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MYCOTOXINS IN GRAINS AND FEED – CONTAMINATION AND TOXIC EFFECT IN ANIMALS[1]

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Review paper

Abstract: Mycotoxins are secondary metabolites of moulds that represent a significant food safety issue and pose a risk to health and wellbeing of humans and animals, having a negative impact on economies all over the world. They can cause intoxication in animals and affect the less productive performance and nutritional value of animal feed. Also, mycotoxins may contaminate different types of food through directly and indirectly way and cause negative health effects in humans. To estimate the potential of their harmful effect it is necessary to know the terms of mycotoxins occurrence and frequency, toxicity and biotransformation in different animal species and humans, possibility of contamination prevention, and their inactivation or reduction in the case when the incidence is inevitable. The relatively small number of comparable data on the incidence of mycotoxins in feed from European countries show the necessity of systematic control and the application of validated analytical methods in their determination. This paper gives the overview of incidence of grains and feed contamination with most important and common mycotoxins and the evidenced toxic effects in animals.

Keywords: mycotoxins, grains, feed, contamination, mycotoxicoses, Europe

Introduction

Grains that are highly represented in human and animal diet, as also in industrial food and feed, may become contaminated by moulds that produce mycotoxins. So far, about 400 different mycotoxins were described as produced mostly by moulds from genera of *Aspergillus*, *Penicillium*, *Fusarium* and *Trichotecium*, among which a smaller number is associated with the occurrence of acute or chronic intoxications. It is very difficult to unique systematize them,

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because of their different chemical structure, biochemical pathway, origin and biological effects but most important are aflatoxin B_1, ochratoxin A, zearalenone, deoxynivalenol, fumonisin B_1 and T-2 toxin *(CAST, 2003; Bryden, 2012)*.

Grains such as maize, wheat, barley and oat, that balancing the nutrition by virtue of providing a low-fat diet, has a number of advantages. At the same time, grains are a common source of mycotoxins which under favourable conditions may produce mycotoxins before and/or during harvest, handling, shipment and storage. Conditions favourable to mould growth and mycotoxin production, such as extreme weather conditions (temperature and humidity), grain damage, presence of insects and poor storage of grain and feed, cause the inevitable occurrence of mycotoxins in a large number of samples and intoxications in larger scale. The Food and Agriculture Organization (FAO) estimated that 25% of the world crops are contaminated with mycotoxins as generally stable substances resistant to the different methods of food processing *(Pleadin et al., 2014a)*.

Contaminated feed represents the main source of mycotoxin infestation of farm animals, which get to be contaminated through parasites living on plants even prior to harvesting or on stored harvested crops *(Gareis and Wolff, 2000)*. As fodder, cereals and seeds used for feeding are inevitably in contact with yeasts and filamentous fungi, and contamination of these raw materials frequently occurs already in the field. Contamination can also occur during harvesting, transport and storage of cereals and their products, as well as due to post-harvest mishandling that can lead to rapid feed spoilage. Due to possible occurrence of mycotoxins in natural grains that are used in the production of food and feed, there is a possibility of their entrance into human food chain. Literature data show that products of animal origin, such as meat and meat products, may also contribute to the entry of mycotoxins in food, whether as a result of indirect transmission from domestic animals, which are used for the production of food of animal origin, exposed to contaminated feed materials and compounds (carryover effect), but also through the mixture of spices used in their production *(Pleadin et al., 2013; Perši et al., 2014)* or direct contamination with moulds which under certain conditions can produce mycotoxins *(Gareis and Wolff, 2000; Pleadin et al., 2015a,b)*.

This paper gives an overview of the mycotoxin contamination of grains and feed and observed toxic effects with special focus in animals for those that are considered as most important and common occurred in the European countries in the last decades.

Toxic effects and natural occurrence

Mycotoxicosis, the disease resulting from exposure to a mycotoxin, may be manifested as acute to chronic, and ranges from rapid death to tumor formation. More occult disease may occur when the mycotoxin interferes with immune

processes, rendering the patient more susceptible to infectious diseases. The general effects of mycotoxins on health and productivity of animals are dependent on dose, and generally the young of a species is more susceptible to the effects of mycotoxins than adults are *(CAST, 2003)*. Ruminants, such as cattle and sheep, are generally more resistant to mycotoxins than most animals, especially pigs, as ruminal microbial population plays a role in detoxification process. This assumption is based on the finding that rumen flora is able to convert a number of mycotoxins into metabolites that are less potent or even biologically inactive at common exposure levels *(Kiessling et al, 1984)*. In animals intended for meat production, which had consumed contaminated feed, the ingestion of mycotoxins leads to substantial degradation of meat quality *(Bonomi et al, 1994)*.

Accumulation of mycotoxins before and after grains harvesting largely reflects actual climate conditions. *Fusarium* toxins are known to be produced during cereal harvesting under high moisture conditions, whereas pre-harvest aflatoxin contamination of crops is associated with high temperatures, insect-mediated damage and prolonged drought. Chronic contamination occurs in warm, humid, tropical, and subtropical growing environments. The degree of moisture mostly depends on the water content available at the harvesting point, but also on the frequency and extensiveness of drying, aerating, and turning of the grain before and during storage, and the respiration of insects and microorganisms harbored by the stored grain *(Bryden, 2012)*. Since *Aspergillus* can tolerate lesser water activity than *Fusarium*, these contaminations may occur both pre- and post-harvesting, whereas *Fusarium* contamination is more specific for the pre-harvesting period. Stored cereals may become infested with fungi and insects; such an infestation is also affected by climatic factors such as temperature and humidity, geographical location, type of storage container, and handling and transport procedures *(Chelkowski, 1991; Krnjaja et al., 2013)*.

Aflatoxins. Among food and feed contaminants, aflatoxins are of current concern. They are known to be produced by two species of *Aspergillus* genus, specifically *Aspergillus flavus* and *Aspergillus parasiticus*, and represent highly toxic, mutagenic, teratogenic and carcinogenic compounds that exhibit an immunosuppressive activity, causing both acute and chronic toxicity in humans and animals *(EFSA, 2004)*. Among them, aflatoxin B_1 (AFB_1) is the most potent liver carcinogen known in mammals, and is classified by the *International Agency for Research on Cancer (IARC)* as Group 1 carcinogen (*IARC, 1993*).

