ash (CA) content in silages changed from 3.28 to 10.30 % in DM. Highest ash content was measured in the silage of whole corn plant and pumpkin as a consequence of the presence of higher content of corn stalks. CA in whole plant maize silages changed from 4.64 to 7.94% in DM (Demirel et al., 2011). Djordjević et al. (2009) reported that CA content in dry matter of maize silages is 5.04%. Filya (2004) reported that CA content in maize silages in different stages of fermentation ranged from 4.02-13.57% in DM. CA content in

carrot is on average 7.4% (3.1-10.4%) in DM (Nonaka et al., 1994). Enishi et al. (2004) reported that fresh pumpkin contains about 7.9% CA in DM.
Calcium (Ca) and phosphorus (P) content in investigated silages is displayed in Table 2. The chemical analysis cleared that these essential macro elements for animal nutrition were found in abundant amounts being, on DM bases in the range of 900-4300 mg/kg and 1600-2200 mg/kg, respectively. The highest content of Ca was measured in vitaminized silage of whole corn plant and carrot. Whole corn plant content is 800 mg Ca/kg DM (Galila et al., 2012). Calcium and phosphorus content in fresh carrot roots are on average 3800 mg/kg and 2900 mg/kg DM, respectively (Nonaka et al., 1994). Idi et al. (2005) reported that Ca and P content in dry matter of carrot is 0.36% and 0.26%, respectively. The pumpkin Ca and P content is on average, 3900 mg/kg and 2600 mg/kg in DM, respectively (Enishi et al., 2004).

CONCLUSIONS

With reference to the nutritional composition, investigated vitaminized silages represent a potential resource that might be used in bovine diets. However, it is necessary to consider that the composition of these silages only gives an idea of its potential use for bovine feeding, but that their real nutritive value could be established after evaluation in trials with animals. The acceptability, voluntary intake and percentage of inclusion in diets are factors that must be carefully considered.

ACKNOWLEDGEMENTS

This work is supported by a grant from the Ministry of Education, Science and Technological Development, Republic of Serbia, Project number TR 31071.

REFERENCES

Balzer, I. (1961). Analitičke metode određivanja kvalitete silaže. Krmiva, *2*, 41-44.
Barnett, A.J.G (1954). Silage Fermentation, Butterworths, London.
Demirel, R., Akdemir, F., Saruhan, V., Sentürk Demirel, D., Akinci, C., Aydan, F. (2011). The determination of qualities in different whole-plant silages among hybrid maize cultivars. African Journal of Agricultural Research, 624, 5469-5474.
Djordjević, N., Grubić, G., Lević, J., Sredanović, S., Stojanović, B., Božičković, A. (2009). The quality of silages from lucerne, whole maize plant and maize cobs prepared with various additives.Proceedings of XIII International Symposium Feed Technology Symposium FEDD-TO-FOOD/1st Workshop FP/REGPOT-3, Novi Sad, Serbia, 146-152.
EC: Regulation No 1804/1999 from European Commission, supplementing regulation No 2092/91, 1999.
Enishi, O., Yoshioka, T., Nakashima, K.., Saeki, M., Kawashima, T. (2004). Analysis of *in situ* ruminal digestive characteristics and nutritive value of pumpkin and carrot juice residue for ruminant feeds. Grassland Science, 50 (4), 360-365.
European Standard (EN ISO) (2010). Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content - Part 1: Oilseeds and animal feeding stuffs. EN ISO 16634-1:2010, European committee for standardization, Management Centre: rue de Stassart, 36 B-1050 Brussels.
Filya, I. (2004).Nutritive value and aerobic stability of whole crop maize silage harvested at four stages of maturity. Animal Feed Science and Technology 116, 141–150.
Galila, A.M.A.Darwish, Bakr, A.A., Abdallah, M.M.F. (2012). Nutritional value upgrading of maize stalk by using *Pleurotus ostreatus* and *Saccharomyces cerevisiae* in solid state fermentation. Annals of Agricultural Science, 57 (1), 47-51.
Idi, A., Permin, A., ChristensenJ.P., Steenfeldt, S., Engberg, R.M., Fink, M. (2005). Effect of carrots and maize silage on colinization of hens by *Ascaridia galli* and *Salmonella enterica* servoar Enteritidis. Helminthologia, 42 (3), 121-131.
Horrocks, R.D., Vallentine, J.F. (1999). Harvested Forages. Academic Press, San Diego.
Hurley, W.L., Doane, R.M. (1989).. Recent developments in the roles of vitamins and minerals in reproduction. J.Dairy Sci. 72, 784–804.
International Standard Organization (ISO), (1978). Animal Feeding stuffs – Determination of crude ash content. ISO 5984, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (1999). Animal Feeding stuffs – Determination of moisture and other volatile matter content. ISO 6496, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (1999). Animal Feeding stuffs – Determination of fat content. ISO 6492, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (2001). Animal Feeding stuffs – Determination of calcium content. ISO 6490-2, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (2002). Animal Feeding stuffs – Determination of phosphorus content. ISO 6491, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (2008). Animal Feeding stuffs – Determination of crude fiber content. ISO 6865, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (2008). Animal Feeding stuffs – Determination of calcium content. ISO 6490-2, International Organization for Standardization, Geneva, Switzerland.

International Standard Organization (ISO), (2010). Animal Feeding stuffs – Determination of calcium content. ISO 6490-2, International Organization for Standardization, Geneva, Switzerland.

Jenkins, H. K. (2010). Feed Value of Alternative Crops for Beef Cattle. NebGuide, Index: Beef Feeding and Nutrition, G2036, University of Nebraska-Lincoln Extension, Institute of agriculture and Natural Resources.

Kamalak A., Gürbüz, Y., Finiaayson, H.J. (2002). Comparasion of in vitro DM degradation of four maize silages using the Menke Gas Production method. Turkish Journal of Veterinary amd. Animal Sciences, 26, 1003-1008.

Kawashima, C., Nagashima, S., Sawada, K., Schweigert, F.J., Miyamoto, A., Kida, K (2010). Effect of β-carotene supply during close-up dry period on the onset of first postpartum luteal activity in dairy cows. Reproduction in Domestic Animals, 45:282-287.

Mogensen, L., Kristensen, T., Søegaard, K., Jensen, S.K., Sehested, J (2012). Alpha-tocopherol and beta-carotene in roughages and milk in organic dairy herds. Livestock Science, 145: 44-54.

Morel d'Arleux, F. (1990). Les sous-produits en alimentation animale. Réseau national Expérimentation Démonstration (RNED), ITEB, 1990.

Nonaka, K., Nakui, T., Shinoda, M (1994). Conservative and nutritive values of carrot silage. Research Bulletin of the Hokkaido Agricultural Experiment Station, 159, 73-85.

Nozière P., Graulet, B., Lucas, A., Martin B., Grolier, P., Doreau , M. (2006). Carotenoids for ruminants: From forages to dairy products. Animal Feed Science and Technology 131, 418–450.

Pravilnik o metodama uzimanja uzoraka i metodama fizičkih, hemijskih i mikrobioloških analiza stočne hrane (1987). Određivanje pH vrednosti, Službeni list SFRJ, metod 15.

Rust, S., Buskrik, D. (2008). Feeding Carrots or Sugar Beets to Cattle. In Cattle Call, Michigan State University Extension Newsletter, 13 (4), http://beef.msu.edu/Link-Click.aspx?fileticket=KjL6uucd9Mg%3d&tabid=537.

Schweigert, F.J., Rambech, W.A., Zucker, H. (1987). Transport of β-carotene by the serum lipoproteins in cattle.Journal of Animimal Physiology and Animimal Nutrition, 57, 162–167.

Vranić, M., Knežević, M., Matić, I., Turčin, D. (2008). Utjecaj dodatka kukuruzne silaže travnoj silaži različitih rokova košnje na *ad libitum* konzumaciju obroka. Mljekarstvo, 58 (1), 69-84.

Yang, A., Tume, R.K. (1993).. A comparison of beta-carotene-splitting activity isolated from intestinal mucosa of pasture-grazed sheep, goats and cattle. Biochememistry and Molecular Biology International, 30, 209–217.

Živkov-Baloš M., Jovičin M., Apić J., Mihaljev Ž., Prica N., Obradović S. (2013): Potentials and benefits of vitaminized silages in the diet of dairy cows. Proceedings of 23rd International Symposium "New Technologies in Contemporary Animal Production", Novi Sad, 85-87.

PRESENCE OF AFLATOXINS, ZEARALENONE, OCHRATOXIN A AND TRICHOTHECENES IN CORN (ZEA MAYS) IN REPUBLIC OF SERBIA

Dragana Ljubojević[1]*, Sandra Jakšić[1], Milica Živkov-Baloš[1], Željko Mihaljev[1], Nikola Puvača[2], Nadežda Prica[1], Miloš Kapetanov[1]

[1]Scientific Veterinary Institute "Novi Sad", Rumenački put 20, 21000 Novi Sad, Serbia
[2]Department of Animal Science, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: ljubojevic.ljubojevicd.dragana@gmail.com; dragana@niv.ns.ac.rs

ABSTRACT

Mycotoxins are toxic metabolites produced by a range of fungal species which common occurrence in food and feed and present a threat to the humans and animals health. The purpose of the current study was to examine the content of total aflatoxins (AFs), zeralenone (ZEN), ochratoxin A (OTA), deoxynivalenol (DON) and T-2 toxin in corn intended for animal nutrition, which was sampled during the year 2013. Content of mycotoxins was analyzed by Enzyme-Linked ImmunoSorbent Assay (ELISA). Among 70 analyzed corn samples, AFs were detected in 59 (84.3%) samples, in the concentration range from 2.17 to 88.85 µg/kg with the mean level of 18.15 µg/kg and 4 samples (5.7%) had AFs concentration greater than 90 µg/kg. AFs content above 50 µg/kg was found in 15.7% samples making them inappropriate for animal consumption by Serbian regulations. Content of ZEN, OTA, and DON was examined in 28 samples and content of T-2 in 29 samples. DON, ZEN, T-2 and OTA were detected in 7 (25.0%), 10 (35.7%), 11 (37.9%) and 11 (39.3%) samples, respectively. OTA, ZEN, T-2 and DON content ranging from 5.03 to 12.0 µg/kg, from 25.8 to 130 µg/kg, from 54.7 to 374 µg/kg and from 82.0 to 792 µg/kg, respectively. Republic of Serbia is one of the biggest exporters of corn in Europe, and there are remarkably economic consequences of mycotoxins contaminated corn. Moreover, continuous monitoring is necessary for prevention of occurrence of mycotoxins in order to protect humans and animal health.
***Keywords**: aflatoxins, zearalenone, ochratoxin A, trichothecenes, corn*

INTRODUCTION

Corn is a greatly required commodity and the most widely available feed energy source in Republic of Serbia. Some limitations on the use of corn in livestock nutrition are due to its contamination with mycotoxins. They are secondary metabolites of molds, with diverse chemical structure and biological effects, and harmful for the health of animals (Janković et al., 2006; Jakšić et al., 2012). Direct consequences on animals due to consumption of mycotoxin-contaminated feed include reduced feed intake, decreased feed conversion ratio, impaired body weight gain as well as increased incidence of disease due to immunosuppression (Pestka, 2007; Rashedi et al., 2012). Furthermore, the mentioned consequences lead to economic losses (Wu, 2004).

Aflatoxins (AFs) are extremely toxic, carcinogenic and mutagenic mycotoxins produced by the fungal *Aspergillus flavus* and *Aspergillus parasiticus* (Whitlow and Hagler, 2005), which accumulate mostly in liver, but also in muscle, gizzard, kidney, adipose tissue, as well as in meat and eggs (Hussain et al., 2010; Yang et al., 2012).The degree of accumulation of AFs in corn and other crops, changes seasonally and is highly dependent on weather (Kos et al., 2013).

Zearalenone (ZEN) is an estrogenic mycotoxin produced by the fungal *Fusarium graminearum* and *Fusarium culmorum*, which is often present in corn and other cereals and causes hyperestrogenism in livestock, especially in the pigs (Rashedi et al., 2012).

Ochratoxin A (OTA) is also extremely toxic mycotoxin and it is produced primarily during storage by the fungus of the genera *Aspergillus*, primarily *A. ochraceus*, especially in warmer regions and by *Penicillium verrucosum*, in temperate and colder regions (Duarte et al., 2010).

OTA represents a potential threat to animal production due to its nephrotoxic, immunotoxic, mutagenic, teratogenic and carcinogenic effects (Duarte et al., 2011).
Deoxynivalenol (DON) and T2-toxin are trichotecenes. DON causes body weight loss, decreased body weight gain and production, suppression of immune system, liver and kidney damage, and even death (Richard, 2007; Sabater-Vilar et al., 2007; Chen et al., 2008).
T-2 toxin is a cytotoxic and immunosuppressive toxin, which can cause acute intoxication or chronic diseases in livestock (Zhou et al. 2008). The symptoms of acute intoxication in animals are decreased production of milk or eggs, increased number of cracked eggs and oral lesions in poultry (Morgavi and Riley 2007).
Due to these adverse effects of mycotoxins on livestock production the purpose of the current study was to examine the content of AFs, ZEN, OTA, DON and T-2 in corn intended for animal nutrition, which was sampled during the year 2013.

MATERIAL AND METHODS

Total of 70 corn samples that were brought to the laboratory during the year 2013 were analyzed. The samples grown in 2012 were collected from a different feed manufacturer, which obtained the samples from different regions of Vojvodina province. Approximately, 1.000 g of each sample was homogenized by grinding in a laboratory mill and analyzed immediately for mycotoxins presence or content. Subsamples of 5.000 g were extracted with 25 ml of methanol/water mixture (70:30, v/v) for total AFs, ZEN, and T2 toxin; with 12.5 ml of methanol/water mixture (70:30, v/v) for OTA and with 100 ml of distilled water for DON analysis and shaken for 10 minutes on laboratory shaker. After that, extracts were filtered through a filter paper and obtained filtrates were collected and used for further analysis. The content of AFs, ZEN, OTA, DON and T2 in corn samples was determined by validated methods using ELISA tests (R-Biopharm, Germany): Ridascreen® AflaFast Art. No. R5202; Ridascreen® FAST ZEASC, Art. No. R5505; Ridascreen® FAST Ochratoxin A, Art. No. R5402; Ridascreen FAST®DON SC, Art. No. R.5905 and Ridascreen® FAST T2, Art. No. R5302, with detection limits: 1.7 µg/kg, 60 µg/kg, 5 µg/kg, 74 µg/kg, 33 µg/kg, respectively. All performed tests were similar. Namely, free mycotoxins in the samples and standards are allowed to compete with enzyme-labeled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate is added, which reacts with the bound conjugate to produce blue color. The intensity of the color is inversely proportional to the concentration of mycotoxins in the samples or standards. Intensity of the color in each well was measured at 450 nm in a microwell reader (Thermo scientific Multiscan Fc, Shangai, China). Analysis of results was performed according to the manufacturer's instructions by Rida®Soft Win program (R-Biopharm, Germany).

RESULTS AND DISCUSSION

The obtained results are presented in Tables 1 and 2. The corn samples were presented as grouped based on the different mycotoxins content compared to maximum permitted concentrations according to Serbian national regulation (4/2010), as well as according to the Regulation which was adopted in the meantime (27/2014).
Among 70 analyzed corn samples, AFs were detected in 59 (84.3%) samples, in the concentration range from 2.17 to 88.85 µg/kg with the mean level of 18.15 µg/kg and 4 samples (5.7%) had concentration greater than 90 µg/kg. AFs content above 50 µg/kg was found in 15.7% samples making them inappropriate for animal consumption by Serbian regulations. Content of ZEN, OTA, and DON was examined in 28 samples and content of T-2 in 29 samples (Table 1). ZEN, OTA, DON and T-2 were detected in 10 (35.7%), 11 (39.3%), 7 (25.0%) and 11 (37.9%) samples, respectively. ZEN, OTA, DON and T-2 content ranging from 25.84 to 130 µg/kg, from 5.03 to 11.99 µg/kg, from 82 to 792 µg/kg and from 54.7 to 374 µg/kg, respectively (Table 2).

Table 1. Number of analysed and positive samples

Mycotoxin	Number of analyzed samples	Number of positive samples*	%
AFs	*70*	*59*	*84.3*
ZEN	*28*	*10*	*35.7*
OTA	*28*	*11*	*39.3*
DON	*28*	*7*	*25.0*
T2	*29*	*11*	*37.9*

*AFs- Aflatoxins, ZEN – Zearalenone; OTA - Ochratoxin A; DON – Deoxynivalenol; T2- T2 toxin *The samples were the levels of mycotoxins were above the limit of detection of performed methods %- percentage of positive samples*

Information on massive contamination of corn with AFs was exposed to Serbian public recently (Kos et al., 2013). The contamination of corn with AFs, as one of the most receptive crop, occurs in a cyclic manner. Extreme weather conditions with high temperature and drought, with relative atmospheric humidity below 18% favored AFs contamination of corn in several regions in the USA (Wu et al., 2011). According to Serbian Regulation (4/2010) the maximum prescribed level of AFs in corn was 50 µg/kg. Regulation 27/2014 regulates the maximum allowed level of 30 µg/kg for Aflatoxin B1 (AFB1), instead of AFs. AFs content above 50 µg/kg was found in 15.7% samples making them inappropriate for animal consumption by Serbian regulation 4/2010. According to EU Commision Directive (2002/32/EC) maximum permitted level of AFB1 in corn intended for animal consumption is 20 µg/kg.

The concentrations of ZEN were between 25.84 and 130.0 µg/kg (Table 2). The ZEN limit in corn has not been regulated by as well as previous Serbian Regulation 4/2010 while new Serbian Regulation (27/2014) precribe maximum permitted level of 4000 µg/kg for ZEN in corn . The obteined ZEN concentrations in this study were lower than maximum permitted level provided by the Regulation 27/2014 as well as by the EU Commission Recommendation (2006/576/EC). The EU Commission Recommendation (2006/576/EC) for the cereals that are intended for animal feeding is 2000 µg/kg.

The concentrations of OTA in corn samples were between 5.03 and 11.99 µg/kg (Table 2). The maximum permitted level of OTA in corn has not been regulated by Regulation 4/2010; and Regulation 27/2014 prescribes maximum permitted level in corn is 250 µg/kg. The OTA levels in all analysed samples were lower than that provided by the Regulation 27/2014 as well as by the EU Commission Recommendation (2006/576/EC). The EU Commission Recommendation (2006/576/EC) for the cereals that are intended for animal feeding is 250 µg/kg, which is agreed with the our national Regulation (27/2014).

Table 2. - AFs, ZEN, OTA, DON and T2 levels (µg/kg) in corn

Mycotoxin	Mean	SEM	SD	Min	Max	4/2010	27/2014
AFs	18.15	2.750	21.12	2.170	88.85	50.00	* 30.00
ZEN	73.34	11.17	35.32	25.84	130.0		4000
OTA	8.050	0.750	2.500	5.030	11.99		250.0
DON	239.0	94.53	250.1	82.00	792.0		8000
T2	113.4	27.96	92.73	54.70	374.0		

Mean levels were calculated according to number of positive samples; AFs, ZEN; OTA A; DON; T2 SEM- standard error of the mean, SD- standard deviation, 4/2010- maximum permitted concentrations according to Serbian national regulations, 27/2014- maximum permitted concentrations according the new Regulation

The DON limits in corn for feed have not been regulated by the Regulation 4/2010 in Serbia. According to Regulation 27/2014, maximum prescribed level in corn is 8000 µg/kg. However,

DON levels in analysed corn samples were lower than that provided by the Regulation 27/2014, as well as maximum prescribed level (8000 µg/kg) by EU Commission Recommendation (2006/576/EC).
The T-2 toxin levels in analysed corn were between 54.7 and 374 µg/kg. Although the T-2 toxin level in corn has not been regulated by the Regulation 4/2010 and Regulation 27/2014, , some of these concentrations were higher than the toxic levels of T-2 toxin that have been reported as 100 µg/kg for animals by Mabbett (1999) but lower than 500 µg/kg as recommended by EU (2013/165/EU) products for feed and compound feed.

CONCLUSIONS

Republic of Serbia is one of the biggest exporters of corn and there are remarkably economic consequences of mycotoxins contaminated corn. A continuous monitoring of corn is necessary for quick and effective response in conditions of livestock production practice and for prevention of occurrence of mycotoxins in feed mixtures intended for animal nutrition. It is for now the only way of successful prevention of the harmful effects of mycotoxins.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Research Program, Ministry of Education, Science and Technological Development, Republic of Serbia (TR31071).

REFERENCES

Chen, F., Ma, Y., Xue, C., Ma, J., Xie, Q., Wang, G., Bi, Y., Cao Y. (2008). The combination of deoxynivalenol and zearalenone at permitted feed concentrations causes serious physiological effects in young pigs. Journal of Veterinary Science, 9, 39–44.

Commision Directive amending Annex I to Directive of the European Parliament and of the Council on undesirable substances in animal feed (2002/32/EC), Official Journal of the European Union, p 36, 2003.

Commission Recommendation: On the presence of deoxynivalenol, zeralenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). Official Journal of the European Union, , 229, 7-9, 2006.

Commission Recommendation: On the presence of T-2 and HT-2 toxin in cereals and cereal products (Text with EEA relevance) (2013/165/EU) Official Journal of the European Union, p 15, 2013.

Duarte, S.C., Pena, A., Lino, C.M. (2010). A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiology, 27, 187–198.

Duarte, S.C, Lino, C.M., Pena, A. (2011). Ochratoxin A in feed of food-producing animals: An undesirable mycotoxin with health and performance effects. Veterinary Microbiology, 154, 1–13.

Hussain, Z., Khan, M.Z., Khan, A. (2010). Residues of aflatoxin B1 in broiler meat: Effect of age and dietary aflatoxin B1 levels. Food and Chemical Toxicology, 48, 3304-3307.

Jakšić, S., Abramović, B., Jajić, I., Živkov-Baloš, M., Mihaljev, Ž., Despotović, V., ŠojiĆ, N., Daniela V. (2012). Co-occurrence of Fumonisins and Deoxynivalenol in Wheat and Maize Harvested in Serbia. Bulletin of Environmental Contamination and Toxicology, 89, 615-619.

Janković, V., Škrinjar, M., Vukojević, J., Karan, D., Radmili, M. (2006). Mikotoksini potencijalna opasnost za žive organizme. Tehnologija mesa, 47, 97-103.

Kos, J., Mastilović, J., Janić Hajnal, E., Sarić, B. (2013). Natural occurrence of aflatoxins in maize harvested in Serbia during 2009-2012. Food Control, 34, 31-34.

Mabbett, T. (1999). The mycotoxin menace. Feeding Times, 4, 4-5.

Morgavi, D.P., Riley, R.T. (2007). An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. Animal Feed Science and Technology, 137, 201–212.

Pestka, J.J. (2007). Deoxynivalenol: toxicity, mechanisms and animal health risks. Animal Feed Science and Technology, 137(3–4), 283–298.

Rashedi, M., Sohrabi, H. R., Ashjaazadeh, M. A., Azizi, H., Rahimi E. (2012). Zearalenone contamination in barley, corn, silage and wheat bran. Toxicology and Industrial Health, 28(9), 779–782.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses:
An overview. International Journal of Food Microbiology, 119, 3–10.

Sabater-Vilar, M., Malekinejad, H., Selman, M. H. J, van der Doelen M. A. M, Fink-Gremmels, J. (2007). In vitro assessment of absorbents aiming to prevent deoxynivalenol and zearalenone mycotoxins. Mycopathologia, 163,81–90.

Službeni glasnik Republike Srbije. Pravilnik o kvalitetu hrane za životinje, 4/2010, pp. 30–61.

Službeni glasnik republike Srbije. Pravilnik o izmeni Pravilnika o kvalitetu hrane za životinje, 27/2014, p 19.
Whitlow, L. W., Hagler, W. M. (2005). Mycotoxins in feeds. Feedstuffs, 77, 69–79.
Wu, F. (2004). Mycotoxins risk assessment for the purpose of setting International Regulatory Standards. Environmental Science and Technology, 38(15), 4049–4055.
Wu, F.D., Bhatnagar, T. Bui-Klimke, I. (2011). "Climate change impacts on mycotoxin risks in US maize." World Mycotoxin Journal, 4, 79-93.
Yang, J., Bai, F., Zhang, K. (2012). Effects of feeding corn naturally contaminated with AFB1 and AFB2 on performance and aflatoxin residues in broilers. Czech Journal of Animal Science, 57, 506–515.
Zhou, T., He, J., Gong, J. (2008). Microbial transformation of trichothecene mycotoxins. World Mycotoxin Journal, 1, 23–30.

SUITABILITY OF MAIZE HYBRIDS BIOMASS FOR ANIMAL FEED PRODUCTION

Valentina Semenčenko*, Dušanka Terzić, Milica Radosavljević, Marija Milašinović-Šeremešić, Zorica Pajić, Goran Todorović, Milomir Filipović

Maize Research Institute, Zemun Polje, Slobodana Bajića 1, 11185 Belgrade - Zemun, Serbia

*Corresponding author:
E-mail address: vsemencenko@yahoo.com

ABSTRACT

The objective of the present study was to observe and compare properties of nine maize hybrids biomass in order to determine their suitability for animal feed production. Yields, structure of the dry matter of the investigated maize hybrids, as well as the yield of the digestible dry matter were determined in maize hybrids harvested in the full waxy maturity stage. The content of lignocellulosic fibres, their respective ratios and the *in vitro* dry matter digestibility of the whole maize plant as well as the neutral detergent fibre digestibility (NDFD) were established.
The results indicated significant differences in dry matter *in vitro* digestibility of the whole plant among different hybrids in the most optimal harvest stage. The whole plant dry matter digestibility of the investigated maize hybrids ranged from 59.67 to 65.53%. Very significant positive correlation was determined between NDFD and *in vitro* dry matter digestibility of the whole maize plant (r=0.66).
Biomass of the selected maize hybrids was rated as feed of high quality and can, therefore, be used for animal feed production in different ratios depending on animal species, category and dietary needs.
***Keywords**: maize hybrids, biomass, pepsin-cellulase in vitro dry matter digestibility, animal feed*

INTRODUCTION

Maize (*Zea mays* L.) is a cereal crop grown worldwide in a range of agroecological environments. More maize is produced annually than any other grain. More than 90% of all produced maize is used predominantly for animal feed, food and alcohol production (Semenčenko, 2013). Due to quality of biomass, suitability for silage and diversified utilisation as feed, maize ranks first among forage plants (Terzić *et al*., 2010). Maize is regarded as one of the most important fodder crops in ruminant nutrition due to high level of dry matter content as well as a significant proportion of grain. This grain is a primary energy supplement in dairy diets and can contribute up to 30, 60, and 98% of the diet's protein, net energy and starch, respectively (Mlyneková and Čerešňáková, 2013).
The prospects are that in the near future maize will continue to expand and diversify as a crop for food, feed and biofuel, as an industrial resource as well as a research model (Bennetzen and Hake, 2009; Semenčenko *et al.,* 2013). In Serbia, maize is a traditionally cultivated as a number one field crop (Radosavljević *et. al*, 2010).
The forage part of the maize plant can be utilised in different ways. Some is harvested as whole plant maize silage and can be used as both an energy source and a roughage source in feedlot diets. The second use of maize forage is residue harvest after grain harvest and fed as a roughage source in finishing diets or mixed with wet by-products and fed as an energy source for cattle or beef cows (Klopfenstein *et al*., 2013).
Digestibility is an important factor of the nutritive value of feed. It determines the relation between contents of nutrients and energy that are available to ruminants. Estimates of potential degradability and rates of degradation in the rumen are prerequisites in feed/ration evaluation systems that model rumen dynamics. The most accurate way of obtaining information on digestibility of feeds for ruminants is by conducting *in vivo* digestibility experiments (Pojić *et al.,* 2008). Currently, *in situ* and *in vivo* methods are the main reference methods used in European systems (Chrenková *et al.,* 2014; Hvelplund *et al.,* 2009). However, these methods are expensive, time-consuming not animal-friendly and not suited for routine analysis, there has been a constant search for laboratory methods for routine

prediction of the *in vivo* digestibility of ruminant feeds, in order to implement an adequate system of quality control in the feed industry (Pojić *et al.,* 2008).
The use of incubation of feeds with exogenous enzymes to predict the *in vivo* dry matter digestibility has the aim to mimic the digestive process in the animal. Most enzymatic methods for the digestibility prediction have been developed for forage feedstuffs, with a few used for other feedstuffs, e.g. grains and compound feeds (Aufrére, 2006).
Digestibility of neutral detergent fibre (NDFD) is an important parameter of forage quality because it varies widely in its degradability in rumen and influences animal performance. Evaluation of forages for NDF digestibility is being conducted to aid prediction of total forage digestibility. Research has demonstrated that lactating dairy cows will eat more dry matter (DM) and produce more milk when fed forages that have higher NDF digestibility (Hoffman et al., 2003). Digestibility of NDF *in vitro* or *in situ* is considered a better indicator of dry matter intake of dairy cows than NDFD *in vivo* because forages with high *in vitro* or *in situ* NDFD have shorter rumen retention times, allowing greater dry matter intake at the expense of NDFD *in vivo.* Although many experiments have reported NDFD data, interpretation of results is difficult because of a variety of confounding factors. Experiments comparing forages that differ only in NDFD are rare, and it is difficult to confirm that other factors that could potentially affect animal performance did not vary (Oba and Allen, 1999).
The main goal of this study was to investigate some of the important properties of maize hybrids biomass such as: lignocellulosic fibre contents and their ratios, *in vitro* dry matter digestibility and neutral detergent fibre digestibility (NDFD) in order to evaluate potentials of their use as animal feed.

MATERIAL AND METHODS

Nine hybrids with different genetic background and maturity groups (ZP 341, ZP 377, ZP 434, ZP 444, ZP 505, ZP 560, ZP 600, ZP 606, ZP 677) were tested in the field experiment. The two-replicate trail was set up according to the randomized complete-block design in the experimental plot of the Maize Research Institute, Zemun Polje.
The following traits were determined: lignocellulosic fibres such as NDF-neutral detergent fibres, ADF-acid detergent fibres, ADL-acid detergent lignin, hemicelluloses and cellulose, reducing sugars, sucrose and dry matter digestibility of kernel and the whole maize plants. Plants of each replicate were harvested at the full waxy maturity stage from the area of $7m^2$ (two inner rows). Samples of the whole maize plants were chopped and dried at 60^oC for 48h and then ground in the mill with 1-mm sieves. The modified Van Soest detergent method was applied to determine lignocellulose fibres (NDF - neutral detergent fibres, ADF - acid detergent fibres, ADL - acid detergent lignin) (Mertens, 1992). *In vitro* digestibility of the whole maize plant samples was performed by the Aufréré method (2006) based on enzymatic solubility (ES). The NDFD (NDF digestibility) was calculated by the equation reported by Brenner *et al*., 2010: NDFD=100(ES-(100-NDF))/NDF).
Data reported for tested parameters of the ZP hybrids were assessed by the analysis of variance (ANOVA) and the LSD multiple test was used for any significant differences at the $P<0.05$ level between the means. All the analyses were conducted using the statistical software package STATISTICA 8.1. (StatSoft Inc. USA).

RESULTS AND DISCUSSION

Yield structure of the investigated ZP maize silage hybrids is presented in table 1. Dry matter content of the whole plant determined at harvest ranged from 36.37 to 43.00%. Recommended values for dry matter content of maize plants used for silage preparation range between 30 and 35%. Ensiling plants with dry matter content lower than 28% produces silages that are too acidic and have lower nutritive value (Pejić, 1994). Dry matter yield of the whole plant ranged from 11.6 (ZP 434) to 14.5 $t \cdot ha^{-1}$ (ZP 444); stover from 5.9 (ZP 434) to

7.6 t·ha^{-1} (ZP 444 and ZP 560); ear from 5.7 (ZP 434) to 7.0 t·ha^{-1}. Whole plant digestible dry matter yield varied between 7.5 (ZP 677) and 9.3 t·ha^{-1} (ZP 444).

Table 1. Yield structure of the investigated maize hybrids

Hybrid	Whole plant dry matter content, %	Dry matter yield, t·ha^{-1}				Fraction of the overall dry matter yield, %	
		Whole plant	Stover	Ear	Whole plant digestible dry matter yield	Stover	Ear
ZP 341	37.97	12.7	6.3	6.4	7.7	49.61	50.39
ZP 377	36.58	12.7	6.6	6.1	7.7	52.16	48.03
ZP 434	36.37	11.6	5.9	5.7	7.6	50.86	49.14
ZP 444	41.37	14.5	7.6	6.9	9.3	52.41	47.59
ZP 505	43.00	14.3	7.3	7.0	8.6	51.05	48.95
ZP 560	42.83	14.4	7.6	6.8	9.2	52.78	47.22
ZP 600	39.97	13.5	7.5	6.0	8.5	55.56	44.44
ZP 606	38.09	13.3	7.5	5.8	8.1	56.39	43.61
ZP 677	39.03	12.5	6.6	5.9	7.5	52.80	47.20

Data on the content of NDF, ADF, ADL, hemicellulose and cellulose are presented in table 2.

Table 2. Content of lignocellulose fibres of the whole maize hybrid plants

Hybrid	NDF, %	ADF, %	ADL, %	Hemicellulose, %	Cellulose, %
ZP 341	50.29^{de}	24.52^{c}	1.95^{c}	25.77^{d}	22.57
ZP 377	54.61^{b}	26.70^{b}	2.45^{a}	27.91^{a}	24.25
ZP 434	49.26^{f}	22.75^{e}	1.61^{f}	26.51^{bcd}	21.14
ZP 444	51.53^{c}	24.56^{c}	1.91^{cd}	26.97^{bc}	22.65
ZP 505	49.76^{ef}	23.52^{d}	1.92^{cd}	26.24^{cd}	21.60
ZP 560	50.61^{d}	23.53^{d}	1.68^{e}	27.08^{b}	21.79
ZP 600	50.81^{cd}	24.36^{c}	1.86d^{e}	26.45^{bcd}	22.53
ZP 606	56.76^{d}	28.43^{a}	2.22^{b}	28.33^{a}	26.21
ZP 677	55.12^{b}	27.15^{b}	1.88^{cd}	27.98^{a}	25.27
$LSD_{0.05}$	0.73	0.59	0.10	0.75	/

Means in the same column with different superscripts differ ($p<0.05$)

As shown in table 2 contents of lignocellulosic fibres of the whole maize plant: NDF, ADF, ADL, hemicelluloses and cellulose ranged between: 49.26 and 56.76; 22.75 to 28.43; 1.61 to 2.45; 25.77 to 28.33; and 21.14 to 26.21%, respectively. The differences in lignocellulosic fibres contents were statistically significant. These differences influenced variations in the obtained *in vitro* dry matter digestibility coefficients. The lowest ADF, NDF and ADL content in the whole plant biomass was observed in ZP 434, a dent x flint hybrid. These results are in accordance with those reported by Mlyneková and Čerešňáková (2013a), who found the larger amount of ADF, NDF and lignin in dent than in dent x flint hybrids' morphological parts as well as of the whole plants.

Obtained results on the NDF digestibility and *in vitro* dry matter digestibility of the whole maize plant are presented in table 3.

Neutral detergent fibre digestibility (NDFD) varied between 19.37 (ZP 505) and 31.86% (ZP 560), and *in vitro* dry matter digestibility of the whole maize plant ranged from 59.67 (ZP 677) to 65.53% (ZP 434). Research has demonstrated that lactating dairy cows will eat more dry matter (DM) and produce more milk when fed forages that have higher NDF digestibility (Hoffman et al., 2003; Oba and Allen, 1999).

Differences in digestible NDF content influenced the overall dry matter digestibility of the whole plant.

Table 3. Neutral detergent fibre digestibility and whole maize plant in vitro dry matter digestibility

Hybrid	NDFD, %	Dry matter digestibility, %
ZP 341	21.30^{d}	60.43^{dc}
ZP 377	27.66^{bc}	60.50^{dc}
ZP 434	30.01^{ab}	65.53^{a}
ZP 444	29.61^{ab}	64.00^{b}
ZP 505	19.37^{d}	59.89^{de}
ZP 560	31.86^{a}	65.52^{a}
ZP 600	26.98^{c}	62.90^{c}
ZP 606	30.73^{a}	60.71^{d}
ZP 677	26.84^{c}	59.67^{e}
$LSD_{0.05}$	2.84	1.02

Means in the same column with different superscripts differ (p<0.05)

Values of the ratios calculated between different lignocelluloses fibres contents are presented in table 4.

Table 4. Lignocellulosic fibre content ratios

Hybrid	ADL/NDF	ADL/ADF	ADF/NDF	Hemicellulose/NDF	Cellulose/NDF	Cellulose/Hemicellulose	Hemicellulose/cellulose
ZP 341	$3,88^{b}$	$7,95^{bc}$	$48,75^{bc}$	$51,25^{cde}$	$44,88^{b}$	$87,57^{b}$	$114,21^{ef}$
ZP 377	$4,48^{a}$	$9,16^{a}$	$48,90^{bc}$	$51,13^{de}$	$44,41^{bc}$	$85,67^{bc}$	$116,76^{de}$
ZP 434	$3,27^{e}$	$7,11^{d}$	$46,01^{f}$	$53,82^{a}$	$42,92^{e}$	$79,76^{f}$	$125,39^{a}$
ZP 444	$3,71^{cd}$	$7,78^{bcd}$	$47,65^{d}$	$52,35^{bc}$	$43,95^{cd}$	$83,95^{cd}$	$119,12^{cd}$
ZP 505	$3,85^{bc}$	$8,14^{b}$	$47,27^{de}$	$52,74^{ab}$	$43,42^{de}$	$82,33^{dc}$	$121,48^{bc}$
ZP 560	$3,32^{e}$	$7,14^{d}$	$46,48^{ef}$	$53,52^{a}$	$43,07^{e}$	$80,48^{ef}$	$124,27^{ab}$
ZP 600	$3,59^{d}$	$7,49^{bcd}$	$47,94^{cd}$	$52,06^{bcd}$	$44,35^{bc}$	$85,20^{bc}$	$117,38^{de}$
ZP 606	$3,91^{b}$	$7,79^{bcd}$	$50,11^{a}$	$49,94^{f}$	$46,21^{a}$	$92,52^{a}$	$108,09^{g}$
ZP 677	$3,40^{e}$	$6,91^{cd}$	$49,25^{ab}$	$50,75^{ef}$	$45,84^{a}$	$90,32^{a}$	$110,74^{fg}$
$LSD_{0.05}$	0.16	0.73	1.06	1.10	0.87	2.54	3.48

Means in the same column with different superscripts differ (p<0.05)

Calculated ratios between different fibre constituents of the whole maize plant were statistically significant. These differences between respective ratios also influenced the overall dry matter digestibility of the whole plant.

Results of the statistically obtained correlation coefficients between the investigated properties of nine selected ZP maize hybrids biomass are presented in Table 5.

Significant positive correlation was determined between digestible NDF and hemicellulose content (r=0.55). Very significant positive correlation was determined between NDFD and *in vitro* dry matter digestibility of the whole maize plant (r=0.66). Very significant negative correlations were determined between *in vitro* dry matter digestibility of the whole maize plant and ADF content (r=-0.61); ADF/NDF ratio (r=-0.73); hemicellulose/NDF ratio (r=-0.65) and cellulose/hemicelluloses ratio (r=-0.68). Significant negative correlations between *in vitro* dry matter digestibility of the whole maize plant and NDF content (r=-0.50); ADL content (r=-0.58); cellulose content (r=-0.58), ADL/NDF ratio (r=-0.58) and ADL/NDF ratio (r=-0.51) respectively, were noticed.

Table 5. Correlation coefficients between the investigated properties of silage maize hybrids biomass

	NDFD	Dry matter digestibility
Ear	-0.10	0.13
Plant without ear	0.24	0.09
NDF	0.32	-0.50*
ADF	0.19	-0.61**
ADL	-0.01	-0.58*
Hemicellulose	0.55*	-0.22
Cellulose	0.22	-0.58*
ADL/NDF	-0.30	-0.58*
ADL/ADF	-0.30	-0.51*
ADF/NDF	-0.10	-0.73**
Cellulose/NDF	0.09	0.73**
Hemicellulose/NDF	-0.01	-0.65**
Cellulose/hemicellulose	-0.04	-0.68**
Hemicellulose/cellulose	0.06	0.69**
Dry matter digestibility	0.66**	/

These results are in agreement with those obtained by Čerešňáková et al. (1996) who determined a negative correlation between digestibility of organic matter (dry matter – ash content) and NDF, ADF and hemicelluloses, as well as a significant negative correlation between digestible organic matter and NDF (%) in organic matter. Very significant positive correlations between *in vitro* dry matter digestibility of the whole maize plant and cellulose/NDF (r=0.73) as well as hemicelluloses/cellulose ratio (r=0.69) were observed.

CONCLUSIONS

The results obtained in this study indicated significant differences in dry matter *in vitro* digestibility of the whole plant among different hybrids in the most optimal harvest stage. The whole plant dry matter digestibility ranged from 59.67 to 65.53%. Very significant positive correlations between *in vitro* dry matter digestibility of the whole maize plant and cellulose/NDF (r=0.73) as well as hemicelluloses/cellulose ratio (r=0.69) were observed. Very significant negative correlations were determined between *in vitro* dry matter digestibility of the whole maize plant and ADF content (r=-0.61); ADF/NDF ratio (r=-0.73); hemicellulose/NDF ratio (r=-0.65) and cellulose/hemicelluloses ratio (r=-0.68). Biomass of the selected maize hybrids was rated as high quality feed and can, therefore, be used for animal feed production in different ratios depending on animal species, category and dietary needs.

ACKNOWLEDGEMENTS

Research presented in this paper is a result of the project TR 31068, funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

REFERENCES

Brener E.A., Zein I., Chen Y., Anderson J.R., Weynel G., Ouyunova M., Eder J., Darnhofer B., Frei U., Barriere Y., Lubberstedt T., (2010). Polymorphisms in O-methyltransferase genes are associated with stover cell wall digestibility in European maize (Zea mays L.) BMC Plant Biology, 10, 27. doi:10.1186/1471-2229-10-27.

Chrenková M., Čerešňáková, Z., Weisbjerg M.R., Formelová Z., Poláčiková M., Vondráková M. (2014). Characterization of proteins in feeds according to the CNCPS and comparison to *in situ* parameters. Czech Journal of Animal Science, 59 (6), 288-295.

Čerešňáková Z., Chrenková M., Flak P. (1996). Relation of content and composition of cell walls to the in vitro digestibility of dry matter, organic matter and crude protein in roughages. Journal of Farm Animal Science, 29, 153-158.

Hoffman P.C., Lundberg K.M., Bauman L.M., Shaver R.D. (2003). In vitro NDF Digestibility of Forages: The 30 vs. 48 hour debate. In: Focus on Forage - Vol 5: No. 16, pp. 1-3. University of Wisconsin Board of Regents, 2003 (http://fyi.uwex.edu/forage/files/2014/01/30vs48-FOF.pdf).

Hvelplund T., Larsen M., Lund P., Weisbjerg M.R. (2009). Fractional rate of degradation (kd) of starch in the rumen and its relation to in vivo rumen and total digestibility. South African Journal of Animal Science, 39 (Suppl. 1), 133-136.

Klopfenstein T.J., Erickson G.E., Berger E.E. (2013). Maize is a critically important source of food, feed, energy and forage in the USA. Field Crops Research, 153, 5-11.

Oba M., Allen M.S. (1999). Evaluation of the importance of the digestibility of neutral detergent fiber from forage: effects on dry matter intake and milk yield of dairy cows. Journal of Dairy Science, 82 (3), 589-596.

Pojić M., Palić D., Mastilović J., Janić-Hajnal E. (2008). The introduction of a method for determination of organic matter digestibility in feeds into routine laboratory practice. Food Processing, Quality and Safety, 35 (3), 151-155.

Radosavljević M., Bekrić V., Milašinović M., Pajić Z., Filipovič M., Todorović G. (2010). Genetic variability as background for the achievements and prospects of the maize utilization development. Genetika, 42 (1), 119-135.

Semenčenko V., Mojović L., Đukić-Vuković A., Radosavljević M., Terzić D., Milašinović Šeremešić M. (2013). Suitability of some selected maize hybrids from Serbia for the production of bioethanol and dried distillers' grains with solubles. Journal of the Science of Food and Agriculture, 93, 811–818.

Terzić D., Radosavljević M., Žilić S., Milašinović M., Semenčenko V. (2010). Quality parameters of ZP hybrids biomass. Biotechnology in Animal Husbandry, 26 (spec. issue), 491-497.

Bennetzen J.L., Hake S.C. (eds.) (2009). Handbook of Maize: Genetics and Genomics – Volume II – Genetics and Genomics, The future of maize, Springer Science + Business Media LLC, 2009, p. 771.

Pejić Đ. (1994). Silage maize technology of production and ensilage, Maize Research Institute, Belgrade, 1994, p. 68.

Semenčenko V. (2013). Investigation of various maize hybrids for bioethanol, starch and animal feed production, PhD Thesis. University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia.

Mlyneková Z., Čerešňáková, Z. (2013). Degradation of nutrients in dent and dent x flint maize hybrids in different stages of maturity, 15th International Conference – Forage Conservation, Nový Smokovec, Slovensko, 24.09-26.09. 2013, Conference Proceedings, Poprad Hasman, 2013, ISBN 978-80-89418-29-9, EAN 9788089418299 s. 81.

Mlyneková Z., Čerešňáková, Z. (2013a). Nutrient content of different morphological parts of maize hybrids dent and dent x flint and their rumen degradation. In: NutriNET 2013, Slovak University of Agriculture in Nitra, Department of Animal Nutrition, Conference Proceeding, 2013, ISBN 978-80-552-1065-0.

Aufréré Josephine (2006). Prevision de la digestibilite des fourages et aliments concentres et composes des herbivores par une methode enzimatique pepsine-celulase. AQ, 353, 1-6.

Mertens D.R. 1992. Critical conditions in determining detergent fiber, in Proceedings of the Forage Analysis Workshop, Sept. 16-17, 1992, Denver, Colorado, National Forage Testing Association, Omaha, NE, pp. C1–C8.

STATISTICA (Data Analysis Software System) (2009). v.8.1., Stat-Soft, Inc., USA (www.statsoft.com).

THE IMPACT OF BENURAL S ADDITION ON CHEMICAL COMPOSITION AND QUALITY OF ENSILED GRAPE POMACE

Vesna Maraš[1]*, Nenad Đorđević[2], Aleksandra Martinović[3], Aleksandra Ivetić[2], Danka Drakić[1], Jovana Raičević[1], Bojan Gašović[1]

[1]"13. Jul Plantaže" a.d., Sector for development, Put Radomira Ivanovića 2, 81000 Podgorica, Crna Gora
[2]Faculty of Agriculture,University of Belgrade, Nemanjina 6, 11080 Zemun, Srbija
[3]Faculty for food technology, food safety and ecology, University of Donja Gorica, Donja Gorica, 81000 Podgorica, Crna Gora

*Corresponding author:
E-mail address: vesnam@t-com.me

ABSTRACT

The impact of Benural S (commercial non-protein source of nitrogen) addition and inoculation on chemical composition parameters and quality of ensiled pomace from grape variety Rkatsiteli, was examined. The experiment was set up as a two-factorial (4□2; n=3), wherein the factor A was the dose of Benural S (A_1= control; A_2=10 gkg^{-1}; A_3=20 gkg^{-1}; A_3=30 gkg^{-1}), while the factor B was the inoculation (B_1=without inoculant; B_2=with inoculants). Ensilages were stored in plastic experimental vessels of 120 dm^3 volume. The experimental tanks were opened 126 days from ensiling and representative samples were taken for the chemical analysis. During ensiling, the highest change was determined on unstructured carbohydrates (NFE) that were intensively consumed for bacterial fermentation and synthesis of lactic, acetic and butyric acid. It was determined that addition of Benural S significantly increased the amount of crude proteins and ammonia nitrogen in ensilages, as well as pH values. The amount of crude proteins in ensilages with addition of 30 gkg^{-1} of Benural S was two times increased, while the share of ammonia nitrogen in total nitrogen (from crude proteins) in the treatment A_2B_1 was around 500 gkg^{-1}. At the same time, pH values were below 4.5, which is considered as a border for the occurrence of more intensive butyric fermentation. Inoculation of ensilages modified the fermentation, therefore inoculated treatments had lower content of ammonia nitrogen and higher lactic acid content.
Addition of Benural S at the doses of 10, 20 and 30 gkg^{-1} positively influenced the most parameters of chemical composition and quality of ensilages. At the same time, there is a significant increase of ammonia nitrogen share, which can be negative for productive and reproductive parameters of cows due to intake of large amounts of degradable protein.
Keywords: *grape pomace, ensilage, Benural S, inoculation*

INTRODUCTION

By development of the food industry in the nineteenth century, in the market occur different products, which are partially or completely unusable for human consumption, but for the animals they are an important nutritional potential. There is no competition between animals and humans in their use, and nutritional value of various industrial crops is used to the maximum, while preserving the environment (Đorđević and Dinić, 2007). In this respect, the most important are the following products of mills, sugar, oil, starch, alcohol and fermentation (Đorđević et al., 1998). The importance of nutrients from these groups is particularly important for countries which have deficits in the balance of forage, due to climate and topography. In the Republic of Montenegro, with the intensification of livestock production, it becomes more pronounced shortage of fodder, not only for non-ruminants, but also for ruminants. For this reason there is a great interest of farmers to use additional products from domestic industrial capacity of processed food products intended for human consumption (Maraš et al., 2013).
The company "13. jul Plantaze" (Podgorica, Montenegro) produces huge pomace quantities after the harvest and grape processing. Based on quantitative and qualitative features, this

kind of product can be interesting and important energetic food, especially in areas with less choice of food for ruminants. However, mentioned product contains high percentage of moisture, and because of that it can be used in short period of time, after which the aerobic microbiological process, spoilage and inapplicability occur. The practice of drying this feed in dehydrators, massively used in the preceding decades, today is not a feasible procedure, due to high energy prices. Because of that, surely the most current conservation is by ensiling of this and other feeds with high percentage of moisture. This experiment was planned with the aim to investigate the possibility of ensilage grape pomace with adding commercial supplement based on urea (in order to increase crude protein content) and bacterial inoculant (in order to intensify and direct fermentation of lactic acid type).