Animals are variably susceptible to aflatoxins, depending on such factors as age, species, breed, sex, nutrition, and certain stresses. Pig, cattle, and poultry are farm animals of greatest economic concern in terms of aflatoxicosis. In all species, the evidence of disease is a general unthriftiness and reduction in weight gains, feed efficiency, immunity, and production. More conclusive evidence of aflatoxin involvement in disease includes acute to chronic liver disease with concomitant increases in specific liver enzymes in the serum. AFB_1 can cause liver

dysfunction, reduced milk production and egg production and to reduced immunity of animals. Long-term consumption of containing low concentrations of AFB_1 in feed can also result in embryo toxicity. Usually, young animals are more sensitive to aflatoxin. The clinical manifestations involve digestive disorders, reduced fertility, reduced feed efficiency and anemia. Aflatoxins not only the decline in milk production, but also the transformation of the milk containing aflatoxin M_1 and M_2 (*Dhanasekaran et al., 2011*).

Maize, as the most widely grown crop extensively used for animal feeding and human consumption, represents a particular problem. Due to its nutritional value, a high percentage of the world maize production is destined to animal feeding (*Pleadin et al., 2014b*). Earlier extensive research of commodities, feedstuffs and feed ingredients revealed the maximal AFB_1 levels in samples coming from the Northern Europe to be 60 μg/kg, in samples coming from the Central Europe to be 311 μg/kg, and in samples coming from the Southern Europe and the Mediterranean region to be 656 μg/kg (*Binder et al., 2007*). In this southeast part of Europe, in the last few years, the research performed in Serbia during 2012 also pointed towards maize contamination with AFB_1 and concluded that weather changes might be held liable for such a contamination (*Kos et al., 2013*). Data have shown that, should a grain such as maize be grown at high ambient temperature, especially during drought, such a grain becomes more susceptible to AFB_1 formation. Grains stored under high moisture/humidity (>14%) conditions and at high temperatures (>20 ºC) and/or inadequately dried, can potentially become contaminated. Grains have to be kept dry, free of damage and free of insects (*Richard, 2007*).

Zinedine et al. (2007) reported that the percentage of contamination with aflatoxins is about 66.6%, while the contamination levels of poultry feed samples ranged between 0.05 and 5.38 μg/kg for AFB_1. In study by *Pleadin et al. (2012a)* the determined mean AFB_1 concentrations were 3.22±2.21 μg/kg in poultry feed, 2.32±1.26 μg/kg in pig feed and 3.45±1.42 μg/kg in feed for calves. Further, *Pleadin et al. (2014c)* provided the evidence of high AFB_1 contamination of maize used by Croatian milk producers. In the maize sampled during 2013 AFB_1 was detected in 38.1% of samples, with 28.8% of the samples containing this toxin in levels higher than the maximal permitted levels (MPLs) and maximal observed AFB_1 level of 2,072 μg/kg (about 100 folds higher than the MPL). High maize contamination was associated with weather conditions, as the period of proceeding was extremely warm, dry, and characterised by a very low average rainfall, all of the aforementioned going in favour of mould formation and AFB_1 presence in maize (*Kos et al., 2013; Pleadin et al., 2014c; 2015a*).

Ochratoxins. Ochratoxins, of which ochratoxin A (OTA) is the most prevalent, are secondary fungal metabolites of some toxigenic species of *Aspergillus* and *Penicillium* that can be found in various feed ingredients. Several studies have shown that OTA is nephrotoxic, causing both acute and chronic

lesions of kidneys, and that it is hepatotoxic, carcinogenic, teratogenic and immunotoxic to several animal species (*Pfohl-Leszkowicz and Manderville, 2007*). Immunosuppression occurs with low concentrations of OTA, while high concentrations lead to kidney toxicity. The mechanism of action of OTA until has not yet been fully clarified but it is known that OTA poses a risk for human and animal health when ingested through contaminated food or feed. The *International Agency for Research on Cancer (1993)* has classified OTA in group 2B as a possible carcinogen to humans.

Long-term exposure of farmed animals to OTA typically results in increased mortality, poor feed conversion, poor growth rates and feed refusal (*Marquardt and Frohlich, 1992*), among which pigs are particularly sensitive *(Malagutti et al., 2005)*. OTA is mainly distributed to the kidneys, with lower concentrations in the liver, muscle and fat, and its disappearance from blood is slower than from tissues (*Perši et al., 2014*). It was found to be immunosuppressive in humans and carcinogenic and teratogenic in laboratory animals and that the DNA lesions induced by OTA *in vivo* were no longer repaired in case of repeated exposure. These effects manifest especially in a dose-dependent and dose-time related fashion *(Marquardt and Frohlich, 1992)*. OTA has been primarily recognized as a nephrotoxic mycotoxin that induces significant changes in serum parameters after ingestion in different animal species (*Kumar et al., 2007)*. Investigations in various animal species showed changes in the values of blood parameters to be observed mostly within 1-2 months of OTA exposure, and to depend on the dose applied and length of treatment (*Marquardt and Frohlich, 1992; Mir and Dwivedi, 2010*).

Literature data revealed that in raw materials OTA was detected in 22-70% of the samples ranged from 4 to 2248 μg/kg (*Griessler et al., 2010; Almeida et al., 2011; Grajewski et al., 2012; Rodrigues and Naehrer, 2012; Streit et al., 2012*). In Italy, the OTA was detected in samples of poultry feed in the range 0.04 to 6.50 μg/kg (*Schiavone et al., 2008*) and in Spain it was detected in 33% of samples of food and raw materials (*Jaimez et al., 2004*). In Croatia, OTA was detected in 39% of samples of maize (*Domijan et al., 2005*), 8% of pig feed samples with mean concentration of 1.53±0.42 μg/kg (*Pleadin et al., 2012b*) and also in low levels, all according to the recommended values, were determined in poultry, pig and calves feed (*Pleadin et al., 2012a*).

Fusarium mycotoxins. As the consumption of *Fusarium*-contaminated products may cause mycotoxicosis and induce teratogenic, carcinogenic, neurotoxic, estrogenic or immune-suppressive effects, contamination of food, feed and their ingredients can significantly affect human and animal health (*IARC, 1993*). Mycotoxins of *Fusarium* species have been found to cause major damage, especially of grains, and could frequently be associated with pre-harvest cereal contamination (*Creppy, 2002*). Their presence has traditionally been associated with temperate cereals, since these fungi require somewhat lower growth and

mycotoxin production temperatures than aflatoxigenic *Aspergillus* species (*Placinta et al., 1999*). Major *Fusarium* mycotoxins that can occur in cereal grains and cereal-based products are zearalenone (maize, wheat), deoxynivalenol (occurring mainly in wheat, maize, barley, oat and rye), fumonisins (maize) and T-2/HT-2 toxins (oat, wheat, and barley). Their biosynthesis can be affected by a number of factors including temperature, humidity, oxygen level, mechanical cereal damage and the presence of mould spores. Level of contamination is linked to climate conditions and widely varies across different world climate zones (*Sforza et al., 2006*). Maize considered to be one of the most frequent crops often contaminated with *Fusarium* mycotoxins (*Pleadin et al, 2013; Krnjaja et al., 2013*).

Zearalenone. Zearalenone (ZEA) is a secondary metabolite of *Fusarium* moulds which is very often encountered at very high concentrations, especially in maize. It is produced primarily by *F. graminearum*, which occurs naturally in high-moisture maize and has been found in moldy hay and pelleted feeds. ZEA as an estrogenic mycotoxin causes vulvovaginitis and estrogenic responses in pigs. Physiological responses in pig occur when the ZEA level in feed maize exceeds about 1 mg/kg (*Kurtz and Mirocha, 1978*). It can be transmitted to piglets in sows' milk, causing estrogenism in the young pigs.

In farm animals, especially pigs, ZEA causes hyperestrogenism causing severe reproductive and infertility issues. Female pigs are considered to be the most sensitive animal species, while poultry and ruminants show a lower responsiveness to ZEA. Its biotransformation runs along two major pathways, as follows: upon hydroxylation, phase-I metabolites α- and β-zearalenol are formed, while the conjugation of ZEA and its reduced forms with glucuronic acid and sulphate leads to the formation of typical phase-II conjugation products (*Zinedine et al., 2007*). The ratios of ZEA over its metabolites and the susceptibility to ZEA considerably vary across animal species (*Songsermsakul et al., 2006*). As ZEA ingestion by livestock may cause losses in terms of poor performance and poor animal health, and given that this mycotoxin, together with its metabolites, often occurs in plant and animal food products, its investigations are also of interest from the food safety standpoint. Studies of pharmacokinetics and metabolism indicate that following an oral administration ZEA is absorbed in a fairly large amount and can be metabolised in pig and possibly also human intestines (*Zinedine et al., 2007*).

It is widely distributed mycotoxin in different commodities and its production is also favoured by environmental conditions such as high humidity and low temperatures (10-15 °C). Data of the incidence of ZEA in grains and feed shown that it was detected in high percentage of samples (*Domijan et al., 2005; Almeida et al., 2011; Grajewski et al., 2012; Pleadin et al., 2012a; Pleadin et al., 2012c; Krnjaja et al., 2013*). Maize is the cereal at the highest risk of frequent and high-level ZEA contamination, while wheat, oat and soybean products have been

found to be contaminated only occasionally (*Zinedine et al., 2007; Placinta et al., 1999*). In raw maize ZEA was determined at the highest level of 6,492 μg/kg, reported for maize sampled in Italy (*EC, 2003*). In the study by *Binder et al. (2007)*, the highest concentration of ZEA in feed (2,348 μg/kg) was determined in the Southern Europe and the Mediterranean region.

Deoxynivalenol. Deoxynivalenol (DON) is primarily produced in cooler climates by *F. graminearum* and *F. culmorum*, and is an important contaminant of maize in many European countries (*IARC, 1993*). Epidemics of *F. graminearum* infection in crops can occur, when relatively warm temperatures and rain coincide with maize silk emergence (*CAST, 2003*), causing *Fusarium* head blight in wheat, and *Gibberella* or pink ear rot in maize. DON is the most common of this group causing animal disease and effects range from feed refusal and vomition to immunosuppression and loss of productivity. Pigs are considerably more sensitive to DON than poultry are, and cattle are quite insensitive (*Prelusky et al., 1994*).

Studies revealed that athough DON can be acutely lethal when ingested in large quantities, moderate- to low-level ingestion of the toxin can cause poor performance and altered immune function. Ruminants are relatively insensitive to DON because rumen microorganisms are able to metabolize/ detoxify this toxin whereas in pigs caused decreased body weight in animals and increase in serum IgA and IgM, cytokine tumor necrosis factor alpha and caused damage including necrosis, blood vessel thickening and hemorrhage (*Chen et al., 2008; Pinton et al., 2010*). At 1.3 mg/kg DON in diet, feed intake by growing pigs is significantly decreased, followed by complete feed refusal at 12 mg/kg and vomiting at 20 mg/kg. The most common signs of acute DON exposure are abdominal distress, increased salivation, and malaise; however, vomiting has been reported at higher dietary concentrations. Extensive lesions are not typically documented in field cases, because pigs regulate toxin ingestion by adjusting their feed intake (*Chavez and Rheaume 1986*).

In the report of experts on collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU Member States which included data on trichothecenes from twelve countries showed that 89% of maize samples were positive on DON (*EC, 2003*). In the report prepared by the *Joint Expert Committee on Food Additives (JECFA)* (*2001*), DON was detected in 41% samples with mean concentrations in the range 3-3,700 μg/kg. Similar data on the occurrence of these mycotoxins are also available from other European researchers. *Schothorst and van Egmond (2004)* reviewed the occurrence of *Fusarium* mycotoxins in 12 European countries and also found that the most common trichothecene mycotoxin in cereals is DON, with 57% of positive samples. Among cereals, maize showed the highest level of contamination. *Tanaka et al. (1990)* reported on contamination of 90% and 62% samples of cereals with DON with average concentrations for positive samples of 221 μg/kg. In Poland, extremely high values (up to 927 μg/kg) of DON were recorded for maize grain

(*Placinta et al., 1999*). *Binder et al. (2007)* published the results of research of this mycotoxin in Europe with regards to their geographical origin. In this research, DON was detected in 70% maize samples with the maximum level of 10.626 mg/kg in Asia and the Pacific region and 5,510 μg/kg in Europe and the Mediterranean. *Pleadin et al. (2012c)* concluded that due to the incidence of *F. graminearum*, high occurrence of ZEA in grains, and specificity of climate in this southeast part of Europe, it can be supposed that DON is often contaminant of grains and feed.

Fumonisins. Fumonisins have been found worldwide, primarily in maize, with more than 10 compounds that have been isolated and characterized. They are metabolites of moulds *Fusarium* (*F. Verticillioides*, *F. proliferatum*) and *Alternaria* and *Aspergillus niger*. Fumonisins B_1, B_2 and B_3 are the major fumonisins produced. The most prevalent is fumonisin B_1 (FB1), which is believed to be the most toxic, i.e. nephrotoxic and hepatotoxic (*Voss et al., 2007*). High concentrations of fumonisins are associated with hot and dry weather, followed by periods of high humidity. High concentrations may also occur in raw maize that has been damaged by insects.