MATERIAL AND METHODS

In the experiment, the influence of the commercial non-protein nitrogen source addition (Benural S) and inoculation (inoculant BioStabil Mays) on the parameters of the chemical composition and quality of grape pomace of variety Rkaciteli were examined. The experiment was set up as a two-factorial (4×2, n=3), where the factor A was the dose of Benural S (A_1= control; A_2=10 gkg^{-1}; A_3=20 gkg^{-1}; A_3=30 gkg^{-1}), while factor B was inoculation (B_1=no inoculant, B_2=with inoculant). As a source of non-protein nitrogen it was used Benural S-containing 42% urea, 2% sulfur and bentonite which provides slow release of ammonia in the rumen and a more efficient utilization by rumen micro-organisms, bind some gases and toxic substances and contains some important alkali elements (K, Na, Mg, etc.). Sulfur contained in Benural S enables effective microbial synthesis of certain amino acids (methionine, cystine). Used inoculants containing homofermentative BioStabil Mays lactic acid bacterias (*Bacillus plantarum* and *Enterococcus faecium*) intensify and direct a fermentation and heterofermentative lactic acid bacteria (*L. brevis* and/or *L. kefiri*) which produces increased aerobic stability of silage. Plastic barrel of volume of 120 dm^3, closed with a screw caps was used for silage. Grape pomace was ensiled immediately after receiving from the presses in the microvinification winecellar of "13. Jul Plantaže" Podgorica-Montenegro. The compression degree was the same for each barrel, of about 100 gdm^{-3}, which is considered to be optimal for providing anaerobic conditions from the beginning.
In order to determine the nutritional value and quality of silage, samples for laboratory analysis were taken 126 days after ensiling. Chemical analysis of silage samples of grape pomace were performed in the laboratory of animal nutrition at the Agricultural Faculty of the University of Belgrade. The parameters of the chemical composition were determined according to AOAC (2002) methods; the amount of lactic, acetic and butyric acids were determined by distillation method according to Wiegner (1926); the amount of ammonia nitrogen by a modified Kjeldahl's method (Dulphy and Demarquilly, 1981). The quality and usage of silage were evaluated using the DLG (1987) methods. Statistical analysis was performed by software Statsoft (2006), where the analysis of variance examined the significance of the factors, and for the significance of the factor interaction between treatments Tukey test was used.

RESULTS AND DISCUSSION

Initial material of grape pomace from variety Rkatsiteli contained significantly less protein level compared to Pirmohamaddi et al. (2007), i.e. less protein and ash content and more lipids and nitrogen-free extractive substance comparing to Mirzaei-Agsaghali et al. (2011), what can be interpreted by influence of the variety, area, year etc. In contrast, all parameters of the initial material chemical composition were very similar to the values established by Maras et al. (2013), given that it is a similar material from the same grape variety and from the same cellar but in different years. Compared to the initial material, all silages had a few percentages higher moisture level, which is explained by the influence of added water as a solvent for used inoculant, but also because of partially volatile substances (free acetic and

butyric acid, and the ammonium nitrogen) loss during the sample drying at 105 ºC, in order to determine the dry matter content. In all silages the determined dry matter content was higher than 300 gkg^{-1}, by which was initially prevented juices extraction and reached maximum control of butyric type fermentation (Table 1).

Table 1. Chemical composition of starting material and silages, gkg^{-1} DM

Treatment		Dry matter, gkg^{-1}	Crude proteins	Crude lipids	Crude fiber	NFE	Ash
Starting material							
		412,500	105,80	99,09	227,60	525,53	41,98
Silages							
A_1	B_1	324.03	146.41	74.17	346.67	369.36	63.60
	B_2	355.73	126.51	86.00	313.57	415.98	57.94
A_2	B_1	325.77	185.21	78.45	264.70	403.75	67.89
	B_2	334.23	169.53	86.23	281.23	396.65	66.36
A_3	B_1	333.97	234.37	87.05	324.29	281.73	72.56
	B_2	317.07	245.49	83.36	326.23	267.96	76.96
A_4	B_1	335.60	246.82	84.93	314.83	272.03	81.34
	B_2	343.23	216.35	85.51	325.84	257.91	96.39
Average for A_1		339.88^{c}	136.46^{a}	80.06^{a}	330.02^{c}	392.67^{c}	60.77^{a}
Average for A_2		330.00^{b}	177.37^{b}	82.34^{b}	272.97^{a}	400.20^{d}	67.13^{b}
Average for A_3		325.52^{a}	$239,93^{c}$	85.20^{c}	325.26^{bc}	274.85^{b}	74.76^{c}
Average for A_4		339.42^{c}	231.59^{c}	85.22^{c}	320.34^{b}	264.97^{a}	88.86^{d}
Average for B_1		329.84^{a}	203.20^{b}	81.15^{a}	312.57	331.72	71.35^{a}
Average for B_2		337.57^{b}	189.47^{a}	85.27^{b}	311.72	334.63	74.41^{b}

a,b,c,d,A,B- Values in the same colon, for different factors and with different letters are significantly different ($p<0.05$)

The all investigated silages, an increase of crude protein, crude fiber and minerals, as well as reducing the amount of crude lipids and nitrogen-free extractive matters, compared to the starting material were determined. These changes are part of the absolute character (increasing of crude protein content due to the addition of commercial sources of non-protein nitrogen) or part of the relative character (due to spending of fermentable carbohydrates and loss of volatile matters during the samples silage drying in aim of preparation the standard chemical analysis).

The amount of crude protein was consistently increased in all silage treatments, in accordance with the increase of the dose of commercial adding of the non-protein nitrogen based on urea. The treatments A_4B_1 and A_4B_2 amount of the crude protein is increased to more than 100% compared to the starting material. This is explained by the fact that the urea contains 42% nitrogen, which is equivalent to 263% of protein. The treatments A_1B_1 and A_1B_2 amount of crude protein was increased compared to the starting material, although it is not used supplement Benural S, which can be interpreted as a consequence of the volatile matter loss (drying samples) and changes in the relative ratio of nutrients.

The amount of crude lipids in silages was slightly lower than in the initial sample, which could be explained by the relative changes ratio in the chemical substances in the silage, primarily due to a significant absolute increase in the share of nitrogen and mineral substances. For silages prepared of green plants is common that the amount of crude lipids increases compared to the starting material, which is mainly the result of the extraction of lactic acid (a non-volatile), diethyl ether, used for the determination of crude lipids according to Soxlet (Đorđević et al., 2003). However, the amount of lactic acid in investigated silages was much lower than in the silage prepared of green plants, and thus the impact that reduced amounts of volatile fatty acids in the share of crude lipids was negligible.

The amount of crude fiber in all treatments was a few percent higher than in the initial material, which is also a consequence of the relative change in the relationship of the individual components. The amount of crude fiber in this experiment was significantly lower than the statements of Nikolić et al. (1980).
The amount of nitrogen-free extractives matters is reduced in all silages, in relation to the initial material, that is a result of their utilization in the process of microbial fermentation process, but also because of the increase in dose of Benural S.
All grape pomace silages contained higher proportion of total minerals, with the constant increase with the increase of the commercial sources of non-protein nitrogen share. This is a consequence of the direct impact of bentonite as a part of commercial mineral sources of non-protein nitrogen urea, which is basically aluminosilicate clay, composed of colloidal and plastic clay, mainly of the minerals montmorillonite (Track et al., 2004).
The initial material of grape pomace was characterized by the low pH (pH = 3.83), which was initially provided an excellent conditions for the lactic fermentation, either spontaneous nature (on the basis of the content of the natural lactic acid bacteria) or on the basis of inoculants used in the experiment. In silages without adding Benural S there has been some reduction in the pH by activity of natural microflora or used inoculants.
However, in the following treatments is recorded a constant increase in the pH value, which is a consequence of alkaline character of ammonia released by hydrolysis of used addition of non-protein nitrogen based on urea ($p < 0.05$). The highest pH values (4.42 and 4.45) were found in the treatment with the maximum dose of Benural S. Determined pH values were on the verge of benefits for activity butyric acid bacteria as a highly undesirable microorganisms silage. The minimum requirements for the development and activity of harmful butyric clostridia is moisture of ensiling material higher than 700 g kg-1 and a pH value higher than 4.5. It should be emphasized that proteolytic strains of butyric acid bacteria are especially harmful; during their activities proteins degrade to ammonia as the final product (Dinić et al., 2012).
The amount of ammonium nitrogen, expressed in gkg^{-1} of total nitrogen of silage (originating from the nature protein, as well as from the addition of commercial non-protein nitrogen based on the urea), was highest in the treatments A_2B_1 and A_2B_2, which contained the lowest amount of commercial addition based on urea, and then decreased by increasing the share of commercial sources of non-protein nitrogen. This could also be explained by relative changes in the share of different nutrients. The proportion of ammonia nitrogen is shown in relation to the share of total nitrogenous matters (crude protein) in the silage. Given that the proportion of crude protein (part from silage and the other part from the used commercial addition) is constantly growing, there has been a decreased trend in the share of ammonia nitrogen. In contrast, treatments without the commercial addition based on urea (A_1B_1 i A_1B_2) contained the lowest amount of ammonia nitrogen, and since they are calculated in relation to the highest share of nature protein. At the same time, their degree of hydrolysis was small. Ammoniacal nitrogen in the silage is the main indicator of the protein degradation and it occurs by the activities of proteolytic enzymes produced from microorganisms and plant cells, primarily butyric acid clostridias. The presence of ammonia in the silages which do not contain butyric acid is a result of plant enzymes activities (McDonald et al., 1991). The grape pomace silage supplemented with Benural S that is also the result of urea hydrolysis ($CO(NH_2)_2$) on CO_2 and $2NH_3$. However, if the quantity (total) ammonia nitrogen exceeds 100 gkg^{-1} of total, it is bad for the quality of the silage, because it leads to an increase in its pH value. Such a trend was detected in all tested silages, with or without the addition of the above, indicating the nature of the protein of grapes.

Table 2. Parameters of biochemical changes in silages (gkg^{-1} DM)

Treatment		pH	NH_3-N, gkg^{-1} N	Lactic acid	Acetic acid			Butyric acid		
					Free	Bonded	Total	Free	Bonded	Total
Starting material										
		3.83	-	-	-	-	-	-	-	-
Silages										
A_1	B_1	3.68	157.13	27.86	3.96	0.77	4.73	0.00	0.00	0.00
	B_2	3.77	151.49	27.25	3.99	1.85	5.84	0.00	0.00	0.00
A_2	B_1	4.05	500.40	20.60	8.74	5.44	14.18	0.00	0.00	0.00
	B_2	4.09	411.54	20.67	6.79	0.71	7.50	0.00	0.00	0.00
A_3	B_1	4.29	377.19	20.07	5.24	5.20	10.44	0.00	0.00	0.00
	B_2	4.18	345.96	21.56	7.26	7.22	14.48	0.00	0.00	0.00
A_4	B_1	4.42	320.56	21.66	5.37	6.13	11.56	0.00	0.57	0.57
	B_2	4.45	344.89	19.21	2.91	6.79	9.70	1.40	0.11	1.51
Average for A_1		3.73a	154.31^{a}	27.56^{b}	3.98^{a}	1.31^{a}	5.28^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Average for A_2		4.07b	455.97^{c}	20.64^{a}	7.76^{d}	3.08^{b}	10.84^{b}	0.00^{a}	0.00^{a}	0.00^{a}
Average for A_3		4.23c	361.58^{b}	20.82^{a}	6.25^{c}	6.21^{c}	12.46^{c}	0.00^{a}	0.00^{a}	0.00^{a}
Average for A_4		4.44d	332.72^{b}	20.44^{a}	4.14^{b}	6.46^{c}	10.63^{b}	0.70^{b}	0.34^{b}	0.54^{b}
Average for B_1		4.11	338.82^{B}	22.55	5.83	4.38	10.23	0.00^{A}	0.14^{B}	0.14
Average for B_2		4.12	313.47^{A}	22.18	5.24	4.14	9.38	0.35^{B}	0.03^{A}	0.13

a,b,c,d,A,B = Values in the same colon, for different factors and with different letters are significantly different (p<0.05)

The highest quantities of lactic acid (absolute and relative) were determined in the treatments without the addition of Benural S. However, in the subsequent treatments, there was a reduction in lactic acid production, in absolute and relative terms, which may be explained by inadequate (higher) pH values. In such circumstances, the use of inoculants did not affect the production of lactic, but led to higher percentages of acetic acid synthesis, which is considered as "desirable" due to increased aerobic stability of silage. The treatments A_4B_1 and A_4B_2 smaller amounts of lactic acid is not only a consequence of the reduced activity of LAB bacteria in unfavorable conditions (high pH values), but degradation of lactic acid due to the activities of butyric acid clostridia. Namely, in these treatments was detected butyric acid, which indicates on specific activity of butyric acid clostridia. The occurrence of butyric acid in the treatment of A_4B_1 and A_4B_2 is in large part a consequence of the high pH values, since the dry matter content (335.60 and 343.23 gkg^{-1}) was certainly inappropriate for the activity of butyric acid clostridia.

For evaluation of silage quality, DLG method was used, which considers the pH value and the percentage of total lactic, acetic and butyric acids. According to the applied methods, all tested silage were rated with the highest score (I class), what is a result of relatively favorable pH values in fhe firts place, as well as a high percentage share of lactic acid.

Table 3. Relative ratio of acids and quality class by DLG method

Treatment		Relative ratio of acids, %			Points	Class by DLG
		Lactic	Acetic	Butyric		
A_1	B_1	85.49	14.51	0.00	49	I
	B_2	82.35	17.65	0.00	49	I
A_2	B_1	59.23	40.77	0.00	45	I
	B_2	73.38	26.62	0.00	50	I
A_3	B_1	65.78	34.22	0.00	47	I
	B_2	59.82	40.18	0.00	45	I
A_4	B_1	64.10	34.21	1.69	45	I
	B_2	63.15	31.89	4.96	45	I

CONCLUSION

Based on the results of this experiment, the conclusion is that the grape pomace can be ensiled with no use of inoculant, which start and intensify the fermentation, since this is a material with a large amount of fermentable carbohydrates, small amounts of buffering substances of the base character (proteins and minerals) and a low initial pH. Using Benural S in the aim of "breeding" grape pomace silage with crude protein has led to an increase in crude protein content, while at the same time there has not been a significant increase in pH, and thus the decline in the quality of silage. However, supplement used resulted in a significant increase in the share of ammonia nitrogen in the silage, which can pose a danger to the health, fertility and as well as to the life of the animal.

The general conclusion is that supplements used (commercial source of non-protein nitrogen and inoculant) did not affected on the change of class quality, but only on chemical composition and nutritive value of silage.

Due to the experience from this experiment, for further research, it is recommended the use of small amounts of Benural S (to 5 gkg^{-1}), by which it could be expected a limited increase in the amount of crude protein in the silage, without a significant increase in the concentration of ammonia nitrogen and pH.

REFERENCES

AOAC (2002): Official Methods of Analysis of AOAC international. 17th ed. Association of Official Analytical Chemists, Washington, DC.

Dinić, B., Đorđević, N., Lugić, Z. (2012): Significance of proteolytic processes in silage for modern nutrition of ruminants. XI international feed technology symposium „FEED – TO FOOD"/COST „FEED FOR HEALTH" joint workshop, Novi Sad, 03-05. october, 2012. proceedings, 94-102.

DLG (1987): Bewertung von grunfutter, silage und heu. DLG-Merkblatt, pp: 224.

Dulphy, J. P., Demarquilly, C. (1981): Problemes particuliers aux ensilages. Prevision de la valeur nutritive des aliments des Ruminants, I.N.R.A.

Đorđević, N., Koljajić, V., Dujić, D. (1998): Efficiency of ensiling different by-products from vegetable and field crop production. Review of research work at the faculty of agriculture, 43, 1: 99-105.

Đorđević, N., Grubić, G., Jokić, Ž. (2003) : Osnovi ishrane domaćih životinja (praktikum). Univerzitet u Beogradu, Poljoprivredni fakultet.

Đorđević, N., Dinić, B. (2003): Siliranje leguminoza. Institut za primenu nauke u Poljoprivredi SRBIJA, Beograd.

Đorđević, N., Dinić, B. (2007): Hrana za životinje. Cenzone Tech Europe – Aranđelovac, Srbija.

Mcdonald, P., Henderson, A. R., Heron, S.J.E. (1991): The biochemistry of silage (second edition). Chalcombe Publications.

Maraš, V., Gašović, B., Drakić, D., Raičević, J., Djaković, J., Kodzulović, V., Šucur, S., Djordjević, N. (2013): Influence of inoculation on grape pomace silage quality supplemented with non-protein nitrogen. 10. International Symposium "Modern trends in livestock production", Institute for Animal Husbandry, Belgrade, Serbia, October 02-04, 2013. Proceedings, p. 1133-1141.

Mirzaei-Aghsaghal, A., Maheri-Sis, N., Mansouri, H., Ebrahim Razeghi, M., Shaddel, A., Aghajanzadeh-Golshani, A. (2011): Estimation of the nutritive value of grape pomace for ruminant using gas production technique. African Journal of Biotechnology, 10 (33): 6246-6250.

Nikolić, A., Negovanović, D., Stoićević, Lj. (1980) : Mogućnosti povećanja hranljive vrednosti grožđane komine. Krmiva, 22, 11: 239-242.

Pirmohammadi, R., Golgasemgarebagh, G., Mohsenpur Azari, A. (2007): Effects of ensiling and drying of white grape pomace on chemical composition, degradability and digestibility for ruminants. Journal of animal and veterinary advances, 6, 9: 1079-1082.

Statsoft, Inc (2006): STATISTICA (data analysis software system), version 7.1.www.statsoft.com.

Trackova, M., Maltova, L., Dvorska, L., Pavlik, I. (2004): Kaolin, bentonite, and zeolites as feed supplements for animals: health advantages and risks. Veterinary Research Institute, Brno, Czech Republic. Review 389, p. 389–399.

Wiegner, G. (1926): Anleitung zum qaantitativenagrikulturchemischen Praktikum. Berlin, Gebriider Borntraeger.

INFLUENCE OF STORAGE CONDITIONS ON DEOXYNIVALENOL LEVEL IN MAIZE

Radmilo Čolović[1]*, Đuro Vukmirović[1], Jovana Kos[1], Jovanka Lević[1], Ferenc Bagi[2], Vera Stojšin[2], Dragana Budakov[2]

[1]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author
E-mail address: radmilo.colovic@fins.uns.ac.rs

ABSTRACT

Mycotoxins are toxic chemical compounds produced by moulds. They can occur in a wide variety of commodities such as raw agricultural products, processed foods, animal products (meat, milk, eggs), imported products, etc. Contamination with mycotoxins is an additive process, meaning that it begins in the field and increases during harvest, drying, and storage. The level of contamination depends on geographic region (climatic conditions), availability of water, inoculum concentrations, mechanical damage, etc. The aim of this study was to investigate the influence of storage conditions on deoxynivalenol (DON) level in maize. Samples of infected maize were stored for 2 months under the warehouse conditions (air temperature of 20°C, relative humidity 50%) as well as for 20 days in a laboratory climatic chamber (air temperature of 20°C, relative humidity 80%) in order to study the influence of relative humidity increase on DON level. The influence of form of maize (kernel vs. milled) on DON production was investigated by the storage in the laboratory climatic chamber. It was noticed that after the storage period of 2 months in warehouse DON level in maize increased from 5 to 15 %. DON level in samples of maize kernels stored in the climatic chamber for 20 days increased up to 2.5%, although relative humidity was very high. On the other hand, DON level in milled maize increased from 15 to 60% when compared with the whole kernel maize. Milled maize was more susceptible to DON contamination than unground maize.
***Keywords**: mycotoxin, deoxynivalenol, storage conditions*

INTRODUCTION

Mycotoxins are toxic chemical compounds produced by moulds. They can occur in a wide variety of commodities such as raw agricultural products, processed foods, animal products (meat, milk, eggs), imported products, etc (Marguardt, 1996). Mycotoxins can severely affect human and animal health by causing a toxic response when ingested. Contaminated material potentially induces acute and chronic effects, which may result in carcinogenic, teratogenic, and immune-suppressive effects (Wu, 2004; Jajić et al., 2008).

Mycotoxins are thermostable molecules, and can be totally destroyed only on very high temperatures. These temperatures cannot occur in the grain chain "from field to fork". Therefore, due to stability of mycotoxin molecules, contamination with mycotoxins can be considered as an additive process, meaning that it begins in the field and increases during harvest, drying, storage, etc. (Dänicke et al., 2004, Avantaggiato, 2012, Čolović et al., 2013).

The level of mycotoxin contamination depends on geographic region (climatic conditions), availability of water, inoculum concentrations, mechanical damage, etc. Thus, when weather changes occur, mycotoxins contamination will be affected. Mycotoxin contamination is climate-dependent, plant and storage-associated problem, and can be also affected by non-infectious factors (e.g., insect damage, bioavailability of micronutrients) that are in turn determined by climatic conditions. Climate represents driving force for fungal colonisation and mycotoxin production (Magan and Lacey 1984; Sanchis and Magan, 2004).

DON is a trichothecene mycotoxin associated produced by *Fusarium graminearum* (Gibberella zeae) and *Fusarium culmorum*. Temperature in particular plays an important role in *Fusarium* species. Small changes in temperature may subsequently influence the incidence and severity of disease. DON has toxic effects in humans and animal species.

These toxic effects can cause reduced performance, immune suppression, diarrhea, hematological and carcinogenic disorders (D'Mello et al. 1999; Jennings et al. 2000; Magan et al. 2002; Ramirez et al. 2004).
Mycotoxin contamination in the milling process may be redistributed and concentrated in certain milling fractions, such as germ and bran. Visconti et al. (2004) showed that outer layers of infected whole wheat kernel, such as wheat bran, have much higher concentration of DON when compared with fine middlings. However, there is no sufficient information about influence of physical form of cereal grains on mycotoxin contamination during storage. The aim of this study was to investigate the influence of storage conditions, as well as physical form of kernels (whole kernel vs. milled) on DON level in maize.

MATERIAL AND METHODS

Maize kernels contaminated with DON were obtained from commercial warehouses within Vojvodina province (Northern Serbia). For the storage of contaminated maize, the warehouse storage as well as the controlled laboratory conditions in the climatic chamber were used. For the warehouse storage, experiment warehouse at the Institute of Food Technology in Novi Sad was used in which contaminated maize was stored in kernel form. Air temperature during the storage was at 20 °C, and relative humidity at 50%. The samples were stored for 60 days.
For the climatic chamber storage experiment, temperature and relative humidity controlled laboratory climatic chamber was used (Model KBF 240, Binder, Germany) (Figure 1).

Figure 1. Laboratory climatic chamber

Prior to storage, each sample was divided in two parts. One part was stored as is (whole kernels), and the other was milled on laboratory hammer mill (Model 11, ABC Inženjering, Pančevo) equipped with sieves with 4 mm diameter of openings. Air temperature in the climatic chamber was set at 20 °C, and relative humidity at 80%. Contaminated maize was stored in laboratory climatic chamber for 20 days, and the samples for the analysis were collected on 10th, 15th, and 20th day.
DON analysis was performed before and after the storage of the samples in order to determine possible changes in DON concentration during the storage. Determination of DON was carried out by isocratic reverse-phase liquid chromatography using Agilent 1200 (Agilent Technologies Inc., USA) system equipped with diode array detector (DAD) and column (Eclipse XDB-C18, 1.8 µm, 4.6 x 50 mm).The mobile phase consisted of an isocratic mixture of water/acetonitrile (84:16, v/v) and flow rate was 0.30 ml/min. DAD detection was performed at 220 nm.

Maize samples were prepared according to the instructions for Mycosep ® 225 Trich cleanup columns given by the manufacturer (Römer Labs, Tulln, Austria).

RESULTS AND DISCUSSION

Concentration of DON in the maize before and after the warehouse storage is shown in Figure 2. All samples had higher concentration after 60 day storage test, meaning that storage conditions caused increase in DON content. DON increase (%) within the storage period of 60 days in the warehouse has been shown in Figure 3. It can be seen that applied storage conditions caused DON increase of approximately 5 to 18 %.