Earlier investigations conducted on different cereals revealed high frequency of FB_1 cereals positive samples (*Domijan et al. 2005*). FB_1 is toxic and carcinogenic to rodents and there are data suggesting that fumonisins or *F. verticillioides* cause esophageal cancer or other human health problems. However, an *International Agency for Research on Cancer (IARC, 1993*) working group on fumonisins concluded that there is "inadequate evidence" for carcinogenicity in humans from oral exposure to FB_1 and a role for fumonisins in any other human disease has likewise not been proven.

Contamination of feed with FB_1 resulted in a diverse range of damage to animal tissues, including lesions to the esophagus, gastrointestinal tract, liver, lungs, and brain. In the animals hepatotoxic, nephrotoxic, neurotoxic and carcinogenic effects were observed. It is considered highly toxic to horses and pigs, while in poultry significantly higher levels generally not cause a change in the production characteristics. When the consumption of feed contaminated with large quantities of fumonisin were caused, slow growth and feed conversion, increased weight of liver, kidney and pancreas, increased levels of aspartate aminotransferase and droped leves of serum alkaline phosphatase, cholesterol and hemoglobin were observed. However, in the presence of other mycotoxins, toxic additive interactions are proven, resulting in poorer production results and the appearance of the above symptoms (*Leeson et al., 1995*).

Extremely high values of fumonisin recorded *Griessler et al. (2010)* in the analysis of samples originating from Portugal, Spain, Italy, Greece and Cyprus. Of the total of 416 samples tested, the average concentrations of fumonisin in the finished feed was 1,411 μg/kg, while in maize originating from Italy concentration

of 36,390 μg/kg was evidenced. Such high levels were not found in other grains such as barley and wheat (*Almeida et al., 2011; Rodrigues and Naehrer, 2012*).

T-2 and HT-2 toxins. T-2 and HT-2 toxins are type A of trichothecene mycotoxins produced by *F. poae, F. sporotrichioides, F. kyushuense* and *F. langsethiae* among which the major producer of T-2 and HT-2 toxins is *F. sporotrichioides* (*Richard, 2007; Creppy, 2002*). The presence of T-2/HT-2 toxin is described in the world, and toxin production is greatest in a wide temperature range (0 to 32 °C, the optimum at 5-15 °C), in the food with a moisture content of 13-22%, in hot and humid climates and in damaged grains. Due to the broad prevalence of this fungus, many different crops including maize, oat, barley, wheat, rice and soya beans can be infected with T-2 and HT-2 toxins.

T-2 toxin is a very potent cytotoxic and immunosuppressive toxin, which can cause acute intoxication and chronic diseases in both humans and animals. The symptoms of acute intoxication are nausea, vomiting, abdominal pain, diarrhea, bloody stools and weight loss. In animals, symptoms also include decreased production of milk or eggs, increased incidence of cracked eggs and oral lesions in poultry (*Morgavi and Riley, 2007*). The major effect of T-2 toxin is inhibition of protein synthesis, which leads to secondary disruption of DNA and RNA synthesis (*Richard, 2007; Creppy 2002*). The immune system is also a target of T-2 toxin, and the effect includes changes in leukocyte count, delayed hypersensitivity, depletion of selective blood cell progenitors, depressed antibody formation, allograft rejection and blastogenic response to lectins (*Creppy, 2002*). HT-2 toxin is a metabolite of T-2 toxin and is formed in microbial transformation *via* deacetylation reaction. This reaction is performed by several intestinal microorganisms in different animals (*Young et al., 2007*).

In Europe, there is very little research conducted on the occurrence of T-2 in feed, which bearing in mind the explicit toxicity of this mycotoxin needs further data collection and the legal values of the maximum allowed level need to be defined (*Vulić et al., 2011*). Earlier performed investigations of T-2 toxin by *Sokolović and Šimpraga (2006)*, over the period 1998 to 2004, determined the values of 100 to 500 μg/kg whereas in an investigation by *Vulić et al. (2011)* the highest concentrations of T-2 was determined in cattle feed (67.68 μg/kg). The highest concentration of T-2 toxin (1776 μg/kg) was determined in Northern Europe (*Binder et al., 2007*). In study by *Pleadin et al. (2012a)*, the lower mean concentration was determined in poultry feed (18.2±8.31 μg/kg) and the highest mean concentration in feed for calves (32.4±15.1 μg/kg).

Mycotoxins reduction

As the presence of moulds and/or mycotoxins in food can be dangerous for human health and represents a huge economic problem, there is a huge space for

the implementation of new methods providing for a safe food production. Methods of control can be classified into two categories: (1) prevention of mould contamination and growth, and (2) detoxification of contaminated products. The prevention of mould growth can be achieved either through pre- or post- harvesting strategies. The applied mycotoxins reduction procedure must effectively inactivate or remove the toxin, maintaining at the same time both nutritional and technological properties of the product and not generating reactive toxic products (*López-García and Park, 1998; Pleadin et al., 2014b*).

Investigation into the methods of inactivation in contaminated food and feed has revealed that pre-harvest contamination can be reduced by virtue of proper curing, drying, sorting and storage, all of the aforementioned limiting the growth of fungi. However, the implementation of unique, totipotent method of mycotoxin reduction, capable of effectively performing in any given biological material, is virtually impossible. The efficiency of the methods of reduction depends on many parameters such as the nature of food and feed, their moisture content and composition, and the level of contamination. Some studies have attempted to achieve detoxification of, or toxin inactivation in, mycotoxin-contaminated feedstuff using gamma irradiation, thermal inactivation, physical separation, microbial degradation and different chemical treatments (*Rustom, 1997*).

Methods of reduction can be divided into chemical, biological and physical. In biological reduction, microorganisms including bacteria, yeasts and acid-producing moulds are used to metabolize and inactivate mycotoxins, and *Flavobacterium aurantiacum* being the most active among them. Production of mycotoxins is also inhibited by lactic acid bacteria, *Bacillus subtilis* and many moulds. Inactivation using physical methods involves extraction with solvents, adsorption, and heat- or irradiation-based inactivation. Contamination can be reduced in stored goods using physical procedures such as color sorting, density flotation, blanching and roasting (*Pleadin et al., 2014b*). The use of chemicals to inactivate or remove mycotoxins has been studied using different chemicals such as propionic acid, ammonia, copper sulfate, benzoic acid, urea, citric acid and some other chemicals, but they have generally been labeled as impractical for application on food as they include drastic conditions in terms of temperature and pressure. Also, they are considered as unsafe because of toxic residues and unfavorable since leading to degradation of nutritional, sensory and functional properties of the product so particular chemical methods are only used for mycotoxins reduction in animal feed (*Rustom, 1997*).