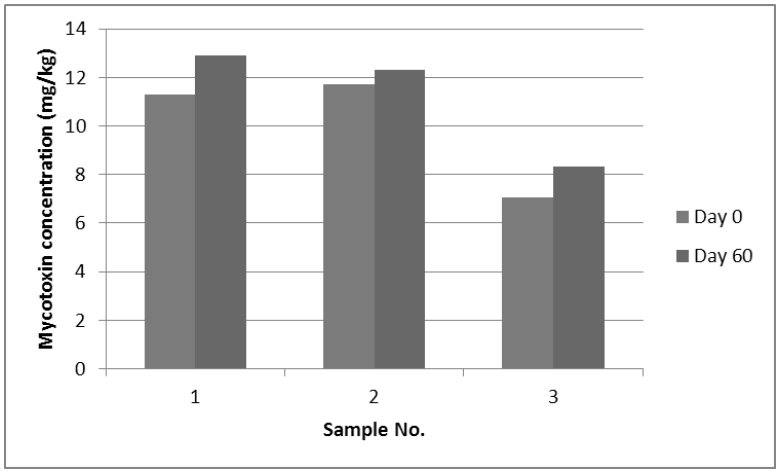

Figure 2. DON concentration (mg/kg) in the maize before and after the warehouse storage

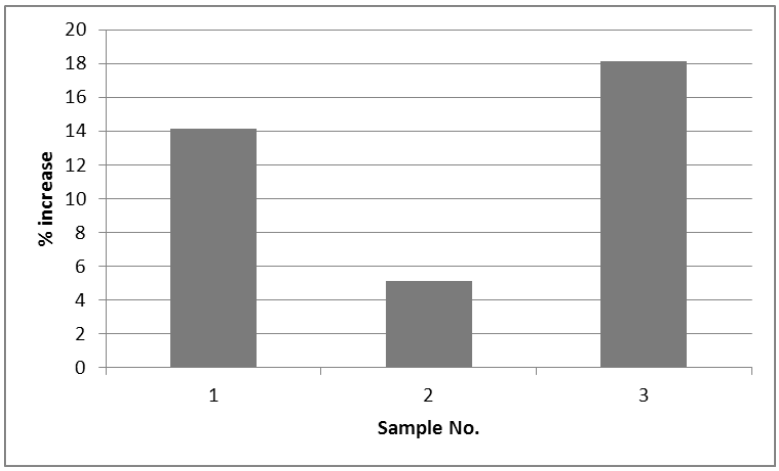

Figure 3. Effect of storage conditions on DON increase (%) in the warehouse stored maize

DON content in the maize before and after the climate chamber stability test storage has been presented in Figure 4, and the effect of storage conditions on DON increase (%) in the climatic chamber stored maize has been presented in Figure 5.

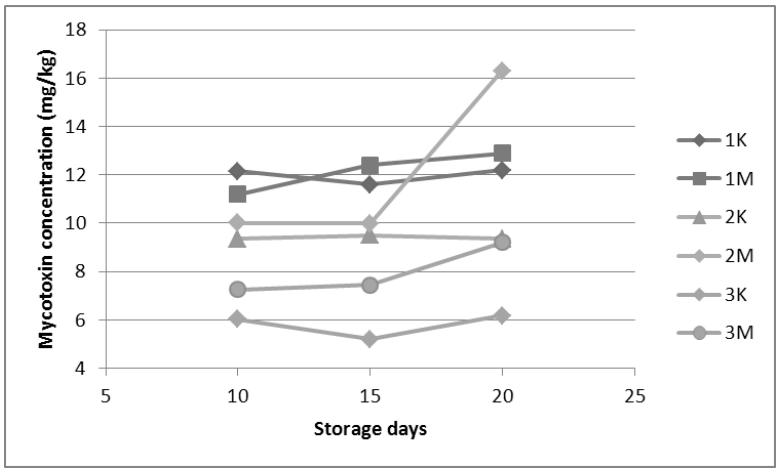

Figure 4. DON concentration (mg/kg) in the maize before and after climate chamber storage (1-3 Sample No., K-kernel, M-milled)

From Figure 4 it can be seen that there was a difference in DON concentration between stored samples of kernel and milled maize. DON concentration in kernel stored maize was not significantly ($p < 0.05$) changed during 20 days of storage, while there was constant increase in DON concentration in milled maize during the 20 days of storage in climatic chamber. Reason for this is that outer layers of grains (pericarp) have protective role for the inner parts of the kernel. This is in line with the results of Visconti et al. (2004) who found that during the dry milling of DON contaminated durum wheat, DON was distributed to different fractions. The highest concentration of DON in dry milled durum wheat was found in wheat bran and germ.

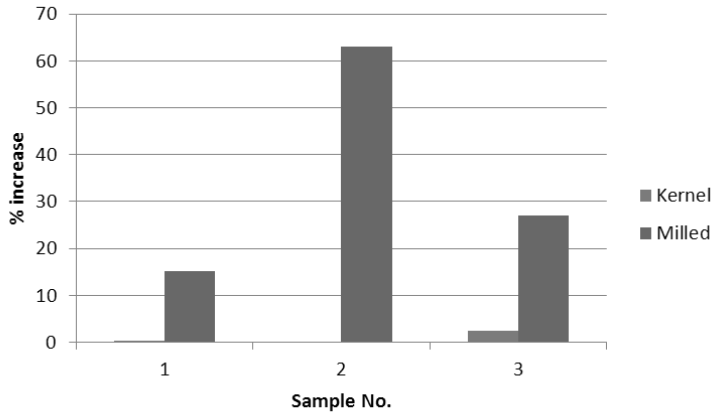

Figure 5. Effect of storage conditions on DON increase (%) in the climatic chamber stored maize

When comparing DON concentration in kernel stored maize samples from both storage places, the warehouse and the climatic chamber, it can be seen that slightly higher concentration of DON was found in the warehouse stored maize, although relative humidity was higher in the climatic chamber (50% compared to 80%). The reason for this might be the duration of storage, which was three times longer in the warehouse storage test (60 days compared to 20 days). On the other hand, up to 70% DON increase was observed in milled stored samples.

Hope et al. (2005) investigated the effects of water activity and temperature (environmental conditions) on the production of DON in wheat grains. These authors showed that temperature and water activity play significant role in DON production. The highest concentration of DON was found in the samples stored at 25 °C, and maximum water activity. These authors also observed that DON production increased during the storage

(maximum storage period was 40 days). Within first 20 days, DON concentration in grain, i.e. kernel, was not significantly changed, which is in line with the results of this study. Marin et al. (1999) also investigated the influence of temperature and water availability on fumonisin B1 production. These authors also found that these factors have significant influence on mycotoxin production, where maximum fumonisin production was found in 15-25 °C temperature range.

CONCLUSIONS

Storage period of 60 days in warehouse at the temperature of 20 °C and the relative humidity of air caused increase in DON level in maize from 5 to 15%. DON level in samples of maize kernels stored in the climatic chamber for 20 days increased only for 2.5%, although the relative humidity was very high, due to short storage period. DON level in milled maize increased from 15 to 60% when compared with whole kernel maize, for the same environmental conditions.

ACKNOWLEDGEMENTS

These results are a part of the research within the project III46012, financed by the Ministry of Education, Science and Technological Development, Republic of Serbia, and the IPA project HUSRB 1002/122/062, financed by EU.
Additionally, the authors are expressing their gratitude to the Provincial Secretariat for Science and Technological Development for the financial support.

REFERENCES

Avantaggiato, G. (2012). Mycotoxin decontamination in the food/feed chain. Detection and decontamination techniques for mycotoxins in the food/feed chain, Training Course, Bari, Italy.

Čolović, R., Bursić, V., Vuković, G., Vukmirović, Đ. Lazić, S., Lević, J., Bagi., F. (2013). Reducing the level of mycotoxins in corn by remova of fines. Zbornik Matice Srpske za prirodn nauke, 124, 67-75.

Dänicke, S., Valenta, H., Döll, S., Ganter, M., Flachowsky, G. (2004). On the effectiveness of a detoxifying agent in preventing fusario-toxicosis in fattening pigs. Animal Feed Science and Technology, 114, 141-157.

D'Mello, J.P.F., Placinta, C.M. and Macdonald, A.M.C. (1999). Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology, 80, 183-205.

Hope, R., Aldred, D., Magan, N. (2005). Comparison of environmental profiles for growth and deoxynivalenol production by Fusarium culmorum and F. graminearum on wheat grain. Letters in Applied Microbiology, 40, 295-300

Jajić, I., Jurić, V., Glamočić, D., Abramović, B. (2008). Occurrence of Deoxynivaenol in maize and wheat in Serbia. International Journal of Molecular Sciences, 9, 2114-2126.

Jennings, P., Turner J.A. and Nicholson, P. (2000). Overview of Fusarium ear blight in the UK – effect of fungicide treatment on disease control and mycotoxin production. The British Crop Protection Council. Pests Dis 2000 2, 707-712.

Magan, N., Hope, R., Colleate, A. and Baxter, E.S. (2002). Relationship between growth and mycotoxin production by Fusarium species, biocides and environment. European Journal of Plant Pathology, 108, 685-690.

Magan, N., Lacey, J. (1984). Effect of water activity, temperature and substrate on interactions between field and storage fungi. Transactions of the British Mycological Society, 82, 83–93.

Marın, S., Magan, N., Bellı´, N., Ramos A.J., Canela, R. and Sanchis, V. (1999). Two-dimensional profiles of germination, growth and fumonisin B1 production by Fusarium moniliforme and Fusarium proliferatum in relation to environmental factors and potential for modelling toxin formation in maize grain. International Journal of Food Microbiology, 51, 159–167.

Marquardt, R.R. (1996): Fungi Effects of molds and their toxins on livestock performance: A western Canadian perspective. Animal Feed Science and Technology, 58, 77-89.

Ramirez, L., Chulze, S. and Magan, N. (2004). Impact of environmental factors on growth and deoxynivalenol production by Fusarium graminearum isolates from Argentinian wheat. Crop Protection, 23, 117-125.

Sanchis, V., Magan, N. (2004). Environmental conditions affecting mycotoxins. In: Mycotoxins in Food Detection and Control, Editors: Magan, N, Olsen, M., 174-189. Cambridge, UK: Woodhead 10 Publishing Ltd.

Visconty, A., Haidukowski, E.M., Pascale, M., Silvestry, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicology letters, 153, 181-189.
Wu., F. (2004). Mycotoxin risk assesment for the purpose of setting International Regulatory Standards. Environmental Science and Technology, 38, 4049-4055.
STATISTICA (Data Analysis Software System), v.12.0 (2013). Stat-Soft, Inc, USA (www. statsoft.com).

EFFECT OF FEEDING PROGRAMS WITH DIFFERENT PROTEIN AND ENERGY LEVELS ON THE PERFORMANCE AND CARCASS QUALITY OF BROILERS

Dragan Milić[1], Nataša Tolimir[2]*, Marina Vukić Vranješ[2], Marijana Maslovarić[2], Vladislav Stanaćev[3]

[1]"Vinfeed", 21 000 Novi Sad, 16/2 Djordja Nikšića Johana St., Novo Naselje, Serbia
[2]Institute for Science Application in Agriculture, 11000 Belgrade, 68b Blvd. despota Stefana, Serbia
[3]Meat Industry "Matijević", 21000 Novi Sad, 86 Rumenački put, Serbia

*Corresponding author:
E-mail address: ntolimir@ipn.co.rs

ABSTRACT

The aim of this paper was to investigate the effect of feeding programs, i.e. the effect of diets with different protein and energy levels on broiler production performance and carcass quality.
The 42-day-experiment was conducted on 1.200 chicks of Ross-308 provenience, separated by sex. Standard technology was used. The four groups (treatments) differed in a type of a feed mash. T1 (control) group was fed mashes containing 23%, 19% and 18% crude proteins (CP) and 12.76, 13.49 and 13.49 MJ/kg metabolizable energy (ME), from Day 1 to Day 14, Day 15 to Day 35, and Day 36 to Day 42, respectively. T2 group was fed mashes containing the same CP level but a reduced level of ME: 13.28 MJ/kg from Day 15 to Day 35 and 13.28 MJ/kg from Day 36 to Day 42. T3 group was fed mashes containing the CP level reduced in the starter period (22% CP) and the same ME content as T1 group. T4 group was fed mashes containing the CP level reduced in the starter period (22% CP) and ME reduced in the period from Day 15 to Day 35 (13.28 MJ/kg) and from Day 36 to Day 42 (13.39 MJ/kg). Slaughtering performance was investigated on six male and six female chicks for each of the treatments.
The obtained results showed that the differences in average final body weights, feed conversion ratios and mortality of broilers between the control (T1) and experimental groups were not statistically significant. The investigated feeding programs did not have a significant effect on "traditionally dressed" and "ready to grill" carcass yields. The lowest abdominal fat was recorded for T4 group (1.98%) - for each sex separately and for both sexes, while a statistical significance ($P < 0.05$) was recorded only when compared T4 to T3 group.
The obtained results showed that energy and protein levels in broiler diet can be slightly reduced without having an adverse effect on production performance and carcass yield. However, they can have a significant effect on the reduction of abdominal fat content.
Keywords: *broilers, protein level, energy, carcass yield, abdominal fat*

INTRODUCTION

Diet is one of the most important factors of broiler production economic efficiency, since feed costs constitute about 70% of total cost of production (Marcu et al., 2013). Modern broiler production raises the need for development of feeding programs that will maximize production performances with minimum cost. Special attention is therefore paid to the protein component of broiler diets, which together with energy sources constitutes the largest share of feed costs (Moosavi et al., 2011.).
The research of Roy et al. (2010) shows that feeding with optimal nutrient level diets result in lower feed costs, reduced environmental pollution, maximized growth and better feed conversion ratio, thus economising the broiler production.
The effect of protein and energy levels and their ratio were the subject of research of numerous authors, who wanted to investigate the effect of different feeding programs on broiler production performances (Nikolova et al., 2007, Moosavi et al., 2012, Silva et al. 2001). Some authors also investigated the effect of different feeding programs on slaughtering performances (Albuquerque et al., 2003, Maiorka et al., 2004.).

The aim of this paper was to investigate the effect of feeding programs with different protein and energy levels on basic production performances and carcass quality of Ross-308 broilers.

MATERIAL AND METHODS

The experiment was conducted on 1.216 chicks of Ross-308 provenience. The experimental period lasted for 42 days, during which the standard feeding technology was applied, with respect to all technological standards for intensive broiler fattening. Feeding and watering were ad libitum. There were four groups (treatments) with eight repetitions. The facility was divided into 32 pens, each pen containing 38 chicks. The treatments and repetitions were conducted according to a randomized block design. Each treatment comprised 304 chicks. The groups (treatments) differed in the type of a feed mash.

- T1 – control group fed mashes containing 23%, 19% and 18% crude proteins (CP) and 12.76, 13.49 and 13.49 MJ/kg metabolizable energy (ME) from Day 1 to Day 14, Day 15 to Day 35, and Day 36 to Day 42, respectively.
- T2 – containing the same level of CP as T1 and reduced energy levels – 13.28 MJ/kg from Day 15 to Day 35 and 13.28 MJ/kg from Day 36 to Day 42.
- T3 – containing a reduced level of CP in the starter period, i.e., from Day 1 to Day 14 (22% CP) and the same level of energy as T1 group.
- T4 – containing a reduced level of CP in the starter period, i.e., from Day 1 to Day 14 (22% CP) and a reduced level of energy in the period from Day 15 to Day 35 (13.28 MJ/kg) and from Day 36 to Day 42 (13.39 MJ/kg).

Control weighing was done with a precision balance on Day 1 and later on a weekly basis. Based on the amount of consumed feed and the average body weight recorded for each fattening period, feed conversion rate was calculated. During the experimental period, the death of chicks was recorded on a day-to-day basis. The obtained production parameters were used to calculate production index (PI). PI = (vitality (%) * body weight (kg) / feeding days * feed conversion rate).

The investigation of slaughtering performances was conducted on 12 chicks (six male and six female) per treatment. The fabrication was done according to the Rulebook on Poultry Meat Quality (1981*)* – into "traditionally dressed", "ready to roast" and "ready to grill" carcasses. When compared thus fabricated carcasses with the body weight prior to slaughter, the authors calculated the yields of "traditionally dressed" and "ready to roast" carcasses. During fabrication, abdominal fat content was also measured and calculated in carcass weight.

The data were processed with the computer program Statistica (version 5) Stat.Soft.Inc, 2006.

RESULTS AND DISCUSSION

The data given in Table 1 show that at the end of the fattening period (after 42 days) the highest body weight (2.306 g) was obtained by T1 group, which had consumed the diet containing the highest protein and energy levels. The lowest body weight was obtained by T4 group, which had consumed the diet with a reduced level of protein in the first 14 days and then with a reduced level of energy till the end of the fattening period. Differences in final body weight among the groups (feeding treatments) were not significant.

The obtained results indicate that the difference in final body weight was most pronounced when energy and protein levels were reduced simultaneously (T4 group). This is in line with the research of Moosavi et al. (2012), in which the diet containing low levels of CP and a constant ME:CP ratio had an adverse effect on the growth.

Table 1. Body weight

Period (days)	Body weight, g			
	Feeding treatments			
	1	2	3	4
7	150	158	149	142
14	404	414	400	392
21	814	822	812	801
28	1.242^{ab}	1.253^{a}	1.242^{ab}	1.213^{b}
35	1.756	1.746	1.738	1.732
42	2.306	2.292	2.295	2.268

Having analysed the feed conversion ratios (Table 2), the authors could conclude that the obtained results were similar, and the differences among the groups not significant. T1 and T3 group had a better feed conversion ratio (1.95) than T2 and T4 group (1.98). The better feed conversion ratios achieved by the groups that consumed diets with higher energy levels are in line with the research of Nikolova (2007). In this research chicks were fed with different feed mashes (energy-protein mashes) and the group that was fed the diet containing about 3200 kcal/kg in the final fattening period also achieved a better feed conversion ratio than the group fed the diet containing lower energy levels. Albuquerque et al. (2003) also investigated the effect of diets with different energy levels at the final fattening period. Their results showed that chicks fed the diet containing 3200 kcal/kg achieved better production performances.

Table 2. Feed conversion ratio, mortality (%) and production index (PI)

Period (days)	Feeding treatments			
	1	2	3	4
Feed conversion ratio				
1-14	1.22	1.22	1.23	1.23
15-35	1.98	2.05	1.99	2.01
36-42	2.43	2.41	2.37	2.50
1-42	1.95	1.98	1.95	1.98
Mortality (%)				
1–42	3.7	3.7	3	4.3
Production index				
1-42	271.15	265.42	271.81	261.00

The vitality of the broilers was satisfactory, which indicates that the used feeding programs had not affected this parameter. The obtained results correspond with the research of Marcu et al. (2013) in which feeding programs with different protein and energy levels did not have effect on broiler mortality.

The results show that the groups fed the diet containing increased levels of energy achieved a higher PI. Investigating the effect of different energy and protein level diets on production and slaughtering performances of broilers, Moosavi et al. (2012) indicated that the production index was lower when chicks had been fed lower protein and ME level diets.

Table 4 shows the data on "traditionally dressed" and "ready to roast" carcass yields, depending on sex and treatments used.

Having analysed the data on "traditionally dressed", "ready to roast" and "ready to grill" carcasses, the authors came to a conclusion that there were no significant differences between the male and female chicks from T1 and experimental groups, and when compared the groups one with another. Moreover, no effect of different protein and energy level diets on carcass yields was determined in the research of Leandro et al. (2003).

Table 3. Carcass yield (%)

Carcass yield (%)				
Sex	Feeding treatment			
	1	2	3	4
"Ready to roast" carcass yield (%)				
Male	85.47	86.18	85.05	85.25
Female	85.68	86.84	86.17	85.58
Both	85.57	86.51	85.61	85.41
"Ready to grill" carcass yield (%)				
Male	70.15	70.81	69.23	70.71
Female	71.42	71.94	70.68	71.42
Both	70.78	71.37	69.95	71.04

Table 4 shows the yield and percentage of abdominal fat in the carcasses, given by sex and treatment. The female chicks of all groups had a higher percentage of abdominal fat than the male chicks. When observed the chicks of the same sex, however, the differences among the treatments were not statistically significant. Nevertheless, when observed the chicks of both sexes, it could be noticed that the chicks from T4 group had the lowest percentage of abdominal fat, and the difference between T4 and T3 group was statistically significant.

Table 4. Abdominal fat (%)

Abdominal fat (%)				
Sex	Feeding treatments			
	1	2	3	4
Male	2.02	2.09	2.21	1.47
Female	2.56	2.56	2.96	2.49
Both	2.29^{ab}	2.32^{ab}	2.56^{a}	1.98^{b}

The results indicate that a lower energy level diet given to T4 group led to a lower percentage of abdominal fat in both male and female chicks. To some extent, this complies with the research of Deaton and Lott (1985), who found that higher energy level diets led to increased abdominal fat. According to Nikolova (2007), chicks fed lower energy diets had a lower abdominal fat percentage. Nevertheless, if we observe energy levels solely, we could expect that the chicks from T2 group also have the lowest percentage of abdominal fat. Given this parameter is also affected by the protein:energy ratio, as implied by Nikolova (2007), the result we have obtained is not fully in line with the previous researches. Therefore, further research is needed.

CONCLUSIONS

The results show that the investigated feeding treatments, i.e. the different protein and energy level diets, had no significant effect on final body weight, feed conversion ratio and broiler mortality. There was also no statistically significant effect on "traditionally dressed" and "ready to grill" carcass yields. T4 group had the lowest percentage of abdominal fat - for each sex separately and for both sexes. Statistical significance was determined only when compared to T3 group.

It could be concluded that a slight reduction in energy and protein levels in diets does not have a significant effect on broiler production performance and carcass yield, yet has an effect on reducing abdominal fat content.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Education, Science and Technological development of the Republic of Serbia, as part of Integrated and Interdisciplinary Research Project No. III 46012.

REFERENCES

Albuquerque, R., de, Faria, D.E., de, Junqueira, O.M., Salvador, D., Faria Filho, D.E., de and Rizzo, M.F. (2003). Effects of Energy Level in Finisher Diets and Slaughter Age of on the Performance and Carcass Yield in Broiler Chickens. Revista Brasileira de Ciência Avícola - Brazilian Journal of Poultry Science, 5, 99–104.

Deaton, J.W., and Lott, B.D. (1985). Age and Dietary Energy Effect on Broiler Abdominal Fat Deposition. Poultry Science, 64 (11), 2161-2164.

Leandro, N.S.M., Café, M.B., Stringhini, J.H., Filho, R.M., Moura, K.A. and Silva Júnior, R.P. (2003). Feeding Program with Different Dietary Crude Protein and Metabolizable Energy Levels, for Broilers. Revista Brasileira de Zootecnia - Brazilian Journal of Animal Science, 32 (3), 620-631. (Abstract in English)

Maiorka, A., Dahlke, F., Santin, E., Kessler, A.M. and Penz Junior, A.M. (2004). Effect of Energy Levels of Diets Formulated on Total or Digestible Amino Acid Basis on Broiler Performance. Revista Brasileira de Ciencia Avicola - Brazilian Journal of Poultry Science, 6 (2), 87-91.

Marcu, A., Vacaru-Opriş I., Marcu, A., Dănăilă L., Kelciov, B. (2013). Effect of Feed Protein and Energy Levels on Economic Efficiency of Broiler Chickens Growth. Proceedings of the 23rd International symposium "New Technologies in Contemporary Animal Production", 19-21 June, Novi Sad, Serbia,

Moosavi, M., Moosa, E., Morteza, C. and Boujarpour, M. (2011). Economic Value of Diets with Different Levels of Energy and Protein with Constant Ratio on Broiler Chickens. Journal of Animal and Veterinary Advances, 10 (6), 709-711.

Moosavi, M., Chaji, M., Boujarpour, M., Rahimnahal, S., Kazemi, A.R. (2012). Effect of Different Levels of Energy and Protein with Constant Ratio on Performance and Carcass Characteristics in Broiler Chickens. International Journal of Applied and Basic Sciences, 3 (12), 2485-2488.

Nikolova, N. (2007). Uticaj genotipa, uzrasta i sastava hrane na proizvodne parametre i kvalitet trupa brojlerskih pilića (Effect of Genotype, Age and Diet Composition of Broiler Production Parameters and Carcass Quality), PhD Thesis, Faculty of Agriculture, University of Novi Sad, Serbia. (In Serbian)

Nikolova, N., Pavlovski, Z., Milosevic, N., Peric, L. (2007). The Effect of Different Energy and Proteins Levels in Diet on Production Parameters of Broiler Chicken from Two Genotypes. Biotechnology in Animal Husbandry, 23 (5-6), 551-557.

Pravilnik o kvalitetu mesa pernate živine (Rulebook on Poultry Meat Quality) (1981). Official Gazette of SFRY, No. 1/81 and 55/88. (In Serbian)

Roy, S.C., Alam, M.S., All, M.A., Chowdhury, S.D. and Goswami, C. (2010). Different Levels of Protein on the Performance of Synthetic Broiler. Bangladesh Journal of Veterinary Medicine, 8 (2), 117 – 122.

Silva, J.H.V., da, Albino, L.F.T, and Nasaimento, A.H. do (2001). Energy Levels and Metabolizable Energy: Protein Ratio for Male Broilers Chicks from 22 of 42 Days of Age. Revista Brasileira de Zootecnia - Brazilian Journal of Animal Science, 30 (6), 1791-1800. (Abstract in English)

STATISTICA (Data Analysis Software System) (2006). v.5.1., Stat-Soft, Inc., USA (www.statsoft.com).