Conclusion

Literature data indicate a high presence of mycotoxins in grains and feed. Special attention should be focused on the particular raw materials in order to reduce the exposure of animals to different mycotoxins that could have adverse

effects on animal health and production results, and consequently indirectly endanger human health and the occurrence of residues in products of animal origin. Data obtained for some mycotoxins suggest teratogenic and genotoxic effects in humans and animals as also their synergistic effects, however the literature does not contain sufficient relevant evidence related to that. In order to ensure safe food production and prevent economic problems of large-scale contamination, continued development and application of modern specific and selective methods in its detection and reduction of contamination are required, as well as the systematic implementation of national monitoring on a representative number of samples of grains and feed.

Mikotoksini u žitima i hrani za životinje – kontaminacija i toksično dejstvo kod životinja

J. Pleadin

Rezime

Mikotoksini su sekundarni metaboliti plesni koji predstavljaju značajan problem za bezbednost hrane, opasnost po zdravlje i dobrobit ljudi i životinja i imaju negativan uticaj na ekonomiju širom sveta. Oni mogu da izazovu trovanje životinja i da utiču na manje produktivne performanse i hranljivu vrednost hrane za životinje. Takođe, mikotoksini mogu kontaminirati različite vrste hrane kroz direktan i indirektan način i da izazovu negativne posledice po zdravlje ljudi. Da bi se procenio potencijal njihovog štetnog dejstva, neophodno je znati uslove pojave i učestalosti mikotoksina, toksičnost i biotransformaciju u različitim vrstama životinja, kod ljudi, mogućnost prevencije zagađenja, kao i njihove inaktivacije ili smanjenja u slučaju kada je pojava neizbežna. Relativno mali broj uporedivih podataka o učestalosti mikotoksina u hrani za životinje iz evropskih zemalja pokazuju neophodnost sistematske kontrole i primene validiranih analitičkih metoda u njihovom određivanju. Ovaj rad daje pregled učestalosti kontaminacije žita i hrane sa životinje sa najvažnijim i najučestalijim mikotoksinima i dokazano toksično dejstvo kod životinja.

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IDENTIFICATION OF RISK FACTORS FOR *SALMONELLA* SPP. IN PIGS AND CONTROL MEASURES DURING MANAGEMENT AND TRANSPORT OF ANIMALS

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Review paper

Abstract: Pigs and pork meat products are common source of human salmonellosis. Salmonella can enter the food chain at any point such as the livestock feed, via the on-farm production site, at the slaughterhouse or packing plant, as well as during manufacturing, processing and retailing of food, or through catering and food preparation at home. The understanding of epidemiology of *Salmonella* sp. at all stages of production chain is of crucial importance. The production of „Salmonella free pigs“ would reduce the risk for the occurrence of human infections Also, production of „Salmonella free pigs“ is difficult to achieve due to a number of practical and financial reasons. However, serological status of particular pig farm can be determined based on the analysis of blood- or meat juice samples taken from slaughtered pigs. This procedure enables the identification of Salmonella free farms“. The basic actions for preventing salmonellosis in humans should involve the following: preventing the entrance of Salmonella to the farm, reducing the number of infected animals and preventing the spread of the infection. The best way to prevent the disease is to keep the infection away from the farm. In order to successfully resolve the problem of human salmonellosis associated with infected pork meat or meat products, control measures need to be simultaneously implemented at all levels of meat production chain.

Key words: salmonellosis, pigs, risk factors, control measures

Introduction

Salmonella spp. is considered one of the leading food borne pathogens that for humans. The sources of infection include infected animals, contaminated food, stables, equipment, manure, rodents, etc. The term salmonellosis describes a range of different forms of infection. The most common form of infection is the carrier

status, where the carrier does not show any symptoms of the disease. Such carrier-animals are of importance for breeding of animals intended for meat production because they can serve as infection reservoirs that facilitate disease spreading via animal excreta and thus can cause the contamination of final product.

Pigs and pork meat products are very often the source of human salmonellosis. Salmonella can enter the food chain at any phase of meat production process - feeding or animals on the farm, in the slaughter house, during packaging of meat products or even during preparation of food in the households (*Stojanov et. al., 2005).* Contamination of pork meat can be reduced by decreasing the contamination at the level of primary production. Proper understanding of Salmonella epidemiology within the entire production chain is the prerequisite for successful implementation of such measures (*Vidić et al., 2014a*). A whole range of studies worldwide have addressed the epidemiology of salmonellosis in pigs. Pork meat and products are an important source of salmonellosis in humans. Precise determination of exact number of cases is difficult to accomplish even in developed countries. The estimated rate of human salmonellosis associated with pork meat and related products in Denmark and Netherlands range between 10 and 15% (*Berends et al. 1998*).

Production of „Salmonella free pigs“ is highly complex and intricate for many practical and financial reasons. However, determination of serological status of the pig farm is feasible and is based on the analysis of blood or meat juice samples collected from slaughtered pigs. This procedure enables identification of a „Salmonella free farms“. The control models for pig salmonellosis differ from country to country; however, all of them include strict control of feed and its components as well as bacteriological and serological monitoring of certain percentage of animals (piglets, sows and fatlings) in defined time frames (*Davis and Funk, 1999*). In order to successfully resolve the problem of human salmonellosis associated with consumption of infected pork meat or meat products, simultaneous implementation of appropriate control measures at all levels of meat production chain is necessary.

In most of EU countries, programs for control and continuous examination are applied throughout all stages of pig production chain at the national level. In Denmark for example, application of defined control programs resulted in significant reduction of salmonellosis rate in pigs, which has dropped from 3.5% in 1993 to 0.7% in 2000, and to even 0.4% in 2014. Such a decreasing tendency is also associated with the reduced incidence of salmonellosis in humans, which was for the same period reduced ten times on annual level (*Mousing et al. 1997, Berends et al. 1998).* In our country, the analysis was performed applying ELISA test and the presence of Salmonella infection was confirmed on the pig farms. The study encompassed 628 blood serum samples of fatlings from 5 different farms. Positive findings for Salmonella were obtained in 46.5% animals, for the cut off at 10%. The S/P value ranged between 0.25 and 3.147 (*Grgić et al. 2004.*). Analysis

of 256 serum samples from sows and boars using ELISA test revealed positive finding in 25.9% animals (*Vidić et al., 2008b*).

Risk factors

Feed

Feed and feed components can be contaminated with Salmonella and as such can represent a potential source of salmonellosis. Large amounts of feed mixture are produced on a daily basis and they are transported and stored for the purpose of pig breeding. Even very low rates of contamination with Salmonella pose substantial risk of infection for many farms. The processes of control and decontamination, such as heat treatment, can be applied at this level to avoid the contamination of feed mixtures. However, according to the research done in Denmark, there are no significant differences in the level of pig infection with *S. enetericae* at the end of fattening period, depending on weather the pigs were fed with pelleted or unpelleted food. *Davies and Wrey* (1997) have found a very high level of contamination with Salmonella on the cooling equipment inside the manufacturing building, on the fresh feces of wild birds collected in the warehouse and the crane for unloading in some mills. This strongly indicates that the contact of final products with infection reservoirs (birds, rats, etc.) must be prevented as well as their potential contamination in the transportation trucks (*Fedorka-Cray, et al., 1997*), warehouses, and mills, which have to be safe and secured.

Epidemiology of Salmonella during pre-harvest stage

Basic goals of control strategies and epidemiology of salmonellosis include prevention of infection introduction into the farm and its transmission and maintenance as well. Farms are not closed systems - constant intake of feed and introduction of new animals represent the potential source of infection as was reported in several studies on the risk factors for salmonellosis (*Dahl et al., 1997; Kljajić et al., 2006*). Besides these two potential entry portals for Salmonella infection, there are many more, some of which have already been confirmed and some are still hypothetical. Based on potential risks and sources of salmonellosis, a number of preventive and control options have been suggested. Feed mixtures treated with heath can help in prevention of salmonellosis in serologically negative herds, but this measure cannot help much in herds where salmonellosis is already present. A range of studies reported on protective effects of feed with low pH (in form of added organic acid, whey or fermented additives) against Salmonella infections (*van der Wolf et al., 2001; Dahl et al., 1999).*

Control of birds, flies and rodents is necessary in pigsties and warehouses but also keeping small animals, such as cats and dogs, out of the facility. Avoiding transmission of bacteria by dust and aerosol inside the facilities and prevention of

contact with infected wild animals is of crucial importance. Appropriate hygienic measures should be provided inside the facilities and drains. Purchase of new animals should be done from the certified and Salmonella free herds. New animals should be quarantined and subjected to relevant health monitoring. The purchase of animals should be performed from limited number of farms. Through washing and disinfection in line with predefined procedures has to be performed at every turnover and movement of animals on the farm. Sanitary rooms and facilities for hand washing as well as changing rooms must also comply with standard operative procedures in order to avoid spreading of Salmonella and other pathogens.

In cases when Salmonella has already been present in pig herd, acidification of feed mixture or even drinking water by adding organic acids or whey was included into the control program. The changes in feeding strategy can help to reduce the exposure to Salmonella and increase resistance to pig infection *(van der Wolf et al., 2001; Dahl et al., 1999*). Application of highly placed pens and partitions can be useful for preventing infection spreading between boxes and buildings. Moreover, a separate facility for keeping sick animals should be planed *(Pedersen, 1997).* Application of only one single control measure is not effective enough to prevent Salmonella infection, reduce the level of infection or to eliminate the infection from the herd (*Kljajić et al 2010a*). Each farm has to define relevant measures and strategies based on the realistic actual situation on the farm and potential successes of all actions has to be objectively assessed and based on combination of measures to be applied, depending on practical and economic factors. Multifactorial infection such as salmonellosis requires a complex approach to identify the procedures to be defined and applied between farms, within the farm itself and to an individual animal on the farm (*Vidić et. al., 2014b*).

Epidemiology of Salmonella during shipping and transportation of animals

Pigs infected with Salmonella have a subclinical form of the disease and only occasionally shed Salmonella in the feces. Stressful conditions can intensify the bacterial shedding in carrier animals and increase the susceptibility to Salmonella infection in animals, which were not infected before (*Williams and Newell, 1970*). During transportation, the pigs are exposed to many stress factors such as noise, smell, mixing with other pigs that were not in the same object, high density of pigs in a small area, duration of transportation, temperature changes and other ambient changes (*Warriss et al., 1992*). Consequently, transportation and manipulation can significantly influence and increase the number of pigs that are shedding Salmonella at the moment of entry to the farm (*Williams and Newell, 1970; Berends et al., 1996; Rajkowski et al., 1998*). Salmonella-negative pigs can be exposed to infection during transportation in a vehicle that has not been

adequately cleaned and disinfected after transportation of Salmonella-positive animals. Contaminated trucks present a potential source of infection also for other farms and slaughterhouses.

After transportation to the slaughterhouse, pigs are usually kept in a lairage before slaughtering. The period spent in a lairage is variable and influences the level of pig infection. The waiting area at the slaughterhouse allows pigs to recover from stress caused by transportation and manipulation of animals. Many of the same stress factors related to transportation are also present during waiting period and the number of pigs which excrete Salmonella is increased also depending on the time spent in the waiting areas (*Morgan et al, 1987b).* Further on, this space or hall is usually cleaned only at the end of the day and represents a potential source of infection for Salmonella negative or weakly infected pigs. Those pigs can easily pick up Salmonella from other pigs or from the environment by oral or nasal intake or even through skin. The longer the time that pigs spend in the waiting area the higher the possibility of contamination and ending up as positive trunks (*Morgan et al., 1987a*).

In order to avoid spreading of infection during transportation or waiting period, appropriate control measures should be taken. Mixing of animals from different farms should be avoided and pigs should be treated as quietly and gently as possible (*Williams et al., 1970, Warriss et al., 1992).* If possible, groups of pigs should be delivered directly to the slaughterhouse in separated trucks (*Morgan et al. 1987b*). Trucks should be cleaned and disinfected between different transports. (*Rajkowski et al., 1998, Swanenburg et al., 2001a*). Waiting time should be as minimal as possible at least for the pigs that are Salmonella negative without mixing animals with other herds (*Morgan et al., 1987b, Swanenburg et al., 2001b*). Pigs should be kept in smaller groups of less than 15 animals and waiting space should be cleaned between different groups of pigs and at the end of slaughtering (*Morgan et al., 1987a, Berends et al., 1998).* Procedures for cleaning and disinfection should undergo constant visual and bacteriological control (*Morgan et al., 1987a, Swanenburg et al., 2001b).*

Control measures

There are three basic strategies to fight against salmonellosis: preventing Salmonella to enter the farm, reducing the number of infected animals and stopping the spread of the disease.