THE INFLUENCE OF PIG DIET ENRICHED WITH n-3 POLYUNSATURATED FATTY ACID ON FATTY ACID COMPOSITION IN MEAT

Tatjana Tasić[1], Predrag Ikonić[1], Rade Jovanović[2], Dušica Čolović[1], Ljiljana Kostadinović[1], Natalija Džinić[3], Jasmina Gubić[1]

[1]University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]IPN, Bulevar despota Stefana 68b, 11000Belgrade, Serbia
[3]University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: tatjana.tasic@fins.uns.ac.rs

ABSTRACT

The aim of this study was to determine the influence of pig diet enriched with n-3 polyunsaturated fatty acids on fatty acid composition in meat. All pigs included in this investigation were of the same genetic origin and initial weight. Control group (C) was fed with standard mixture for pigs, while experimental group (E) was fed with the same mixture but with the addition of extruded flaxseed rich in polyunsaturated fatty acids. Fatty acid composition in meat was investigated on *M. semimembranosus* (SM) and *M. longissimus dorsi* (LD).
Most prevalent fatty acid was oleic acid (C18:1 n-9) ranging from 43.30 % (LD, E group) to 49.75 % (SM, C group). The content of alpha-linolenic acid (ALA, 18:3, n-3) varied from 0.22 % (SM, C group) to 1.26 % (SM, E group) and was higher in experimental group compared to the control group.
In SM PUFA/SFA ratio was above WHO/FAO recommended value, but higher in group fed with the addition of extruded flaxseed rich in polyunsaturated fatty acids, while in LD was lower than recommended values in both groups.
Content of n-3 fatty acids was higher in the experimental group compared to control group for both muscles. In SM this content in the experimental group was 3.18 times higher than in control group, while in LD this ratio was 2.32.
In the present study n-6/n-3 ratio was higher than recommended and ranged from 8.16 (LD, E group) to 25.60 (SM, C group). Both muscles (SM and LD) from experimental group had lower n-6/n-3 ratio compared to muscles from control group.
Based on the obtained results it can be concluded that the use of a well-balanced diet enriched with n-3 polyunsaturated fatty acids resulted in the functional pork or pork with modified fatty acid composition, i.e. meat with a higher content of n-3 fatty acids and better n-6/n-3 ratio.
***Keywords**: pig diet, pork meat, fatty acid composition*

INTRODUCTION

Modern way of life followed by speed and deadlines, tension and stress, as well as health problems and disorders, is setting new requirements in all its fields, especially in the diet. Therefore, in recent years, food science is intensively engaged in research of specific ingredients that positively affect the human health or have a preventive effect on increasing diseases of modern populations. Thus, functional food or food that besides the basic nutritional components contains ingredients that have a positive effect on people's health is more and more present in everyday nutrition. Meat industry, as well as other food industries is starting with production of functional products in accordance with modern knowledge, trends and market demands (Siróa *et al.,* 2008; European Commission 2010; Henchion *et al.,* 2014; Font-i-Furnols and Guerrero 2014).
A very important group, among ingredients with benefits on human health are polyunsaturated fatty acids. The beneficial effects of these compounds, with particular significance of n-3 polyunsaturated fatty acids, on human health and wellbeing have been known for years (Simopoulos 1998; Rose and Connolly 2002; Gurr *et al.,* 2002; Gebauer 2006; Gu 2013). Omega-3 polyunsaturated fatty acids, especially the long chain (LC) fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are well recognised

for their protective effects on hypercholesterolemia, cardiovascular disease, inflammatory diseases (such as rheumatoid arthritis), their role in brain health (improve cognitive function and mood and possibly protect against dementia) and nervous sistem, cancer and various other biological functions (Simopoulos 1999; Connor 2000; Simopoulos 2002; Harris 2007; Simopoulos 2008; Calder 2009). The omega-3 fatty acids cannot be synthesized in the body of mammalian species and have to be obtained from food (Gu 2013; Jakobsen 1995, Sretenović *et al*., 2009).

Pork is very present in the human diet because of its valuable nutrients such as essential amino acids, proteins of high biological value, vitamins (especially from B group), micronutrients (especially iron) and other bioactive compounds (Higgs 2000; Williamson *et al.,* 2005, Lawrie and Ledward 2006; McAfee *et al.,* 2010).

But pork meat also contributes to the intake of fat, saturated fatty acids, cholesterol, and other substances that, in inappropriate amounts may result in negative physiological effects (Toldrá and Reig 2011).

Way to increase omega-3 fatty acids and to improve their ratio in the human nutrition is modification of meat fatty acid composition. As feed has a direct impact on meat quality it is possible to change fatty acid composition of meat by using feed enriched with omega-3 fatty acids and thus increase nutritional value of meat and reduce its potentially negative physiological effects (Jiménez-Colmenero *et al.,* 2001, Jiménez-Colmenero *et al.,* 2006; Ivanov *et al.,* 2010; Okanović *et al.,* 2010).

In order to maintain and improve the high production and consumption of pork meat from one hand, and to meet modern trends and consumer demands in terms of functional or products with some added value on the other hand, aim of this study was to determine the influence of pig diet enriched with omega-3 polyunsaturated fatty acid from extruded flaxseed on fatty acid composition in meat.

MATERIAL AND METHODS

Samples

All pigs included in this investigation were of the same genetic origin and initial weight. The control group (C) obtained standard feed mixture for pigs and the experimental group (E) was fed with the same mixture but with the addition of extruded flaxseed rich in polyunsaturated fatty acids. Feeding of both groups of animals ended when the average live weight of pigs was 97 – 110 kg. Pigs were slaughtered and processed by standard technological procedure. Fatty acid composition in meat was investigated on *M. semimembranosus* (SM) and *M. longissimus dors*i (LD).

Fatty acid composition

Total lipids were extracted from 2 g samples of SM and LD using chloroform: methanol (2:1, v/v) solution (GC-grade) (Folch *et al., 1957*). The extracts were used for preparation of fatty acid methyl esters by transmetilation method as recommended method for this type of substrates. This method requires the use of 14% wt. boron trifluoride/methanol solution (Sigma Aldrich, MO, USA) (Hayat *et al.,* 2009). Fatty acid composition analyses were done on a gas chromatographer Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detector (GC-FID), auto-injection module for liquid, equipped with fused silica capillary column (DB-WAX 30 m, 0.25 mm, 0.50 µm). Carrier gas was helium (purity > 99.9997%). Prepared samples (2 µL of each) were injected with helium (flow of 3.5 mL/min), which was programmed for operating conditions such as column oven temperature 220°C for 7.5 minutes, split ratio (50%) with injector and detector temperatures (260°C). The standards of fatty acids methyl esters purchased from Sigma-Aldrich were also run under the same conditions. Peak areas and total fatty acids profile percentages were calculated for each sample by retention time using Agilent Chem. Station software comparing with retention times of authentic standards and were expressed as percentages of total fatty acid methyl esters.

Statistical analysis

One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 12 for Windows, Stat Soft, Tulsa, Oklahoma, USA, 2009. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Fatty acid composition of *M. Semimembranosus* (SM) and *M. longissimus dorsi* (LD) from pigs fed with standard (C) and diet enriched with omega-3 polyunsaturated fatty acids (E) is shown in Table 1.

Table 1. Fatty acid composition of M. semimembranosus (SM) and M. longissimus dorsi (LD) from pigs fed with standard (C) and diet enriched with omega-3 polyunsaturated fatty acids (E)

Fatty acid	% of fatty acid in total fatty acids			
	C	E	C	E
	SM		LD	
C12:0	0.07^{a}±0.00	0.06^{a}±0.01	0.59^{a}±0.55	0.05^{a}±0.00
C14:0	1.42^{ab}±0.15	1.59^{b}±0.01	1.38^{ab}±0.18	1.27^{a}±0.07
C16:0	28.4^{a}±1.59	28.0^{a}±1.42	28.7^{a}±0.38	24.7^{b}±0.71
C16:1	0.33^{a}±0.05	0.28^{a}±0.00	0.79^{a}±0.26	2.65^{b}±1.00
C17:1	0.01^{ab}±0.01	0.00^{a}±0.00	0.07^{b}±0.04	0.17^{c}±0.04
C17:0	0.05^{a}±0.05	0.00^{a}±0.00	0.29^{b}±0.13	0.28^{b}±0.08
C18:0	3.98^{a}±0.86	5.17^{a}±0.06	5.13^{a}±0.35	12.7^{b}±0.78
C18:1 n-9	49.8^{b}±3.20	44.3^{a}±1.55	48.0^{ab}±2.84	43.3^{a}±2.35
C18:2 n-6	10.5^{a}±0.16	12.5^{b}±0.20	10.1^{a}±0.99	9.20^{a}±0.79
C20:0	0.28^{a}±0.04	0.40^{c}±0.01	0.51^{d}±0.01	0.33^{b}±0.01
C20:1	0.87^{a}±0.03	1.29^{b}±0.07	0.87^{a}±0.00	1.35^{b}±0.06
C18:3 n-3	0.22^{b}±0.03	1.26^{a}±0.09	0.39^{c}±0.10	1.16^{a}±0.05
C18:3 n-6	0.21^{a}±0.01	0.28^{b}±0.00	0.51^{d}±0.04	0.37^{c}±0.02
C20:2	0.3^{7a}±0.01	0.53^{a}±0.00	0.53^{a}±0.04	0.64^{a}±0.32
C21:0	0.00^{a}±0.00	0.00^{a}±0.00	0.01^{a}±0.00	0.00^{a}±0.00
C20:4 n-6	1.29^{a}±0.08	1.81^{c}±0.10	1.39^{ab}±0.25	1.65^{bc}±0.14
C20:3 n-6	1.15^{b}±0.07	1.41^{c}±0.01	0.00^{a}±0.00	0.00^{a}±0.00
C20:3 n-3	0.10^{b}±0.00	0.16^{c}±0.01	0.00^{a}±0.00	0.00^{a}±0.00
C22:2	0.88^{a}±0.04	0.78^{a}±0.17	0.57^{a}±0.50	0.04^{b}±0.00
C20:5 n-3	0.05^{c}±0.00	0.04^{b}±0.00	0.01^{a}±0.00	0.02^{a}±0.01
C22:6 n-3	0.14^{a}±0.01	0.15^{a}±0.03	0.19^{a}±0.04	0.19^{a}±0.01

^{a,b,c,d} *In the same raw, different letters means that values are significantly different ($P < 0.05$)*

As it can be seen from the Table 1, most prevalent fatty acid was oleic acid (C18:1 n-9) ranging from from 43.30 % (LD, E group) to 49.75 % (SM, C group). Second one was palmitic acid, with similar presence in all examined groups and content from 24.65 % (LD, E group) to 28.71 % (LD, C group). As it is known, pork have relatively high proportion of linoleic acid (C18:2 n-6) (Wood *et al*., 2008) and in present study this was the third most common fatty acid ranging from 9.20 % (LD, E group) to 12.52 (SM, E group).

The content of alpha-linolenic acid (ALA, 18:3, n-3) varied from 0.22 % (SM, C group) to 1.26 % (SM, E group). This content was higher in experimental groups compared to the control groups for both muscles, for SM 5.7 times and for LD 2.97 times. The differences between ALA values for SM and LD from experimental group were not significant ($P > 0.05$), while these values differed significantly ($P < 0.05$) between same muscles from experimental and control groups, as well as between muscles from control group.

The content of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), total unsaturated fatty acids (UFA) and ratios between them of *M. Semimembranosus* (SM) and *M. longissimus dorsi* (LD) from pigs fed with standard (C) and diet enriched with omega-3 polyunsaturated fatty acids (E) is shown in Table 2.

The content of SFA, MUFA, PUFA and UFA was approximately the same in both muscle (SM and LD) and both of the tested groups (C and E). Content of SFA ranged from 34.16 % (SM, C group) to 39.25 % (LD, E group), of MUFA ranged from 45.84 % (SM, E group) to 50.95 % (SM, C group), of PUFA from 13.29 % (LD, E group) to 18.69 (SM, E group) and of UFA from 60.75 (LD, E group) to 65.84 (SM, C group).

An important parameter in meat fatty acid composition analysis is the ratio between PUFA and SFA. The average ratio of PUFA/SFA recommended by the British Department of Health is more than 0.45, and WHO/FAO experts have reported guidelines for a "balanced diet" in which suggested ratio of PUFA/SFA should be above 0.4 (Wood *et al.,* 2008; Wood *et al.,* 2003; HMSO, 1994).

There was significant ($P < 0.05$) difference in PUFA/SFA ratio between SM and LD, as well as between SM from control and experimental group. In SM PUFA/SFA ratio ranged between 0.44 (C group) and 0.54 (E group) what is above WHO/FAO recommended value in both groups, but higher in group fed with the addition of extruded flaxseed rich in polyunsaturated fatty acids. In LD mentioned ratio ranged between 0.34 (E group) and 0.37 (C group), what is lower than the recommended values in both groups.

Table 2. Content of saturated, monounsaturated, polyunsaturated, total unsaturated fatty acids and ratio between them

Fatty acid	% of fatty acid in total fatty acids			
	C	E	C	E
	SM		LD	
SFA[1]	34.16^{a}	35.20^{a}	36.62^{ab}	39.25^{b}
MUFA[2]	50.95^{b}	45.84^{a}	49.71^{ab}	47.46^{ab}
PUFA[3]	14.88^{a}	18.69^{b}	13.67^{a}	13.29^{a}
UFA[4]	65.84^{b}	64.80^{b}	63.38^{ab}	60.75^{b}
MUFA/SFA	1.49^{b}	1.30^{ab}	1.36^{ab}	1.21^{a}
PUFA/SFA	0.44^{b}	0.54^{c}	0.37^{a}	0.34^{a}
omega-3	0.51^{a}	1.62^{c}	0.59^{a}	1.37^{b}
omega-6	13.12^{b}	16.02^{c}	11.98^{ab}	11.23^{a}
omega-6/omega-3	25.60^{c}	9.89^{a}	20.95^{b}	8.16^{a}

^{a,b,c,d} *In the same raw, different letters means that values are significantly different ($P < 0.05$)*

[1] *– saturated fatty acids*

[2] *– mono unsaturated fatty acids*

[3] *– polyunsaturated fatty acids*

[4] *– total unsaturated fatty acids*

Content of omega-3 fatty acids ranged from 0.51 (C group) to 1.62 % (E group) in SM and from 0.59 (C group) to 1.37 % (E group) in LD. As it can be seen, the content of the omega-3 fatty acids was significantly ($P < 0.05$) higher in the experimental group compared to control group for both muscles. In SM omega-3 fatty acids content in the experimental group was 3.18 times higher than in control group, while in LD this ratio was 2.32.

The ratio of omega-6 and omega-3 fatty acids is also one of the very important indicators during fatty acid composition analysis. It has been estimated that the present Western diet is deficient in ω-3 fatty acids, with a ratio of ω-6 to ω-3 of 15-20/1, instead of 1/1 (Simopoulos, 2008, Ivanov *et al.,* 2010). Nutritional advice for today's ω-6/ω-3 ratio is less than 4 (Scollan *et al.,* 2006). In our study this ratio was higher than recommended and ranged from 8.16 (LD, E group) to 25.60 (SM, C group). But, both muscles (SM and LD) from experimental group had significantly ($P < 0.05$) lower ω-6/ω-3 ratio compared to muscles from control group; for SM 2.59 and for LD 2.57 times lower.

CONCLUSIONS

Based on the obtained results it can be concluded that the use of a well-balanced pig diet enriched with omega-3 polyunsaturated fatty acids resulted in the functional pork or pork with modified fatty acid composition, thus meat with a higher content of omega-3 fatty acids and better omega-6 and omega-3 ratio.

ACKNOWLEDGEMENTS

This study was financially supported by Ministry of Science and Technological Development, Republic of Serbia, project No III 46012.

REFERENCES

Calder P. C. (2009). Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale, Biochimie, vol. 91, 791–795.

Connor, W. E. (2000). Importance of n-3 fatty acids in health and disease. American Journal of Clinical Nutrition, 71, 171–175.

European Commission (2010). European Research Area, Food, Agriculture & Fisheries & Biotechnology. Functional Foods. Luxembourg: Publications Office of the European Union,

Folch, J., Lees, M., Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497-509.

Font-i-Furnols, M., Guerrero, L. (2014). Consumer preference, behavior and perception about meat and meat products: An overview. Meat Science, 98, 361–371.

Gebauer, S.K. , Psota, T.L., Harris, W.S., Kris-Etherton, P.M. (2006). n-3 Fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. American Journal of Clinical Nutrition, 83, 1526-1535.

Gu, Z., Suburu, J., Chen, H., Chen, Y. Q. (2013). Mechanisms of Omega-3 Polyunsaturated Fatty Acids in Prostate Cancer Prevention. BioMed Research International, Article ID 824563, 10 pages.

Gurr, M.I., Frayn, K.N., Harwood, J.L. (2002). Lipid Biochemistry, fifth ed. Blackwell Scientific, Oxford.

Harris W. S. (2007). International recommendations for consumption of long chain omega-3 fatty acids, Journal of Cardiovascular Medicine, 8, 50–52.

Hayat Z., Cherian G., Pasha T. N., Khattak F. M., Jabbar M. A. (2009). Effect of feeding flax and two types of antioxidants on egg production, egg quality, and lipid composition of eggs. Journal of Applied Poultry Research, 18, 541-551.

Henchion, M., McCarthy, M., Resconi, V. C., Troy, D. (2014). Meat consumption: Trends and quality matters. Meat Science, 98, 561–568.

Higgs, J. D. (2000). The changing nature of red meat: 20 years of improving nutritional quality. Trends in Food Science & Technology, 11, 85–95.

HMSO, UK Department of Health (1994). Nutritional aspects of cardiovascular disease. London, Report on Health and Social subject, 46, 37 – 46.

Ivanov, D., Lević, J., Sredanović, S. (2010). Fatty acid composition of various soybean products. Food and Feed Research, 2, 65-70.

Jakobsen, K. (1995). Possibilities of enriching meat with n-3 fatty acids. In: 2nd Dummerstorf Muscle-Workshop Muscle Growth and Meat Quality. (Ed. K. Ender). Rostock Germany, 17-19th May, 141-148.

Jiménez-Colmenero, F., Carballo, J., Cofrades, S. (2001). Healthier meat and meat products: Their role as functional foods. Meat Science, 59, 5–13.

Jiménez-Colmenero, F., Reig, M., Toldrá, F. (2006). New approaches for the development of functional meat products. In L. M. L. Nollet & F. Toldrá (Eds.), Advanced Technologies for Meat Processing, 275–308, Boca Raton Fl: CRC Press.

Lawrie, R. A., Ledward, D. A. (2006). Lawrie's Meat Science. Cambridge: Woodhead Publishing Limited and CRC Press LLC

McAfee, AJ., McSorley, EM., Cuskelly, GJ., Moss, BW., Wallace, JM. Bonham, MP., Fearon, AM. (2010). Red meat consumption: An overview of the risks and benefits. Meat Science, 84, 1–13.

Okanović, Đ., Ilić, N., Ivanov, D., Palić, D, Drobnjaković, R., Vukčević, Ć., Ikonić, P. (2010). Influence of linseed enriched diet on omega-3 fatty acids content in pork. Krmiva, 52, 225-231.

Rose, DP., Connolly, JM. (2002). Omega-3 fatty acids as cancer chemopreventive agents. Pharmacology and Therapeutics, 83, 217-244.

Simopoulos P. (2008). The importance of the omega 6/omega 3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med, 233, 674-88.

Simopoulos, AP. (1998). Overview of evolutionary aspects of omega 3 fatty acids in the diet. World review of nutrition and dietetics, 83, 1–11.

Simopoulos, P. (1999). Essential fatty acids in health and chronic disease. American Journal of Clinical Nutrition, 70, 560–569.

Simopoulos, P. (2002). The importance of the ratio of omega-6/omega-3 essential fattyacids, Biomedicine and Pharmacotherapy, 56, 365-379.

Siróa, I., Kápolna, E., Kápolna, B., Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. Appetite, 51, 456–467.

Sretenović, L., Pantelić, V., Novaković, Ž. (2009). Importance of utilization of omega-3 fatty acids in human and animal nutrition. Biotechnology in Animal Husbandry, 25, 439-449.

Toldrá, F., Reig, M. (2011). Innovations for healthier processed meats. Trends in Food Science & Technology, 22, 517–522.

Williamson, C. S., Foster, R. K., Stanner, S. A., Buttriss, J. (2005). Red meat in the diet. Nutrition Bulletin, 30, 323−355.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R, Richardson, R. I., Huges, S. I., Whittington, F. M. (2008). Fat deposition, fatty acid composition and meat quality: A review. Meat Science, 78, 343 –358.

Wood, J. D., Richardson, R. I., Nute, G. R., Fisher, A. V., Campo, M. M., Kasapidou, E., Sheard, P. R., Enser, M. (2003). Effect of fatty acids on meat quality: A review. Meat Science, 66, 21 – 32.

RELATIONSHIP BETWEEN FEED INGREDIENTS PROPERTIES AND PELLET QUALITY PREDICTIVE MODELS BASED ON PRODUCTION DATA

Mia Eeckhout[1], Sigrid Van Geyte[1], Patrick Gouwy[2], Sofie Landschoot[1]

[1]Faculty of Bio-engineering, Ghent University, Dept. applied biosciences,
Valentin Vaerwyckweg 1, 9000 Gent, Belgium
[2]Vanden Avenne Ooigem NV, Oostrozebeeksestraat 160, 8710 Ooigem, Belgium

*Corresponding author:
E-mail address: mia.eeckhout|@ugent.be

ABSTRACT

Low cost formulation of animal feed performed with linear programming (LP) selects feed ingredients on base of their availability, price setting and on certain limitations from a nutritional point of view. Volatile feed ingredients prices and restricted availability result in a large variability of feed compositions of which some of them cause problems at production level resulting in a decreasing pellet quality.
The input for this research was a data set of 101 different pig feed production batches. It seemed that the hardness and durability were highly correlated with some feed ingredient characteristics. The hardness and durability were significantly positively correlated with the water absorption index (WI, defined as the amount of water absorbed to the feed ingredients after a subsequent wetting and centrifugation step) (respectively R = 0.81 and R = 0.66) and were significantly negatively correlated with the amount of fat (respectively R = - 0.62 and R = - 0.57).
In order to find the most suited model for predicting the pellet quality, different modelling techniques were compared. To estimate the model performance the cross-validated R^2 and Mean Squared Error (MSE) were calculated. For the pellet hardness, stepwise linear regression resulted in a predictive model for hardness based on two parameters: fat percentage and WI with R^2 = 0.82 and a MSE of 0.09.
The pellet durability was more difficult to predict, resulting in a lower R^2 and a higher MSE for all models. Stepwise linear regression and lasso regression turned out to be the most suited modelling techniques to predict this variable. The final model was based on the components fat, WI, sugar and ADL with a cross-validated R^2 of 0.60 and a MSE of 0.12.
***Keywords**: pellet quality, water absorption index, pellet hardness, predictive model*

INTRODUCTION

Feed formula is based on Least Cost Formulation with the help of commercial available mathematical programming models. The input data for the software are on the one hand the nutritional requirements necessary to maintain good health and assure weight maintenance or gain; on the other hand, the analytical data for the feed ingredients available at production site and the real time feed ingredients price.
The restrictions mentioned are based on the presence of, for example, anti-nutritional factors affecting digestibility. Other restrictions are made on the basis of experience of the formulator for example the limitation on fat inclusion as the formulator knows this gives problems for pelleting. When all data are available, the computer calculates within certain limits the best formula with the best price (least cost). After all the aim of all feed producers is the production of a quality feed at a reasonable cost which is the key to successful animal production.
Although these programs are widely used and there is a lot of technological experience in the field of feed production, we see that often formulation does not take into account the necessary information to assure the quality of the end product as in the case of pelleted feed. In feed pelletizing the grounded feed ingredients are mixed, conditioned with steam to reach the process temperature and humidity and then pressed through a die to form pellets, which are cooled and dried afterwards (Behnke, 2006) The pellet quality seems to be important for

the feed intake. The pellet quality is quantified as pellet hardness and pellet durability. The methods used to determine the hardness and durability are more or less standardized (Payne, et al, 2001). Proper testing and evaluation of these quality parameters are used by feed mill operators to check if the pellet quality meets the requirements. If these parameters are below the limits, this may result in an increased production of fines during the post-production period. These fines result in a decreased feed intake, resulting in farmer complaints (Brue and Latshaw, 1981; Cutlip et al, 2008; Greenwood, Clark and Beyer, 2004; Hanke et al., 1972; Reece et al, 1985).
Pellet quality is affected by a lot of parameters as feed composition, particle size, conditioning method, including temperature and time, die choice and cooling. Keeping in mind that in a feed mill the technological parameters as particle size, conditioning method, die choice and cooling are kept constant for a specific feed (ex. poultry starter or grower feed) the feed composition in terms of feed ingredients selection gains attention (Briggs et al, 1999; Thomas et al 1996; Thomas et al, 1997).
Due to price fluctuations of the feed ingredients and the cost sensitivity of the feed and animal production, the output of least cost formulation may result in the sudden use of other feed ingredients often resulting in a clear effect at pelletizers' operator level as an effect on the pelletizers' performance quantified as an increase in energy consumption and/or a decrease in pellet quality. Although, theoretically, the operator should be able to change certain process parameters to adjust the process, this is often difficult to perform.
As a result of this one could say that LP formulation should have more feed ingredients restrictions based on the technological knowledge of a specific feed mill. A lot of researchers tried during the last decennia to predict pellet quality based on feed ingredients choice, sometimes expressed as the pelletizing ability of feed ingredients (Payne et al. 2001).
The aim of this study was to find a correlation between the inclusion percentage of feed ingredients and pellet quality based on the nutritional data and production data of pig feed in a feed production plant. This correlation could result in an acceptable prediction of the pellet quality change due to the variation in feed ingredients.

MATERIAL AND METHODS

Statistical data analysis

From a production period of six months in 2013, 101 different pig feed production batches were analyzed with SPSS statistics, a software packet for statistical analysis and modelling. This high-dimensional dataset contains a lot of feed ingredient characteristics that could be used in a predictive model. However, only a small number of variables are truly informative, while others are redundant. Furthermore, many of these variables are highly correlated. Therefore, identifying the truly informative variables is regarded as the first step in predictive modelling. An underfitted model excludes truly informative variables and may lead to severe estimation bias in model fitting, whereas an overfitted model includes the redundant uninformative variables, increases the estimation variance and hinders the model interpretation.
For all feeds, the percentage of feed ingredients was added to the dataset.
The nutritional data used in the dataset were starch, Acid Detergent Lignin (ADL), moisture, crude protein (TCP), fat, sugar, ash, Acid and Neutral Detergent Fiber (ADF and NDF), Crude Fiber (CF), hemicellulose (= NDF - AFD), Non starch polysaccharides (NSP), Digestible Non starch polysaccharides (DNSP).
Additionally, the response variables pellet quality, quantified as the pellet hardness (Kahl-method) and durability (Holmen tester) were added to the dataset.
Furthermore, a new parameter expressing the water absorption capability of the feed ingredients (called Water absorption Index or WI) was introduced. To determine the WI 1.00 gram of grounded product was brought into a Falcon tube after which the total mass was measured on an analytical scale (m_1). Then 20 ml of demineralized water was added. The tube was shaken thoroughly every 5 minutes during a total period of 30 minutes. After 30

minutes the falcon tube was placed in a centrifuge (Sigma 3-18K with 19776-H rotor) at 3000 rpm for 10 minutes. After centrifugation the water was completely decanted and the falcon tubes were weighed (m_2). The WI was calculated as WI = (m_2) - (m_1).
The WI of the feed mash was not determined, but calculated based on the WI of the feed ingredients and their inclusion percentage. This WI was called the Theoretical WI (WI_{the}) while the experimental one was called the WI_{exp}

$$(\text{WI Theoretical})\,(\text{g}) = \sum_{1}^{n} \text{WI experimental } \mathbf{1} * \text{inclusion percentage } \mathbf{1}$$

Where *n* is the number of feed ingredients. For liquids no WI was measured.
The determination of the Pearson correlation coefficient (R) is a measure of the linear relation between a predictor variable (x) and a response variable (y) which is calculated as:

$$R = \frac{\sum[(x_i - \bar{x})*(y_i - \bar{y})]}{\sqrt{\sum(x_i - \bar{x})^2 * \sum(y_i - \bar{y})^2}},$$

with x_i and y_i the values for a certain predictor or response and $\bar{x}$ and $\bar{y}$ the mean values of all x_i and yi values. In this study, x is used for the feed composition variables and y is either hardness, durability or feed composition variable. It is logical that the R value for similar x and y is equal to 1. All other relationships result in values between 1 and - 1. The more the R value reaches + the stronger the positive correlation. One important remark is that Pearson correlation coefficient is based on a supposed linear relationship between the variables.
In order to find the most suited model, different modelling techniques (multiple-stepwise) linear, lasso, ridge regression and regression trees) were compared. Lasso and rigde regression are regression methods that involve penalizing the absolute size of the regression coefficients. To estimate the model performance the cross-validated R^2 and Mean Squared Error (MSE) were calculated.

Pilot scale pelletizing experiment

For the experimental feed production a set of nine feed formula was made. Formula 1 was chosen as a reference (

Table 1). Feed ingredients were mixed in a vertical screw mixer to produce 100 kg of feed. The mash was conditioned at a conditioner outlet set point of 60°C. The products were pelletized on a Labor Monoroll press (Andritz) equipped with a 4 mm die with an effective channel length of 80 mm at a capacity of 300 Kgs/hour. The pellets were cooled in a forced air batch cooler (20 kg) at room temperature.
The WI of the pre-conditioned mash was determined experimentally as well as the WI of the different feed ingredients. The moisture content of the conditioned mash at conditioner outlet was determined by sampling the mash and over drying at 105°C for 4 hours. The pellet hardness was determined with the manual Kahl hardness tester and the durability with the Holmen tester with a 1 minute circulation time.

Table 1 Feed ingredient inclusion percentage for 9 different pig feed batches

	Inclusion percentage								
Barley	35.0	35.0	35.0	25.0	20.0	15.0	10.0	10.0	10.0
Corn	5.0	10.0	15.0		5.0	10.0	15.0	10.0	5.0
Wheat	22.0	17.0	12.0	35.0	35.0	35.0	30.0	35.0	40.0
Wheat tailings							2.0	2.0	2.0
Wheat gluten feed				10.0	7.0	7.0	7.5	10.0	12.5
Bakery by-products	6.5	6.5	6.5	3.0	6.0	5.0	3.0	3.0	3.0
Corn middling				3.0	3.0	3.0	3.0	2.0	1.0
Corn germ meal	1.5	1.5	1.5	1.5	1.5	1.5			
Rapeseed meal				3.0	5.0	6.0	12.5	10.0	7.5
Toasted soy bean	9.0	10.5	12.0	14.0	12.0	10.0			
Soy meal	15.0	13.5	12.0			2.0	3.0	4.0	5.0
Sunflour meal							4.0	4.0	4.0
Chalk	1.0	1.0	1.0	1.5	1.5	1.5	1.0	1.0	1.0
Beet root pulp							1.0	1.0	1.0
Fat-oil	0.5	1.0	1.5	1.5	1.5	2.5	2.5	3.0	3.5

RESULTS AND DISCUSSION

Correlations and predictive models

The Pearson' s correlation coefficients can be visualized in a heat map (Figure 1). The lighter the color of the map, the higher the correlation between the different parameters.
From the heat map we can conclude that, as expected, there is a strong positive relationship between the different fiber fractions i.e. the non-starch polysaccharides (NSP) and between the fiber fractions and the water absorption index WI as these fractions indeed are responsible for the water absorption. Non-starch polysaccharides refer to all carbohydrate fractions and types of dietary fiber, with the exception of lignin (ADL), either soluble or insoluble (Capitra, 2010). The hydration properties of NSP influence its water holding capacity and water binding capacity (Moms, 1992).
There is also a positive relation between the fiber fraction and the hardness and durability. As the NSP and the WI are related parameters the WI could be an interesting indicator to replace NSP and the effect on the hardness. On the other hand there is a rather negative effect between ADL and starch on the pellet quality and a negative correlation between those parameters and the WI.

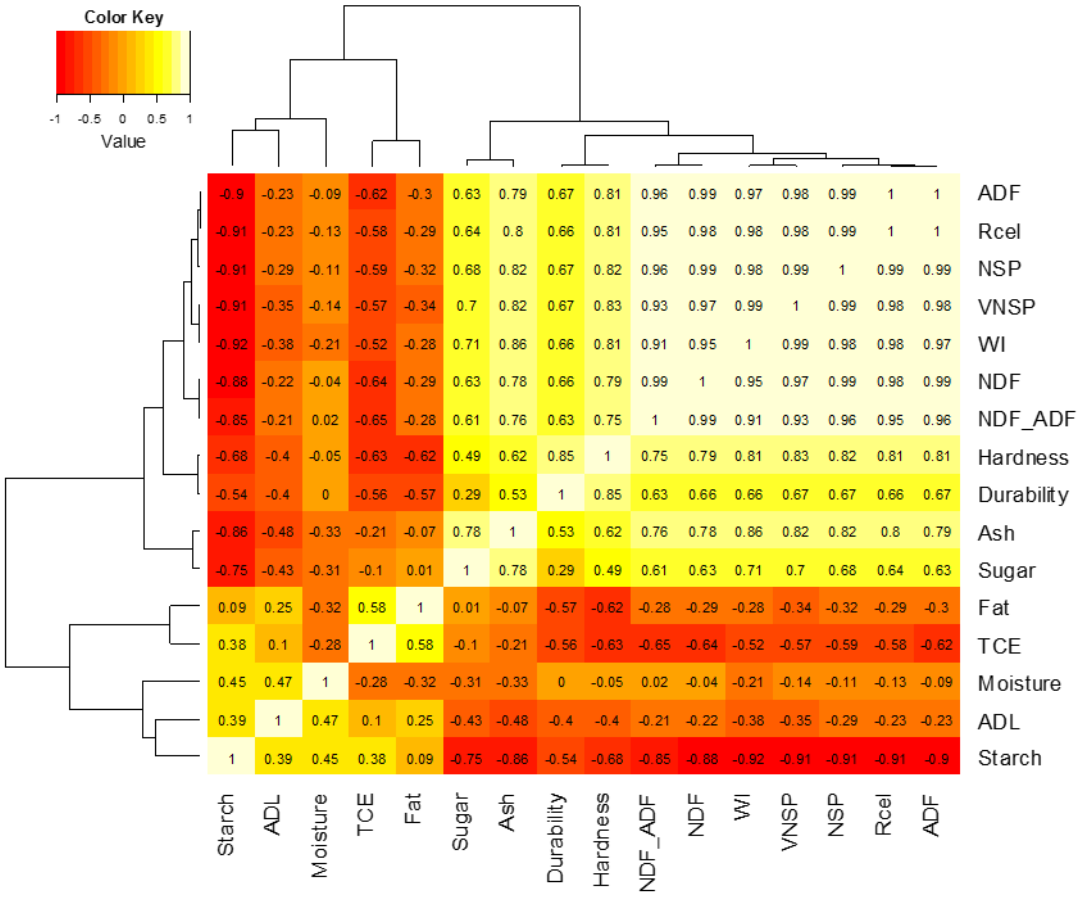

Figure 1. Heat map with Pearson's correlation coefficients for the feed data set

The first model (equation 1) derived by multiple linear regression contains all variables with exception of the six parameters related to the fiber fraction which were substituted by the WI. Although all modelling techniques resulted in comparable R^2 and MSE , the most suited model derived was for the pellet hardness, stepwise linear regression resulting in a predictive model for hardness based on two parameters: fat percentage and WI with $R^2 = 0.82$ and a MSE of 0.09 (equation 2).

KHD = 8.73 – 0.06 *TCE – 0.49*Fat + 0.005*starch + 0.06*sugar – 0.007*ash
– 0.07 *moisture – 0.08*ADL + 0.61*WI (eq 1)

KHD = 6.95 – 0.51*Fat + 0.65*WI (eq 2)

The correlation between the feed ingredients characteristics and the pellet durability were found to be much lower than for the pellet hardness, which makes the pellet durability more difficult to predict, resulting in a lower R^2 and a higher MSE for all models. Stepwise linear regression and lasso regression turned out to be the most suited modelling techniques to predict this variable. The final model was based on the components fat, WI, sugar and ADL with a cross-validated R^2 of 0.60 and a MSE of 0.12.

Feed pelletizing experiments

The pelleting temperatures varied between 56°C and 58°C. The mean increase in moisture content after conditioning was 2.35 ± 0.35 %. The WI_{exp} of the test mixtures showed a mean value of 2.8 g with an upper value of 2.89 and a lower limit of 2.7. The WI_{the} showing the same narrow range.

As the experiments were carried out on a pilot pelletizer with a different die and throughput hus resulting in a different overall hardness of the pellets, the experimental hardness was found to be much lower than the predicted one. Compared to the reference mixture (test 1) all mixtures showed a decrease in the experimental Kahl Hardness (KHD_{exp}) although the predicted value (KHD_{pred}) was much higher giving an extreme overshoot for Test 6 and Test 9. The correlation coefficient being 0.772 for the nine tests. Leaving out Test 9 results in an R of 0.877.

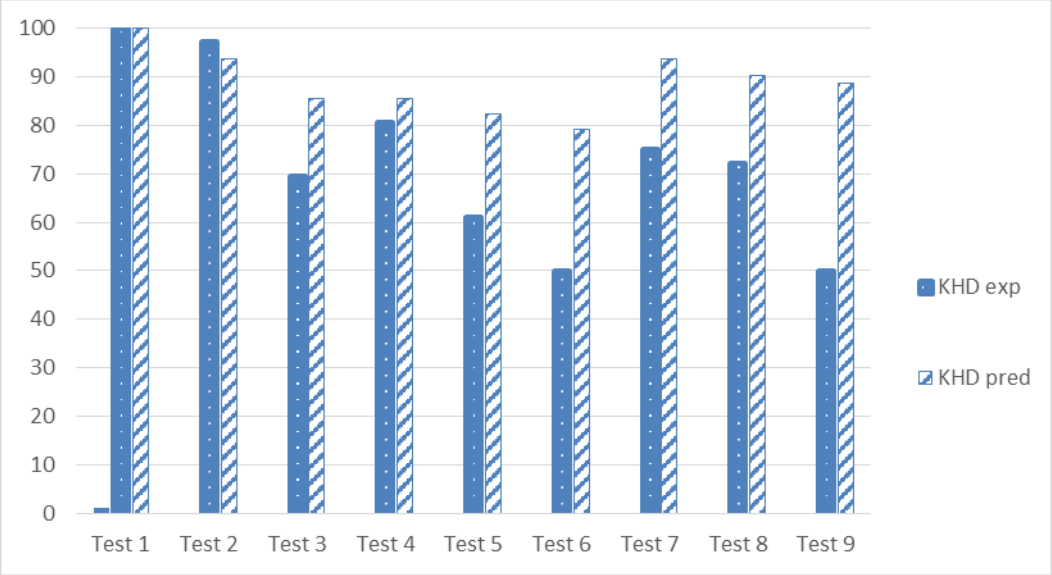

Figure 2. Relative experimental and predicted Kahl hardness (KHD_{exp} resp. KHD_{pred}) for nine experimental pig feed mixtures

For the Holmen durability the predicted durability ranged between 96.4 % (Test 1) and 95.6 % (Test 9), while the experimental showed an upper limit of 88.1 % (Test 1) and the lowest value 65.2 for Test 4.

CONCLUSIONS

Based on 100 different feed mixtures produced during a long production period a model for the prediction of the pellet quality has been calculated. The model was calculated using stepwise regression resulting in a predicted Kahl hardness based on the fat percentage and a theoretical WI of the feed mixtures. The Holmen durability showed less correlation and the best model was derived using more variables and a lower R^2 and larger MSE.
By performing a limited experiment on a pilot scale pelletizer the predicted value was compared to the experimental value for hardness and durability. The experiment showed the predicted decrease in quality is reflected a decrease of the experimental one. There is a moderate correlation between the two values. There is no correlation between de predicted durability and the experimental one.

REFERENCES

Behnke, K.C. 2006. The Art (Science) of Pelleting. In Technical Report Series: Feed Technology, 5-9. Singapore: American Soybean Assn. International Marketing Southeast Asia.

Briggs, J.L., D.E. Maier, B.A. Watkins, and K.C. Behnke. 1999. Effects of ingredients and processing parameters on pellet quality. Poult. Sci. 78: 1464-1471.

Brue, R.N. and J.D. Latshaw. 1981. Growth and energy retention of broilers as affected by pelleting and by density of the feed. Poult. Sci., 60: 1630.

Caprita R., Caprita A. and Calin J. 2010. Biochemical Aspects of Non-Starch Polysaccharides. Scientific Papers: Animal Science and Biotechnologies, 43(1) 368.

Cutlip, S.E., J.M. Hott, N.P. Buchanan, A.L. Rack, J.D. Latshaw, and J.S. Moritz. 2008. The Effect of Steam-Conditioning Practices on Pellet Quality and Growing Broiler Nutritional Value. J. Appl. Poult. Res. 17:249-261.

Greenwood, M.W., P.M. Clark, and R.S. Beyer. 2004. Effect of feed fines level on broilers fed two concentrations of dietary lysine from 14 to 30 days of age. Int. J. Poult. Sci., 3: 446-449.

Hanke, H. E., J. W. Rust, R. J. Mead, and L. E. Hansion. 1972. Influence and source of soybean protein, and of pelleting, on rate of gain and gain/feed of growing swine. J. Anim. Sci. 35:958-962.

Moms, E. R. 1992. Physico-chemical properties of food polysaccharides. In: Dietary Fibre - A Component of Food, Nutritional Function in Health and Disease, T. F. Schweizer, C. A. Edwards (eds.), Springer-Verlag, London, 1992, pp. 41-56.

Payne, J., W. Rattink, T. Smith, T. Winowiski, G. Dearsledy, and L. Strøm. 2001. The Pelleting Handbook. Norway: Borregaard Lignotech.

Reece, F.N., B.D. Lott, and J.W. Deaton. 1985. The effects of hammer mill screen size on ground corn particle size, pellet durability, and broiler performance. Poult. Sci. 65: 1257-1261.

Stark, C. R. 2009. Effect of die thickness and pellet mill throughput on pellet quality. Abstr. T89. Southern Poultry Science Society Meeting.

Thomas, M. and A.F.B. van der Poel. 1996. Physical quality of pelleted animal feed 1. Criteria for pellet quality. Animal Feed Sci. and Tech. 61(1-4): 89-112.

Thomas, M., D.J. van Zuilichem, A.F.B. van der Poel. 1997. Physical quality of pelleted animal feed. 2. contribution of processes and its conditions. Animal Feed Sci. and Tech. 64(2-4): 173-192.

Wood, J. F. 1987. The functional properties of feed feed ingredients and the effect on the production and quality of feed pellets. Anim. Feed Sci. Technol. 18:1.

THE IMPACT OF INCLUSION OF ORGANIC ACIDS AND PHYTOGENIC ADDITIVES INTO DIET ON ECONOMIC RESULTS OF BROILERS PRODUCTION

Đorđe Okanović[1], Vladislav Zekić[2], Radmilo Čolović[1], Predrag Ikonić[1], Tatjana Tasić[1]

[1]University of Novi Sad, Insitute of Food technology, Bulevar cara Lazara 1, Novi Sad, Serbia
[2]University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad, Serbia

*Corresponding author:
E-mail address: djordje.okanovic@fins.uns.ac.rs

ABSTRACT

Production of broilers practically presents the most intensive branch of the animal husbandry. The reproduction process is relatively short, which enables faster turnover of the engaged resources. The influence of phytogenic additives and organic acids addition in broiler diet on the economic results of the production was examined in present paper.
It was concluded that the diet enriched with phyitogenic additives had beneficial effect on the majority of monitored economic parameters compared to diet with organic acids: total benefit (2,396.32 : 1,457.20 €), benefit per broiler (0.29 : 0.18 €), economy (1.10 : 1.06) and profitability (3.33 : 2.02%).
Keywords: *broilers production, phytogenic additives, organic acids, cost*

INTRODUCTION

Animal husbandry presents the most intensive branch of agriculture and has multiple significances, for both producers and consumers. The increase in the production of the meat, milk, eggs, among others is the foundation for the improvement of the nutrition structure of the population with highly valuable animal proteins (Tica et al., 2009). Broilers fattening, as a final phase in the production line of chicken meat within the modern intensive poultry production based on industrial principles, is the fastest and the most rational way of producing poultry meat (Andersen et al., 2005; Okanović et al., 2011). In a floor system and on a deep mat, mainly heavy type proveniences are fattened, that are characterized by intensive growth, good food utilization, excellent carcass conformation, wide and long breast muscles and short leg muscles (Perić et al 2010; Džinić et al., 2011).
The extensive use of antibiotics in animal production has increased the risk of development of resistance in human and animal pathogens (Witte, 1998). Because of concerns about potential negative human health consequences, as well as satisfying consumer demand for a food chain free of drugs, use of antibiotics as growth promoters is forbidden in the European Community (Council Regulation, 1998).
The ban on antibiotic usage in Europe lead to increasing researchers interest in finding alternatives to antibiotics for poultry production such as enzymes, organic or inorganic acids, herbs, essential oils, immunostimulators, microelements, probiotics and prebiotics (Lević et al, 2007; Vekić et al., 2010; Okanović et al., 2014). The beneficial effects of garlic (*Allium sativum L*.) on human and animal organism, which result from its antimicrobial, antioxidative and antihypertensive properties, have been known from ancient times (Stanaćev et al., 2010). Phytogenic additives are a group of natural growth promoters, derived from herbs, spices or other plants (Ristić et al., 2008; Hasmeni et al., 2010). In recent years, the use of phytogenic compounds increased because their potential role as natural alternatives to antibiotic as growth promoters in animal nutrition (Mountzouris et al., 2011). Phytogenic additives enhance broiler performance and health, and have beneficial effects on: feed intake, broiler growth performances, digestive function, feed conversion ratio, gut health parameters, body weight gain. Also, they may have a beneficial effect on carcass and stored meat quality and production economy (Cross et al., 2007).
Organic acids have been used for a long time as food additives to prevent food deterioration and extend the shelf life of perishable food ingredients (Ricke, 2003). The supplementation of

organic acids in the diet of broilers enhanced nutrient utilization, growth, and feed efficiency (Denil et al., 2003), and can prevent bacterial and fungal growth (Heres et al., 2003). Organic acid supplementation have been reported to decrease colonization of pathogens and production of toxic metabolites, improve digestibility of protein and minerals like Ca, P, Mg and Zn. Dietary supplementation of organic acids increases the body weight and feed conversion ratio in broiler chicken (Skinner et al., 1991), as well as increased growth performance, reduced diseases and management problems (Ao et al., 2009).
Thus, the aim of this study was to determine the influence of phytogenic additives and organic acids on the economic aspects of broiler production.

MATERIAL AND METHODS

The experiment was carried on 16480 broilers, provenience ROSS. Broilers were divided in two groups (E1 and E2) and fed under the same conditions. Broilers were fed with commercial feed mixture with addition of: phytogenic additives (Biomin P.E.P) (E1) and organic acids (Biotronic forte) (E2). Fattening of broilers lasted 40 days. Food and water were provided *ad libitum* in the floor fattening system.
Calculation of the expense for feed mixtures has been derived according to the standard of expenses for the preparation of animal food, based on market prices of certain kinds of food and experience normative. The expense for other material has been calculated according to the expenditure made on the observed farm and market prices. Investments into buildings and the equipment have been calculated based on standard investments in objects and equipment. Expenses of the buildings and equipment amortisation have been derived based on the assumed lifetime of the utilized means (Marko et al., 1998). Expenses for salaries were calculated in accordance to realized expenses. Expenses for the energy consumption were calculated on the basis of realized expenditure of the electrical power and fuel. Apart from that, the calculation includes expenses of veterinary and selection services. Calculation of the income was based on clarification of total income from the above mentioned production, whereby the financial result presents the income from the overall production (Andrić, 1998).

RESULTS AND DISCUSSION

During the analysis of the observed production, production results have been followed closely. The main production indicators are given in the table 1.
If we take a look at the Table 1 one can draw a conclusion about the almost minor expenditure of food in group fed a diet with the addition of phytogenic additives. Also, the same group has bigger number of broilers and average carcass mass at the end of the fattening and achieves higher value of the production.
The analysis of the overall economic indicators of the observed production starts from the assessed investment into the farm, in other words investment into the buildings for breading with the following equipment. According to the assessment, the investment into the buildings and equipment amount is up to 370,800.00 €.
Calculation of other expenses (energy, work and additional materials) of the production, has been derived per turn**.** In the distribution, they were divided proportionally to the starting number of chickens, e.g. two equal groups. In accordance to the derived calculations, establishing of the total expense and the price of the fattening chicken has been derived. Calculation of these indicators is presented in the Table 2.

Table 1. Basic production results for two groups of broilers

Category	E1			E2		
		Cost, €	Total, €		Cost, €	Total €
Chickens	8 240	0.40	3 296.00	8 240	0,40	3 296.00
Produced broilers	7 850			7 844		
Produced broilers, kg	19 232.50	1.43	27 502.48	18 590.30	1,43	26 584.13
kg/broiler	2,45			2.37		
Total feed consumption,kg	33 460			33 540		
Starter, kg	5 100	0.4084	2 082.84	5 100	0.4080	2 080.80
Grover, kg	19 300	0.4031	7 779.83	19 380	0.4028	7 806.26
Finisher, kg	9 060	0.3678	3 332.27	9 060	0.3674	3 328.64
Feed conversion ratio, kg/kg	1.740			1.804		
Average daily gain, kg/day	0.061			0.059		

Table 2. Calculation of the total expenses, €

Category	**E1**	**E2**
Feed expense,	16,490,94	16,511,71
Amortization	3,708,00	3,708,00
Salaries	2,158,10	2,158,10
Expenses for energy	1,925,12	1,925,12
Expenses of other and additional materials	824	824
Total	25,106,16	25,126,93

Calculation of the income includes the incomes that farm achieves and it is based on the sale of broilers. On sale, the price that was achieved was 1.43 €/kg. In the accordance to the number of fattened broilers and the sale price, the calculation of the total income was made. Total income was 27,502.48 € for the group fed a diet with addition of phytogenic additives and 26,584.13 € for the group fed a diet with addition of organic acids. Based on that the benefit was calculated as a difference between the income and expense (table 3).
The benefit achieved per one turn amounts up to 2,396.32 € for the group fed with the diet with addition of phytogenic additives and 1,457.20 € for the group fed a diet with addition of organic acids, e.g. 0.29 € and 0.18 € per produced broiler.
If the realized benefit is calculated per kilogram of produced broilers, we get 0.12 €/kg for the group fed a diet with addition of phytogenic additives, and 0.08 €/kg for the group fed a diet with addition of organic acids. The economy calculated from the ratio of total income and total expenses is 1.10 for the group fed a diet with phytogenic additives and 1.06 for the group fed a diet with addition of organic acids.