Prevention of entering Salmonella on the farm

The best way to prevent the disease is to prevent the entrance of the pathogen into the farm (*Kljajić et al., 2008*). Purchase of the pigs should be done

from only one or only few herds that are Salmonella-free. Vehicles must be thoroughly cleaned and disinfected before shipping the pigs for further transportation. Before introducing the newly purchased pigs into the farm, quarantine has to be provided. The entrance to the farm should be strictly controlled. Vehicles and trucks should not enter the farm and the shipping of animals and feed should be done in front of the farm entrance. If the vehicles have to enter into the farm, appropriate disinfection should be performed. Dead animals should be disposed into a secured container, which is regularly disinfected. No other animals should be allowed to go near the pigs of warehouse for feed. Dogs and cats are not welcome to pig farm, especially cats which carry particularly high risk. Rodents have to be under control, nets should be mounted on all entrances and openings and all dishes with feed should be covered. Rodents, birds or cats must have no access to warehouses (*Kljajić et al., 2010b*).

Pigs should be separated from other production animals if there are any. The risk from foxes, birds and other pests should be reduced by collecting dead animals immediately as well as the remains after farrowing. Feeding dishes should be covered and there should be solid fences around the objects in the open air. If objects are close to the roads that people use, they should be at least 5m away with a double fence. All machines and equipment should be cleaned and disinfected both before entering and leaving the farm. Unemployed persons must have no access on the farm or, if their presence is necessary only with protective clothes. Feed and water must be purchased from reliable warehouses and public water supply. If this is not possible, disinfection of water should be done.

Salmonella can be transmitted from pigs to humans, so toilets and bathrooms for personnel are needed on the farm. All workers on the farm have to wash their hands every time they work with a different group of pigs.

Reduction of the number of infected animals and prevention of disease spreading

If Salmonella is present on the farm, the infection will most probably spread across the farm, wherever there is contamination with pig feces and liquid manure. The microorganism will die very soon if the environment is dry, clean (no organic matter), warm and disinfected. Places where Salmonella can persist are dirty dishes, dust and cobweb, corners and wall cracks, taps, tools and machines, dirty boots, clothes, warehouses, feed containers, contaminated roads. Thorough cleaning is of utmost importance because the disinfectants are ineffective on dirty surfaces. The ideal strategy is all in – all out. Facilities that are filled with animals all the time cannot be cleaned in a proper way. However, if the facilities are empty for one-two weeks before the next round of pigs, the infection risk can be reduced applying thorough cleaning and disinfection procedure. Everyday work on the farm

should always start from the youngest pigs and then proceed to older groups. Farrowing facilities should be done first. The facilities for housing of sick animals must be cleaned as last. The golden rule is not to mix pigs of different age (*Vidić et al., 2008a*). If there are any pigs smaller and weaker than the others, it might suggest that they are infected and may be a carrier of Salmonella. Pools for boot disinfection should be positioned near every pigsty.

The choice of feed used on the farm can contribute to protection from salmonellosis and retention of Salmonella in the intestinal tract (*Stojanov et. al., 2005*). Pelleted food is the worst solution, that is, feeding powdered meal should prevail. The meals should be based on barley and not wheat, with some addition of organic acids. Liquid meal, especially fermented one is the best option in the control of salmonellosis. This strategy proved effective in Denmark, Netherlands and UK. Water acidification can be of use with low costs, but it appropriate cleaning of the water system is the prerequisite for a successful outcome

The level of stress has to be reduced to the minimum. Pigs must have enough space, especially near feeding place. Water dishes have to work properly and there has to be enough space for lying.

The use of antibiotics is forbidden in the control of salmonellosis. Antimicrobial drugs do not help in reducing the level of infection and can even increase the rate of shedding of Salmonella, prolong the period of excretion and contribute to the development of resistance to antibiotic treatment. Antibiotics can be used only in sick animals, which is rare in pigs. Vaccines are not an easy option and they are not the usual choice for pigs. However, if nothing else gives results, the use of vaccine can be considered. Vaccines made of Salmonella strains originating from the observed herd usually have not been appropriately tested and thus cannot be used as replacement for other measures, such as cleaning and disinfection. Vaccines administered during the period before slaughter can cause a positive finding during testing of the meat juice.

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Faktori rizika kod salmoneloze svinja i mere kontrole u procesu proizvodnje i transporta životinja

B. Vidić, S. Savić, N. Prica

Rezime

Svinje i proizvodi od svinjskog mesa su često izvor humanih slučajeva salmoneloze. Salmonela može ući u lanac hrane na bilo kojoj tački lanca, od ishrane životinja, proizvodnje na farmi, na klanici ili pakeraju, tokom proizvodnje, prerade proizvoda, u snabdevanju i pripremi hrane u domaćinstvu. Razumevanje epidemiologije salmoneloza u svim fazama proizvodnog lanca je od presudnog značaja. Proizvodnja svinja slobodnih od *Salmonella* spp. redukovala bi rizik za nastajanja i javljanje ove zoonoze kod ljudi. Proizvodnja svinja slobodnih od salmonela teško je izovodljiva iz praktičnih i finansijskih razloga. Međutim, realno je moguće utvrditi serološki status farme svinja baziran na rezultatima pregleda krvi ili mesnog soka uzetog od zaklanih svinja. Ovim postupkom definišu se farme «slobodne od salmonela». Osnovni pravci delovanja su: sprečavanje unosa salmonela na farmu i smanjivanje broja inficiranih jedinki na farmi i zaustavljanje njenog širenja. Najbolji način za prevenciju bolesti je sprečavanje ulaska na farmu. Kako bi efikasno rešavali problem salmoneloze kod ljudi, čiji je uzrok svinjsko meso ili proizvodi, potrebno je da se uvedu mere kontrole istovremeno na svim nivoima u lancu proizvodnje.