Table 3. Calculation of the benefit, economy and profitability, €

Total expenses	25,106,16	25,126,93
Total income	27,502,48	26,584,13
Benefit	2,396,32	1,457,20
Benefit per broiler	0.29	0.18
Benefit per kg	0.12	0.08
Economy	1.10	1.06
Profitability, %	3.33	2.02

Profitability of the production is obtained from the ratio of realized benefit and total investment. Total investment includes investments into the buildings and equipment and investment into the unfinished production within the fattening. Thereat, in total five or six turns are foreseen per year. Binding of means in the form of debits has not been calculated; instead the calculation has been derived with an assumption of advance payment. Profitability of the overall production process in the observed case is not difficult to calculate, since the production is concentrated and monophase. Realized profitability for the group fed mixture diet with addition of phytogenic additives was 3.33% and 2.02% for the group fed a diet with additional of organic acids.

CONCLUSIONS

Lucrativeness and profitability of the production are the most important principles and the basis of rational business in the market economy, which is more and more becoming an imperative for our production too. Economic results of the production of broilers have in the paper been analysed in the paper and what can be concluded is the following:
The profit achieved per one turn amounts to 2,396.32 € for the group fed a diet with addition of phytogenic additives and 1,457.20 € for the group fed a diet with addition of organic acids, e.g. 0.29 € and 0.18 € per produced broiler.
The economy calculated from the ratio of total income and total expenses is 1.10 for the group fed a diet with phytogenic additives and 1.06 for the group fed a diet with addition of organic acids.
Detailed analysis of economic indicators shows good profitability of the production. The realised profitability of the production makes only 3.33% for the group fed a diet with addition of phytogenic additives and 2.02% for the group fed a diet with addition of organic acids.
Regardless of that, all presented indicators point out the justifiability of the usage of phytogenic additives in the preparation of feed.

ACKNOWLEDGEMENTS

The research has been conducted as a part of the Project III 46012 ("The research of modern biotechnological methods in feed production in order to increase competitiveness, food quality and safety") financed by the Ministry of Education, Science and Technological Development of Republic of Serbia, and Project "Medical plant as alternative to antibiotics as growth promoters in the sustainable production of healthy and safe broiler meat", financed by the Provincial Secretariat for Science and Technological Development, Autonomous Province of Vojvodina, No:114-451-4685-/2013-03.

REFERENCES

Andersen, H.J., Oksbjerg, N., Young, J.F., Therkildsen, M.(2005). Feeding and meat quality – a future approach, Meat Science, 70, 543-554.

Andrić, J. (1998). „Troškovi i kalkulacije u poljoprivrednoj proizvodnji", Poljoprivredni fakultet – Zemun, Beograd.

Ao, T., Cantor, A. H., Pescatore, A. J., Ford, M. J., Pierce, J. L. & Dawson. K. A. (2009). Effect of enzyme supplementation and acidification of diets on nutrient digestibility and growth performance of broiler chicks. Poultry Science 88: 111-117.

Council Regulation 98/2821/CEE (1998). Official Gazette of European Community, No. L 351, Luxembourg: Office for Official Publications of the European Communities,29 December.

Cross, D.E., Mcdevith, R.M., Hillman, K., Acamovic, T. (2007). The effect of herbs and their associated essential oils on performance, digestibilities and gut microflora in chickens 7 to 28 d of age, British Poultry Science, 4, 496–506.

Denil, M., Okan, F., & Celik, K. (2003). Effect of dietary probiotic. organic acid and antibiotic supplementation to diets on broiler performance and carcass yield. Pakistan Journal of Nutrition 2: 89-91.

Džinić, N., Okanović, Đ., Jokanović, M., Tasić, T., Tomović, V., Ikonić, P., Filipovic, S. (2011). Carssas and breast meat quality of broilers feed with extruded corn, Biotechnology in animal husbandry, vol 27, 4, 1697-1703.

Hashemi, Sr., Davoodi H. (2010). Phytogenics as new class of feed additive in poultry industry, Journal of Animal and Veterinary, 9, 2295- 2304.

Heres, L., Engel, B., Van Knapen, F., De Jong, M. C., Wagenaar, J. A. & Urlings, H. A. (2003). Fermented liquid feed reduces susceptibility of broilers for Salmonella enteritidis. Poultry Science 82: 603–611.

Lević J., Sredanović S., Đuragić O., Jakić D., Lević Lj., Pavkov S. (2007). New feed additives based on phytogenics and acidifiers in animal nutrition, Biotechnology in Animal Husbandry, 23, 527 – 534.

Marko J., Jovanović M., Tica N. (1998). "Kalkulacije u poljoprivredi", Poljoprivredni fakultet, Novi Sad.

Mountzouris, K.C., Paraskevas, V., Tsirtsikos, P., Palamidi, I., Steiner, T., Schatzmayr, G., Fegeros K. (2011). Assessment of a phytogenic feed additive effect on broiler growth performance, nutrient digestibility and caecal microflora composition, Animal Feed Science and Technology, 168, 223–231.

Okanović, Đ., Zekić, V., Filipović, S., Tica, N. (2011). Influence of the feeding system on the economic results in the production of fatty poultry. Macedonian Journal of Animal Science, vol. 1, No 1. pp 245-250.

Okanović Đ., Karović D., Lilić S., Radović V., Filipović S. (2014). Mineral adsorbents in broilers nutrition – efects on yield and meat quality *Food and Feed Researces*, 41(1), 63-70.

Perić, L., Milošević, N., Žikić, D., Bjedov, S., Cvetković, D., Markov, S., Mohnl, M. Steiner T. : Effects of (2010). Probiotic and phytogenic products on performance, gut morphology and cecal microflora of broiler chickens, Archiv Tierzucht, 53, 350-359.

Ricke, S. C. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science 82: 632–639.

Ristić, M., Damme, K., Freudenreich, P. (2005). Uticaj fitogenih dodataka stočnoj hrani na kvalitet mesa živine. Tehnologija mesa, 46, 1-2, 51-55.

Skinner, J. T., Izat, A. L. & Waldroup, P. W. (2005). Fumaric acid enhances performance of broiler chickens. Poultry Science 70: 1444-1447.

Stanaćev, V., Milošević, N., Kovčin, S., Plavša, N., Bjedov, S., Stanaćev, V. (2010). Effect of different garlic levels in broiler chicken food on production parameters. Savremena poljoprivreda, **59**(1-2), pp. 86-91.

Tica, N., Okanović, Đ., Zekić, V., Filipović, S. (2009). The efect of extruded corn on the economic results of broilers production. Food Processngs, Quality and Safety, 36, 3-4, 59-64.

Vekić, M., Perić, L., Đukić-Stojčić, M., Jotanović, S., Bjedov, S. *И* Mohnl, M. (2010). Effect of probiotics on production and quality of eggs in early phase of laying cycle. Savremena poljoprivreda, **59**(3-4), pp. 325-331.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture, Science, 279, 996–997.

LEFTOVER BREAD AS A RAW MATERIAL IN ANIMAL FEED

Zvonko Nježić*, Jelena Filipović, Olivera Šimurina, Jasmina Živković, Milenko Košutić

Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author
E-mail address: zvonko.njezic@fins.uns.ac.rs

ABSTRACT

Food processing industry is one of the most important industries worldwide. Concerning that population is constantly increasing, lack of food is a growing problem throughout the world as well as in Serbia. There is an evident problem of withdrawn bread in Serbia, in terms of quantity, environmental and health aspects. According to the survey conducted in previous research, there is a significant amount of leftover bread in Serbia. Leftover bread does not represent only an environmental problem, but also potentially valuable raw material for human food and animal feed. Increase in food production for humans and animals can be achieved by use of new technologies in agro-food industry. Nowadays, there are many different ways for thermal processing of cereals: toasting, extrusion, hydrothermal processing, micronization, microwave treatment. The most frequently used processes in Serbia are extrusion and hydrothermal processing. Large quantities of food waste are advisable for utilization as animal feed. One of the possible utilizations of withdrawn bread in food and animal feed was described in this study. Additionally, the directions for leftover bread reutilization have been presented in this paper.
Keywords: *withdrawn bread, waste revalorization, health and safety food*

INTRODUCTION

Bread is the most common product made from grains and also a staple food in many countries. It is made by mixing flour and water and certain secondary materials, which after fermentation, shaping and baking gives the final product. After taking them out of the oven, loaves have to be cooled down, while over the time they become dry and lose their freshness (Marić et al, 2009; Nježić et al., 2010a). One of the habits of population of Serbia is to discard bread which is not consumed in one single day. This raises the question of quantity and quality of leftover bread and its safety for further use. One of the possible utilizations of withdrawn bread in food and animal feed was described in this study. The aim of this paper is to propose the directions for re-use of leftover bread.

MATERIAL AND METHODS

The survey was conducted through questionnaires and interviews of consumers and workers of small bakeries, large industrial bakeries, large retail chains, restaurants, city utility companies on the territory of Vojvodina. Statistical and computer processing of survey data was done using the software package of agency "SMARTLINE" from Novi Sad.
In order to avoid loss of information, finding the finest links and information on non-parametric sizes, the scaling of the data in contingency tables was applied. This process is based on the frequency so that each class is assigned with the real number. Statistical analysis included multi-analysis of variance (MANOVA), discriminative analysis, and other parametric procedures and methods (Data Analysis Software System, 2006). Also, Roy's test, Pearson's Contingency coefficient (c), and multiple correlation coefficient (R) were calculated. Assessment of the statistical significance was done using the P value, where P-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSION

There are no official statistical data on the quantities of leftover and discarded bread in Serbia, whereas in England it is about 0.1% (http://www.magrama.gob.es). According to the survey conducted by the Institute of Food Technology in Novi Sad (FINS) the amount of leftover bread in Serbia ranges from 5-10% of the produced quantity (Nježić et al., 2010b). In Table 1, chemical composition of different types of bread and bakery products is shown. According to the attitudes of consumers key factors for the quality of bakery products (attributes influencing the quality) are:

- Raw materials quality,
- Technology
- Sanitation and
- Baker's knowledge and experiences.

Table 1. Chemical composition of different types of bread

Product type	Flour		Chemical composition, %						Bruto calories per 100 g product
	Rye	Wheat	Water	Proteins	Fat	Carbo-hydrates	Cellulose	Ash	
Rye bread	Whole grain	-	45.5	5.9	1.1	44.5	1.0	2.0	217
Pan bread	Refined	-	42.0	6.2	0.8	49.0	0.5	1.6	233
Wheat bread	-	Whole-grain	43.1	7.0	1.6	45.1	1.2	2.0	228
Wheat Loaf	-	*Wheat flour type 1100	35.8	9.0	1.3	51.4	0.7	1.8	260
Bun	-	**Wheat flour type 850	31.7	9.4	2.0	55.1	0.2	1.6	283
Puff pastry	-	White	35.0	7.6	5.5	50.7	0.2	1.0	290
Toasted rye bread	Whole-grain	-	11.0	11.4	1.4	70.6	1.9	3.7	349

**Ash content: 1.05-1.15% **Ash content 0.80-0.90%*

It was found that from one million of loaves (weight 0.5 kg per loaf) produced in Vojvodina, a minimum of 50,000 loaves are discarded what makes total of 25 tons of bread per day. Leftover bread without the presence of harmful substances can be a very attractive raw material for further processing in food or feed industry instead of being a major health and environmental problem. However, stale bread which is 1-5 days old can be problematic from microbiological safety point of view. Mycotoxins from unsafe leftover bread pose a serious health threat for the part of population who collect food from street dustbins.
A significant amount of leftover bread comes from:

- Households (it is thrown away together with other waste or in separate bags),
- Large bakeries-undelivered products or products returned from supermarkets,
- City dumps where large quantities of bread ends up mixed and contaminated by other waste.

According to the survey carried out among small bakeries, leftover bread does not represent a problem for them. This is due to the fact that small bakeries quite rationally and

economically organize their production. According to them, the basic requirement is to produce safe fresh bread, and the remaining bread will serve as a raw material for further processing.

Special attention should be given to good hygiene practice, which is mandatory for bakery manufacturers. Implementation of HACCP improves the situation on the market, as those who do not fulfil hygienic requirements, will not be allowed to manufacture, whereas those who do, will have a regular quarterly audits.

Leftover bread can be used, under appropriately hygienic conditions, as animal feed, biofuel, as well as in bio fermentation processes for biogas production. Stale bread, which does not contain mycotoxins or other metabolites of molds, can be processed by extrusion and then used as a protein-energy ingredient in animal feed for fish and pets. Possibilities for further processing of leftover bread and its reuse, as proposed by the authors, are shown in Figure 1.

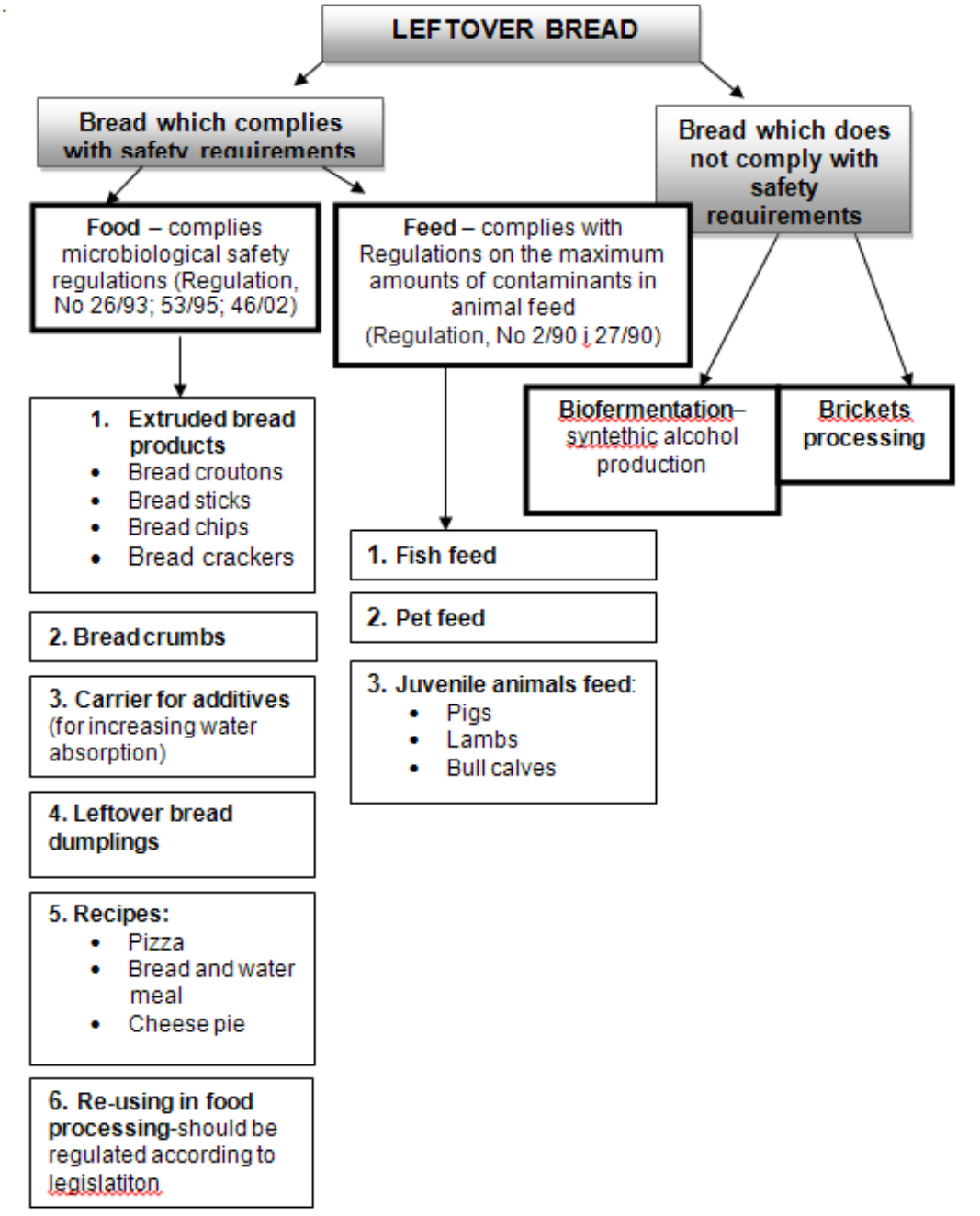

Figure 1. Possibility of processing leftover bread and its re-use

Recommendations for physico-chemical, microbiological and toxicological analysis of raw materials, semi-manufactured and final products

In relation to leftover bread valorisation in feed processing and biobricket production, the following parameters should be analysed:

a) Chemical analysis of fresh and old bread:

– Basic chemical composition, starch content, sugars content, heavy metals content (lead, cadmium, mercury and arsenic).

b) Physical characteristics as test weight.

c) Microbiological and toxicological analysis according to regulation about quantities of pesticides, metals and metalloids and other toxic substances, chemotherapeutics, anabolic and other substances that can be found in food (Official Journal SFRJ 5/92, 11/92 and 32/2002).

d) Energy values (calorimetric bomb).

Quality analysis of final products of leftover bread processing

Quality analysis of following parameters should be done on brickets and animal feed:

a) Brickets:

- Moisture content;
- Test weight;
- Total energy values (calometric bomb).

b) Human food and animal feed:

- Basic chemical composition: starch content, sugars content, heavy metals content (lead, cadmium, mercury and arsenic);
- Physical characteristics as test weight.
- Microbiological and toxicological analysis according to regulation about quantities of pesticides, metals and metalloids and other toxic substances, chemotherapeutics, anabolic and other substances that can be found in food (Official Journal SFRJ 5/92, 11/92 i 32/2002);
- Energy values (calorimetric bomb).

CONCLUSION

There is a significant amount of leftover bread in Serbia, which represents an important ecological, health and safety issue. There is no organized way for collection and distribution of leftover bread for possible further processing. As a result, there is evident increase of environment pollution and other related problems in the food chain.

The main causes of the high percentage of leftover bread are:

- Bread is still a social category,
- Relatively low price of bread,
- Relatively low level of bread quality,
- Habits of consumers, who buy more bread than they usually consume,
- Serbian people do not have habit to re-use leftover bread in their diet.

Leftover bread can be further reutilized as a raw material for human food or animal feed. Previously, its safety has to be assured and appropriate hydrothermal pre-processing has to be performed in order proceed with further processing.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III No.46005) and project of the Provincial Secretariat for Science and Technological Development, AP Vojvodina.

REFERENCES

Marić, A., Arsovski, S., Mastilović, J. (2009). Contribution to the improvment of products quality in baking industry, proceedings of 3rd international quality conference, Kragujevac, Serbia, 21-28.

Nježić, Z., Psodorov, Đ., Živković, J., Palić, D., Cvetković, B. (2010a). Possibility of Using Leftover Bread as Safe and Quality High-quality Food and Feed, Proceedings of 4th ICQM, Kragujevac, Serbia, 165-169.

Data Analysis Software System) (2006). v.7.1., Stat-Soft, Inc., USA (www.statsoft.com).

Nježić, Z., Živković, J., Cvetković, B. (2010b). Possibilities of Utilization of Leftover Bread. Chemical Industry & Chemical Engineering Quarterly, 16 (4) 399−403.

Regulation on microbiological food safety in trade (Official Journal SRJ No. 26/93, 53/95 i 46/02).

Regulations on the maximum amounts of contaminants in animal feed (Official Journal SRJ No. 2/90 and 27/90).

Program to reduce food loss and waste and maximise the value of discarded food "MORE FOOD, LESS WASTE" STRATEGY", Madrid, 2013 (http://www.magrama.gob.es/imagenes/es/Librode%20la%20estrategia_ENG_baja_tcm7-286285.pdf)

EFFECTS OF HEAT PROCESSING ON NUTRITIVE VALUE OF WHOLE COTTONSEED

Yavuz Gurbuz*, O.Latif Demir, M.Furkan Yigit, Erkan Karatas

Depertmant of of Animal Nutrition, Faculty of Agriculture, University of Kahramanmaras Sutcu Imam, Avsar, 9046100 Kahramanmaras, Turkiye

*Corresponding author:
E-mail address: yavuz@ksu.edu.tr

ABSTRACT

Whole cottonseed (WCS) is a by-product of the cotton industry in the world. A lot of cotton seed varieties have been measured. Some WCS are characterized with more gossypol and a higher amount of the (−) isomer than the other WCS. Monogastric animals are sensitive to the toxic effects of gossypol, whereas ruminants are to some extent more resistant. The sign of gossypol toxicity experiential was a raise in erythrocyte fragility (EF) for cows delivery high dietary free gossypol. Pre- and postpartum eating of free gossypol might harm some aspects of calf skeletal development and vitamin metabolism, but long-term performance of cows and calves was not affected. The probable effect of WCS fat on reducing microbial activity and potential gossypol toxicity may bind the amount of WCS that can be supplemented to high-yielding dairy cows. Heat treatment in WSC, may aid in provided that more unaffected WCS fat and CP to the small intestine, and decreasing ruminal CP degradability and increasing post ruminal digestibility. Heat treatment may also be a helpful tool in reducing free gossypol in WCS. Heat treatment may make possible an increase in the supplementation rate of WCS for ruminant rations. Processing of WCS includes heat treatment, extrusion, cracking, expanding, expelling, pelleting, and chemicals treatment. WCS processing may help in providing more unaffected WCS fat and CP to the small intestine, and declining ruminal CP degradability and rising post ruminal digestibility. Heat treatment may also be a helpful device in reducing free gossypol in WCS. Thus, heat treatment may make possible an increase in the supplementation rate of WCS for ruminant rations.
***Keywords**: Ruminant Nutrition; Whole cottonseed; Whole Cotton Seed Processing, Heat Treatment*

INTRODUCTION

Cotton is produced in over 60 countries ın world. Most production of cotton was grown in India, Syria, China, Turkey, the United States, Tanzania and Uganda, respectively. Turkish cotton area and production are 420,000 hectares and 630,000 metric tons (MT) in market year (MY) 2014. However cotton seed production is around 930 MT/year in Turkey. Whole Cottonseed is the seed produced as a by-product of the cotton ginning process. This feedstuff is feeding value for average and high-yielding ruminant (Chandler, 1992). Whole cottonseed is high in fat, protein and fibre and can be fed to cattle, sheep and other ruminants. WCS is high in fat 20 % crude protein CP 23% and neutral detergent fiber NDF, 44 %. This is showed in its high energy content (9.2 MJ of net energy for ruminant nutrition. However, WCS's composition suggests some restrictions because of content in gossipol in its free procedure in animal diets.

Whole cottonseed can be used as a supplement for ruminants or alternatively for cottonseed meal and oil production. The seed can be fed as a supplement to pasture or as an component in feedlot rations. The digestion of whole cottonseed in the rumen causes a slow release of nutrients. With the slow release of nutrients, a component of protein is bypass protein. This bypass protein will be offered for direct absorption by the animal. As the seed contains little starch and the fat provides the energy. Whole cottonseed contains the plant pigment, gossypol. The free form of gossypol is toxic to animals. Cattle, sheep and other ruminants are able to detoxify the free gossypol, up to a certain level. Whole cottonseed typically contains between 0.6% - 1.6% free gossypol. Whole cottonseed provides a energy,

protein, and fiber relative to other feed ingredients. WCS contains 22.5% CP, 38.8% ADF, 47.2% NDF, and 17.8% ether extract in United States (Calhoun et al. 1995).

Table 1. Composition of whole cottonseed in comparision to other feedstuffs,[ab]

Composition	Whole	Cottonseed Meal	Corn
Crude protein, %	28	30-46	9.8
TDN,[c] %	90	75	90
Crude fat, %	22	3	4
Crude fiber, %	20	13	2
NDF,[d]%	52	29	11
ADF,[e] %	30	18	3
Calcium, %	0.2	0.2	<0.1
Phosphorus, %	0.8	1.2	0.3
NEm,[f] Mcal/kg	2.24	1.79	2.44
NEg,[g] Mcal/kg	1.55	1.16	1.55

a- Dry matter basis; typical dry matter concentrations - 92%, 90%, and 90% for whole cottonseed, cottonseed meal, and corn, respectively; b - Source: NRC (2000), Nutrient Requirements of Beef Cattle, National Research Council, Washington DC, USA; c- Total digestible nutrientsd Neutral detergent fiber e Acid detergent fiber f Net energy for maintenance g Net energy for weight gain

The high concentration of energy from oil and fiber from the lint and hull are mainly valuable when formulating rations for high producing ruminant. Whole cottonseed has been made known to be an efficient source of fiber for maintaining rumen function (Clark and Armentano 1993). But the lint causes usage problems in most industrialized handling systems, limiting its use by many feed manufacturers and ruminant producers. Most of the protein in WCS is considered degradable (76.5% of CP) (Arieli et.al.1989); then, treatment to decrease the degradability would improve its value in rations fed to high producing ruminant production. Extruding, pelleting, and roasting have been used to reduce protein degradability, but the production comeback of lactating dairy cows to processed WCS has not been consistent. Several processing methods have been used to improve the handling characteristics of WCS including acid delinting, mechanical delinting, extruding, pelleting, and coating. The effects of processing on nutrient digestion and metabolism vary among processing methods, contributing to the practical variation in animal response. Howover, Processing may also change the concentration, form, and effects of gossypol.

Effects of Processing on Gossypol

Gossypol ($C_30H_{30}O_8$), which is originating throughout the cotton plant, is a naturally occurring toxin. It is a yellow, polyphenolic aldehyde compound, which is present in the highest concentrations in WCS pigment glands (Blauwiekel et al., 1997). Gossypol exists in WCS in both free and bound forms. Most gossypol originate in WCS is in the free form, but some becomes bound due to the heat, moisture and pressure associated with WCS meal extrusion, and other types of WCS processing (Mena et al., 2001; Calhoun, 1995). The bound form of gossypol is well thought-out to be nontoxic to ruminants although. it has been recommended that some bound gossypol (BG) from processed WCS or CS meal may be converted to free gossypol (FG) (Mena et al., 2001; Noftsger et al., 2000; Blauwiekel et al., 1997). Gossypol exists as a mixture of (+) and (−) stereoisomers, with the (−) isomer having the higher biological activity (Joseph et al., 1986). Gossypol free form is toxic to monogastric animals (Alford et al., 1996). Nonruminant animals are sensitive to the toxic effects of gossypol, whereas ruminants are rather more resistant than others. Signs of gossypol toxicosis in nonruminants, preruminants and male ruminants are similar and include labored breathing, dyspnea, decreased growth rate, and anorexia (Randel et al., 1992).

Toxicity has been linked with free gossypol concentrations, but free gossypol intake is not always correlated with toxicity (Calhoun 1995). The negative isomer appears to have the greatest biological activity and is dependable for many of the negative effects. Gossypol that escapes ruminal digestion is more available for absorption than that released during ruminal digestion. Cotton varieties vary in total and free concentrations and isomeric forms of gossypol. Grinding or cracking WCS before feeding has increased plasma gossypol concentrations 30% (Mena et.al. 1997). Processing methods involving heat treatment that crack the seed may decrease free gossypol concentrations in WCS and plasma gossypol concentrations. Whereas processing methods that do not crack the seed may raise the bioavailability of gossypol (Bernard and Calhoun. 1997). The concentration of total and free gossypol was reduced when a mix of WCS (71%) and soybean meal (29%) was roasted and pelleted compared with WCS or RCS. However, plasma concentrations of total gossypol and both negative and positive isomers were significantly higher when RCS was fed to lactating dairy cows at 15% of the ration DM.

Table 2 Effect of processing on gossypol in whole cotton seed and plasma gossypol (Bernard 1999).

	WCS	**RCS**	**PCS**	**ECS**
In seed %				
Total	0,69	0,63	0,24	0,36
Free	0,65	0,52	0,03	0,05
Plasma Yg/mL				
Positif isomer	0,63	0,86	0,53	0,26
Negatif İsomer	0,78	0,99	0,60	0,31
Total	1,41	1,84	1,13	0,56

WCS: Whole Cotton seed; PCS: Roastated and pelletted blend of WSC and soybean meal Cotton Seed; RCS: Roasted Cotton Seed; ECS: Exruted blend of WSC and Soybean meal

Plasma gossypol concentrations were least for ECS, but WCS only represented 50% of the DM provided by ECS (15% of ration DM). These results are consistent with the results of a big pasture study reported by Wedegaertner and Lalor (1997) in which plasma gossypol concentrations were higher when RCS were fed compared with WCS. The amount of heat and heating time required to raise the bioavailability of gossypol are not well-known. However, the conditions used to dry the gelatinized starch coating useful to WCS to get better its handling quality do not come out to alter gossypol bioavailability (Bernard 1999). The little raise in plasma gossypol concentrations practical when WCS + 5S was fed was due to differences in gossypol intake and is of little biological significance. The amount that processing decreases free gossypol concentrations or increases its bioavailability depends on processing temperature, pressure, and time (Calhoun 1995), but the effect of these variables on gossypol concentrations and bioavailability are not well know.

Concentrations of plasma gossypol (PG) reached a raised 28–35 d on treatment, they were highest in cows receiving a diet with high TG and FG. Erythrocyte fragility (EF) differed between treatments and increased with increasing FG intake. PG returned to insignificant concentrations 28 d after removal of cottonseed products from the high TG and FG diet. Urea N increased in diets upper in TG and FG. Feeding high TG and FG (1894 mg/kg TG and 960 mg/kg FG for 84 d) enlarged PG concentrations and EF and resulted in small changes in blood metabolites and enzymes. Indicators of liver, kidney, and muscle cell viability suggest that the higher amounts of gossypol consumed in this study had only small effects on those tissues in dairy cows.

Mena et al. (2001) feeding 15% or 13.5% WCS, Prieto et al. (2003) feeding 12.8% Pima WCS and Santos et al. (2002) feeding 10% WCS to lactating cows, no harmful impact on lactation performance were observed. Gadberry et al. (2005) feeding more than 19% WCS diet to beef steers, DM intake and animal performance were not transformed. In heifers fed

WCS (0, 150 and 300 g/kg) for 430 d, EF was upper for the 300 g/kg diet on days 230 and 430 (Colin-Negrete et al., 1996).

Methods of processing of WSC

Heat treatment has been suggested to reduce ruminal degradability of protein, fairly by blocking reactive sites for proteolytic enzymes and partly by reducing protein solubility (Broderick and Craig 1980; Mabjeesh et al., 1999). Roasting of WCS may reduce in vivo rumen ammonia stage (Pena et al., 1986) when WCS comprises 400 g/kg of the dietary DM. Feeding heated WCS is associated with decreased blood urea levels which may reflect decreased ruminal degradation, as well as higher biological worth AA profile (Roseler et al., 1993). While heat treatment achieved its principle of decreasing ruminal CP degradability and increasing post ruminal digestibility (Mabjeesh et al., 1999). Optimal heat input for WCS would be existed the arithmetical product of heat-treatment period (in h) and treatment temperature above 130 °C (in °C) was 45 °C/h (Arieli et al., 1989). Although WCS was the smallest amount sensitive to heating as compared with almond hulls, safflower seeds and alfalfa, acid detergent insoluble nitrogen (ADIN) in WCS increased with time of heating exposure.
Dry-heating reasoned a linear reduce of ammonia concentration in vitro as heating increased from 140 °C to 180 °C and as display time increased from 20 to 120 min. Dry-heating WCS at 155 °C for 20 min resulted in declined in vitro ammonia production (Smith and Vosloo, 1994). In situ protein degradation was decreased under these conditions from 54 to 30%. Stutts et al. (1988) confirmed the potential of WCS extrusion to decrease ruminal degradation of its protein. The helpfulness of this process was apprised by measuring CP solubility, in vitro ammonia production and in situ CP appearance. The in situ method was finished to be more expressive, because it provides both rate and extent of vanishe. Protein solubility in KOH has been recomended as another good in vitro assay for detecting decreased protein quality due to over-processing of oil-seed meals.

Delinting of WSC

Delinting involves taken away the lint from WCS with acid or by cutting the lint off mechanically. Removing the lint raises the energy and protein concentration by about 10% when compared with WCS that is not delinted (NRC 1989). Acid delinted and Pima WCS, a naturally delinted WCS, propose fewer dietary energy than WCS or mechanically delinted WCS because of an improved rate of passage of whole seed and reduced ether extract digestibility (Coppock, 1985; Sullivan 1982). Nutrient digestibility of mechanically delinted WCS is like to that of WCS (Alfonso 1986) and was informed to kept high milk yield in a field study (Kutchens and Chalupa 1986). Milk production and composition were same for cows fed equal amounts of whole, delinted, or coated WCS in a recent controlled study) (Moore 1998).

Table 3. Effecets Of Fed Processed Cotton Seed On Lactating Dairy Cows(Bernand 1999)

	WSC	RSC	PSC	ESC
DMI, kg/d	20,5	20,2	20,7	19,8
Milk, kg/g	27,3	27	27,6	28,3
Fat, %	3,72	3,67	3,41	3,1
Protein, %	3,09	3,10	3,02	3,04
Change in BW, kg	-5,4	-4,6	3,1	2,9

WCS - Whole Cotton seed; PCS- roastated and pelletted blend of wcs and soybean mealCotton Seed; Roasted Cotton Seed; ECS- exruted blend of WSC and Soybean meal

Pelleting of WSC

Pelleting WCS reduces ruminal protein degradation and to become better handling (Bernard and Amos 1985). Ruminal ammonia concentrations were decresed, and flow of N to the abomasum was higher, for pelleted WCS compared with natural WCS; fiber digestibility was not affected by pelleting. Cows fed diets containing pelleted WCS produced 1.7 kg/d more milk, but the difference was not significant. Sullivan and Richardson (Sullivan and Richardson 1982) did not detect any difference in milk production or composition when pelleted WCS were fed compared with WCS. Inadequate amounts of pelleted WCS are formed because of the cost and mechanical problems associated with pelleting. Processing of WCS may furthermore decrease the chances of gossypol toxicity. High temperature prefers the formation of stable bonds between gossypol and other molecules. Bound gossypol is generally well thought-out to physiologically inactive (Randel et al., 1992). Pelleting and adding together iron sulfate are means of decreasing the toxicity of gossypol in cottonseed products (Barraza et al., 1991). Cottonseed may also be decontaminated by ammonia treatment (Rogers and Poore, 1995). But expanded-expelled cottonseed didn't decrease levels of total plasma gossypol and plasma levels of the negative isomer of gossypol (Noftsger et al., 2000). Heat treatment of WCS is usually expected at dropping protein degradability in the rumen and increasing the amount of WCS protein flood into the intestine. It can be finished that an additional beneficial effect of such a treatment is a decrease in the negative effects of gossypol.

Roasting of WSC

Roasting WCS degres ruminal ammonia concentrations and protein degradability but increases the concentration of ADIN (Arieli et.al. 1989; Pena et al. 1986) observed a reduce in ruminal N solubility from 79 to 37% of total N when WCS was roasted. In vitro ammonia production reduced linearly as the temperature used to roast WCS improved from 140 to 180 ^{0}C and exposure time greater than before from 20 to 120 min. Roasting WCS also affects the digestion of other nutrients. In situ DM appearance reduced as the heating temperature improved above 140 ^{0}C (Tagari 1986). Ruminal ADF digestion was lower for RCS than for WCS, which might have been due to higher ADIN concentrations in RCS (23). Pires et al. (1997) observed lower ruminal digestibility of OM and NDF and biohydrogenation of fatty acids with RCS compared with WCS, but grinding RCS improved total tract digestibility of OM and NDF. Total tract digestibility of DM was lesser, but total fatty acid digestibility was higher, for RCS than for WCS (Bernard and Calhoun. 1997).
These results advise that roasting may negatively affect ruminal fiber digestibility partially because of an raise in the formation of Malliard reaction compounds, but the impact appears to be minimal because bacterial protein synthesis is not affected by RCS in the diet (Pena et.al 1986). The special effects of RCS in the diets of lactating dairy cows have been examined in several trials (Bernard and Calhoun. 1997; Mabjeesh et al., 1999, Mohamed 1988; Pire 1997; Wedegaertner and Lalor 1997). As cited by Wedegaertner and Lalor (1997), experimental an increase in milk yield with RCS compared with WCS; however, no differences were reported in the other studies. Dry matter intake and milk composition were not altered and averaged 21.9 kg DM/d, 3.56% fat, and 3.02% protein for the six trials. These results point out that roasting WCS can get better milk yield, presumably through better amino acid flow to the small intestine. These results also hold the best conditions for roasting WCS as outlined by Hsu et al. (1993). Although roasting improves protein use, it does not get better the handling characteristics of WCS. The possibility of combining roasting with other processes that would increase handling has not been explored.

Extrusion of WSC

Extruding WCS has also been indicate to reduce protein solubility and degradability. Ruminal ammonia concentrations reduced (Pena et.al. 1986) or remained unchanged (Stuts et. al.

1988) for cows fed extruded WCS (EWCS) compared with WCS. Extruding WCS does not show to revise total VFA production but did decrease molar concentrations of propionate, isobutyrate, and isovalerate compared with WCS (Pena et.al. 1986). Apparent digestion of OM and ADF in the rumen was not affected. Some researchers also reported greater quantities of nonammonia N (20%) and amino acids (17%) flowing to, and amino acids (25%) weakening from, the small intestine for EWCS than for WCS. Total tract apparent digestibility of OM and total fatty acids was higher, and digestibility of ADF and NDF tended to be higher, when lactating dairy cows were fed a diet containing accessible extruded blend of WCS and whole soybeans compared with WCS (Bernard and Calhoun. 1997). Milk yield was higher, particularly for high producing cows, but milk protein proportion was lower, though yield was like, for cows fed ECS than for those fed soybean meal (McCoy 1992). Milk yield was not diverse, but milk fat percentage and yield were lower, for cows fed ECS than for cows fed WCS or RCS (Bernard and Calhoun. 1997). However, cows fed the ECS gained BW compared with cows fed WCS, resulting in increased efficiency of dietary energy utilization. Both roasting and extrusion can decrease protein degradation in the rumen and improve amino acid flow to the small intestine. The conditions used for roasting or extruding affect the degree to which protein is protected from ruminal digestion. The better protein utilization observed by Pena et al. (1986) with roasting compared with extrusion was partially due to greater control of the roasting process compared with extrusion. Improvements in processing have been made since the early research was conducted, and additional research is needed to identify the best situation for extruding WCS. Extrusion at temperatures between 131 and 156 °C degreased WCS CP degradability in situ from 80% to 67– 46%, and CP solubility in warm water from 33% to 25– 26%. Pelleting of WCS also resulted in upper abomasal CP healing in steers, which was related with a reduce in rumen ammonia (Bernard and Amos, 1985).

CONCLUSIONS

WCS is a byproduct of the cotton-fiber industry, and it is not a typical feedstuff. There are different varieties of CS Some sotton seed more gossypol and a higher proportion of the (−) isomer than some CS. Monogastric animals are mainly sensitive to the toxic effects of FG, while ruminants are somewhat more resistant, particularly for female. Concentrations of PG and its negative isomer were directly proportional to FG intake in dairy cows. The mark of gossypol toxicity observed was an raise in EF for cows acceptance high dietary FG. Pre-and postpartum consumption of FG might damage some aspects of calf skeletal development and vitamin metabolism, but long-term performance of cows and calves was not affected. Generally, feeding a diet containing higher FG could result in small changes in blood metabolites, antioxidant vitamin and indicators of liver, kidney, and muscle cell viability, but not significantly different. If cottonseed suitably heat treated may improve the nutritional value. In addition, gossypol in cottonseed damage may be reduced. Thus, cottonseed much more efficient and can be use in animal feeding ruminants.

REFERENCES

Alfonso, A. A., M. Ojowi, J. E. Trei, and A. J. Kutches. 1986. Comparative digestibility of whole, delinted, and treated cottonseed. J. Dairy Sci. 69 (Suppl. 1):216. (Abs.).

Alford, B.B., G.U. Liepa, and A.N. Vanbeber. 1996. Cotton seed protein: What does the future hold? Plant Foods for Hum. Nutr. 49:1-11.

Arieli, A., Aharoni, Y., Tagari, H., Mabjeesh, S., Zamwel, S., 1996. The effect of heating and NaOH treatmentof non-linted whole cottonseeds on their nutritive value. J. Anim. Feed Sci. 5, 215–223.

Arieli, A., A. Ben-Moshe, S. Zamwel, and H. Tagari. 1989. In situ evaluation of the ruminal and intestinal digestibility of heat-treated whole cottonseed. J. Dairy Sci. 72:1228.

Barraza, M. L., C. E. Coppock, K. N. Brooks, D. L. Wilks, R. G. Saunders, and G. W. Latimer, Jr. 1991. Iron sulfate and feed pelleting to detoxify free gossypol in cottonseed diets for dairy cattle. J. Dairy Sci. 74:

Bernard, J. K., and H. E. Amos. 1985. Influence of pelleting whole cottonseed on ration digestibility and milk production and composition. J. Dairy Sci. 68:3255.

Bernard, J. K., and M. C. Calhoun. 1997. Response of lactating dairy cows to mechanically processed whole cottonseed. J. Dairy Sci. 80:2062.

Bernard, J.K. 1999. Effect of Processing Whole Cottonseed on Nutrient Digestion, Performance of Lactating Dairy Cows, and Gossypol Bioavailability *The professionel Animal Scientist December 1999 vol. 15 no. 4 224-229.*

Blauwiekel, R., S. Xu, J.H. Harrison, K.A. Loney, R.E. Riley and M.C. Calhoun. 1997. Effect of whole cottonseed, gossypol and ruminally protected lysine supplementation on milk yield and composition. J. Dairy Sci. 80:1358-1365.

Broderick, G. A., and W. M. Craig. 1980. Effect of heat-treatment on ruminal degradation and escape, and intestinal digestibility of cottonseed meal protein. J. Nutr. 110:2381–2389.

Calhoun, M.C. 1995. Assessing the gossypol status of cattle fed cotton feed products. Proc. 13 Pacific Northwest Animal Nutrition Conference. pp. 147A-158A. Portland.

Calhoun, M.C., S.W. Kuhlmann and B.C. Baldwin, Jr. 1995. Cotton feed product composition and gossypol availability and toxicity. Pages 125-145 *in* Proc. National Invitational 18 Symposium on Alternative Feeds for Dairy and Beef Cattle, St. Louis, MO.

Chandler, P., 1992. Whole cottonseed has economic feeding value for dairy cattle. Feedstuffs 64 _53., 10–11.

Clark, P. W., and L. E. Armentano. 1993. Effectiveness of neutral detergent fiber in whole cottonseed and dried distillers grains compared with alfalfa haylage. J. Dairy Sci. 76:2644.

Colin-Negrete, J. ; Kiesling, H. E. ; Ross, T. T. ; Smith, J. F., 1996. Effect of whole cottonseed on serum constituents, fragility of erythrocyte cells, and reproduction of growing Holstein heifers. J. Dairy Sci., 79 (11): 2016–2023

Coppock, C. E., J. R. Moya, J. W. West, D. H. Nave, J. M. LaBore, and C. E. Gates. 1985. Effect of lint on whole cottonseed passage and digestibility and diet choice on intake of whole cottonseed by Holstein cows. J. Dairy Sci. 68:1198.

Gadberry, M. S., Beck, P. A., Kellogg, D. W., & Gunter, S. A. (2005). Digestion characteristics and growth of steers fed a corn-grain based supplement compared to a de-oiled rice bran plus cottonseed supplement with or without extrusion processing. *Animal Feed Science and Technology, 118*(3-4)*,* 267-277.

Hsu, J. T., T. R. Dhiman, and L. D. Satter. 1993. Chemical, in vitro, and in vivo evaluation of roasted cottonseed. J. Dairy Sci. 76 (Suppl. 1):205. (Abs.).

Mohamed, O. E., L. D. Satter, R. R. Grummer, and F. R. Ehle. 1988. Influence of dietary cottonseed and soybean on milk production and composition. J. Dairy Sci. 71:2677.

Joseph, A. E., S. A. Matlin, and P. Knox, 1986. Cytotoxicity of enantiomers of gossypol. Br. J. Cancer 54:511–513.

Kutchens, A. J., and W. Chalupa. 1986. Lactational response to fuzzy and mechanically delinted whole cottonseed. J. Dairy Sci. 69 (Suppl. 1):215. (Abs.).

Mabjeesh, S. J., I. Bruckental, and A. Arieli. 1999. Heat-treated whole cottonseed: Effect of dietary protein concentration on the performance and amino acid utilization by the mammary gland of dairy cows. J. Dairy Res. 66:9–22.

McCoy, G. C., T. H. Klusmeyer, and M. J. Hutjens. 1992. Feeding extruded whole cottonseed and soybeans to dairy cows. In Illinois Dairy Report. p 65. Univ. of Illinois, Urbana.

Mena, H. J., T. Huber, M. C. Calhoun, and M. Tarazon. 1997. Effects of unprocessed and cracked upland and Pima cottonseed on blood gossypol levels and performance of Holstein steers. J. Dairy Sci. 80 (Suppl. 1):186. (Abs.).

Moore, D. N., J. E. Tomlinson, B. L. Clark, R. B. Moore, M. E. Boyd, and W. J. Ricks. 1998. Evaluation of whole cottonseed (fuzzy, mechanically delinted and starch coated) as an ingredient in the diet of high-producing dairy cows. J. Dairy Sci. 81 (Suppl. 1) :1187. (Abs.).

Mena, H., J.E.P. Santos, J.T. Huber, J.M. Simas, M. Tarazon and M.C. Calhoun. 2001. The effects of feeding varying amounts of gossypol from whole cottonseed and cottonseed meal in lactating dairy cows. J. Dairy Sci. 84: 2231-2239.

National Research Council, 2000. Nutrient Requirements of Dairy Cattle. 8th rev. edn. Natl. Acad. Sci., Washington, DC, 157 pp.

Noftsger, S. M.; Hopkins, B. A.; Diaz, D. E.; Brownie, C.; Whitlow, L. W., 2000. Effect of whole and expanded-expelled cottonseed on milk yield and blood gossypol. J. Dairy Sci., 83 (11): 2539–2547.

NRC. 1989. Nutrient Requirements of Dairy Cattle. (6th Rev. Ed.). National Academy Press, Washington, DC.

Pena, F., H. Tagari, and L. D. Satter. 1986. The effect of heat treatment of whole cottonseed on site and extent of protein digestion in dairy cows. J. Anim. Sci. 62:1423–1433.

Pires, A. V., M. L. Eastridge, J. L. Firkins, and Y. C. Lin. 1997. Effects of heat treatment and physical processing of cottonseed on nutrient digestibility and production performance by lactating cows. J. Dairy Sci. 80:1685.

Randel, R. D.; Chase, C. C. Jr. ; Wyse, S. J., 1992. Effects of gossypol and cottonseed products on reproduction of mammals. J. Anim. Sci., 70 (5): 1628-1638.

Rogers, G. M., and M. H. Poore. 1995. Optimal feeding management of gossypol-containing diets for beef cattle. Vet. Med. 90:994−1005.

Roseler, D. K., J. D. Ferguson, C. J. Sniffen, and J. Herrema. 1993. Dietary protein degradability effects on plasma and milk urea nitrogen and milk nonprotein nitrogen in Holstein cows. J. Dairy Sci. 76:525–534.

Santos, J.E.P., M. Villasenor, E.J. DePeters, P.H. Robinson and B.C. Baldwin, Jr. 2002. Type of cottonseed and level of gossypol in diets of lactating dairy cows: Effects on lactation performance and plasma gossypol. J. Dairy Sci. 85:1491-1501.

Smıth, W.A. & Vosloo, L.P., 1994.E ffect of whole cottonseedh, eat treated whole cottonseed and whole cottonseed plus lanolin on milk production and composition. S. A.li J. Anim. lci.24,45.

Stutts, J. A. ; Nipper, W. A. ; Adkinson, R. W. ; Chandler, J. E. ; Achacoso, A. S., 1988. Protein solubility, *in vitro* ammonia concentration and in situ disappearance of extruded whole cottonseed and other protein sources. J. Dairy Sci., 71: 3323-3333.

Sullivan, B. F., and C. R. Richardson. 1982. Dry matter digestibility, rumen variables, milk composition and production in dairy cows fed whole or processed cottonseed. J. Dairy Sci. 65 (Suppl. 1):225. (Abs.).

Tagari, H., F. Pena, and L. D. Satter. 1986. Protein degradation by rumen microbes of heat-treated whole cottonseed. J. Anim. Sci. 62:1732.

Wedegaertner, T. C., and W. F. Lalor. 1997. Roasted cottonseed for dairy cows – research review. In Proc. Beltwide Cotton Conf. p 1602. Natl. Cotton Counc. Am., Memphis, TN.

CIP – Каталогизација у публикацији
Библиотека Матице српске, Нови Сад

636.084 / .087 (082)

INTERNATIONAL Symposium Feed Technology (16 ; 2014 ; Novi Sad)

Proceedings [Elektronski izvor] / XVI International Symposium Feed Technology, 28-30. 10. 2014., Novi Sad ; [editor Bojana Kokić]. – Novi Sad : Institute of Food Technology, 2014. – 1 elektronski optički izvor (CD-ROM) : tekst, slika ; 12 cm

Nasl. sa naslovnog ekrana. - Tiraž 350. – Bibliografija uz svaki rad

ISBN 978-86-7994-044-5

а) Домаће животиње – Исхрана – Зборници
б) Сточна храна – Технологија – Зборници
COBISS.SR-ID 290532615