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REPRODUCTIVE BIOTECHNOLOGY IN ANIMAL HUSBANDRY – CURRENT STATUS AND FUTURE PROSPECTS[1]

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Original scientific paper

Abstract: To date there is still no optimal biotechnology which ensures maximum preservation of the functional parameters of the spermatozoa from buffaloes, boars and dogs. The aim of this research is to study the biological potential of seminal plasma proteins that are specific only to ejaculates with high cryotolerance and good quality parameters of the spermatozoa. The motility and velocity parameters of the spermatozoa were assessed by computer-assisted sperm analysis. Seminal plasma proteins were separated by size-exclusion liquid chromatography and characterized by polyacrylamide gel electrophoresis and mass spectrometry. Based on the results obtained, sperm diluents and methods for biological evaluation of the fertilization potential of the spermatozoa from buffalo bulls, boars and dogs were created and proposed for practical application.

Keywords: buffalo, boar, dog, seminal plasma proteins

Introduction

The preservation of genetic material from breeding animals is a priority for the livestock breeding in most developed countries in Europe and the world. The needs of the practice require the presence of gene banks in order to increase the number of nucleus herds, to conduct planned selection and to preserve gametes from valuable and highly productive animals, as well as endangered species. The successful functioning of a gene bank is always accompanied by an effective reproductive biotechnology for semen cryopreservation and artificial insemination (AI).

Reproductive biotechnologies are always associated with the quality control on reproductive traits of the breeding stock. This control includes both

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exterior parameters of the animal and functional assessment of the gametes**.** Many of the evaluation criteria are based on approved European parameters. Additional specific evaluation criteria are also used. They include precise control on the full reproductive cycle - from gametogenesis and in vitro fertilization to assessment of the fertilization potential of the gametes, as well as analyses on the implantation and pregnancy.

In Bulgaria there are long lasting traditions in the field of reproductive biology and in vitro technologies applicable to different types of farm animals. Biotechnologies for AI with preserved and cryopreserved semen are introduced in practice since mid-last century. Bulgarian scientists have developed one of the first efficient embryo biotechnology and from 1950 to 1990 new Bulgarian breeds were created: 5 in cattle- breeding, 8 in sheep, 1 in buffaloes, 1 in goats, 2 breeds and 3 hybrids in pig breeding. These results were achieved thanks to the rapid breed development and genetic improvement of our livestock and the massive implementation of the technology for AI. All this allowed the use of the elite breeding animals' reproductive potential in the most effective way.

Today, due to the new political and economic situation and after the transition from planned to market economy; there have been a lot of changes that affected in varying degrees the planned breeding and selection in livestock. These changes have affected the average annual production of meat and milk. The average annual milk production per capita declined from 275 kg for the period 1986-1989 to 165 kg for the period 2008-2011, meat production decreased for the respective periods from 90 kg to 30 kg.

Similar is the situation with the application of AI in animals. By 1991-1992, over 90% of the animals in Bulgaria were artificially inseminated. Today, these data are reflected in table 1.

Table 1. Artificial insemination in farm animals (Source: Ministry of Agriculture and Forestry of Bulgaria, direction "Agrostatistics"- survey "Number of livestock in Bulgaria up to 01.11.2002")

Animal species	Artificial insemination:	
	% of farms	% inseminated female animals
Cattles	37.5%	50.0%
Pigs	11.2%	27.8%
Sheep	1.7%	5.3%
Goats	0.8	0.2

In one of the best centers for AI in animals on the Balkan Peninsula, located in the town of Sliven, about 149 bulls were available in 1992-1993. Now the number of bulls is 18. Currently the national genetic reserve stores the following genetic material: 347 913 doses from 405 bulls of 33 breeds and 7300 doses from 23 rams of 10 autochthonous breeds.

This reduction of the milk and meat production is an illustration of the serious fall-off in Bulgarian livestock breeding in the area of planned breeding.
Complex approaches are required to deal with the situation, which can be summarized in the following directions:

- Sustainable development of livestock genetic resources;
- Increased number of animals undergoing selection control;
- Maintenance of an optimal number of animals in nucleus and reproductive herds;
- Expanded use of AI;
- Introduction of innovations in reproductive biotechnology for preservation and cryopreservation of gametes;
- Updated nutrition and breeding;
- Health control;
- Introduction of good manufacturing practices for sustainable production and animal welfare;
- Optimization of production, processing technology and marketing of food of animal origin.

Today, the biotechnology for cryopreservation of male gametes is not yet widely used in some species of farm and domestic animals such as boars, rams, buffaloes and dogs. The reasons for this stand in unresolved issues related to the lack of optimal biotechnology that ensures maximum preservation of the biological potential of the spermatozoa. A lot of additional research is required in these animal species. This is a prerequisite for search for new semen cryoprotectants and cryopreservation media in these species.

In recent years, more and more scientists focus their attention on the role of seminal plasma proteins (SPPs) and their association with the activation of signaling pathways responsible for the sperm functioning *(Furugen et al., 2012; De Lamirade et al., 1984; De Vries et al., 2003)*. It has been reported that some SPPs affect motility and survival rates of male gametes and can affect their fertilization capacity. To this end, there are still many unexplored concerns, especially when it comes to the role of SPPs on the spermatozoa of the boar, ram, buffalo bull and dog *(Daskalova et al., 2015; Kukov et al., 2012; Januskauskas at al., 2003; Martin et al.,2004; Moura 2005; O'Meara et al., 2007; Gradinarska et al., 2015).*

The lack of conclusive data on the mechanism of protection of the SPPs in sperm cryopreservation in these species, and scant information about SPP's role in the process of preservation and capacitation gave us a reason to make an attempt for separation and analysis of the SPPs with protective effects on the spermatozoa, in order to optimize the biotechnology for long-term sperm preservation *(Daskalova et al., 2012; Daskalova et al., 2014; Wysocki et al., 2015; Frazer and*

Strzezek, 2007; Strzezek et al., 2005; Thomas et al., 2006; Bailey et al., 2000; Kirilova et al., 2014; Ardon et al., 2013).

On the basis of this analysis and our resources, we turned our efforts towards the development of an effective biotechnology for long-term semen preservation based on native SPPs present only in ejaculates with high cryotolerance and good quality parameters of the spermatozoa. Based on these studies were created and proposed for practical application sperm diluents and methods for biological evaluation of the fertilization potential of spermatozoa from buffalo bulls, boars and dogs.

Materials and Methods

For the studies we used semen from elite breeding animals- 10 ejaculates from boars, 16 from Bulgarian Murrah buffalo bulls, 10 from dogs.

Buffalo bulls' semen is the property of Executive Agency on Selection and Reproduction in Animal Breeding (EASRAB) - Sofia and Sliven.

Boar ejaculates were collected using the gloved-hand technique from 10 Polish Larger White (average age of 2 years) used for breeding purposes in insemination centers in Olsztyn, Poland.

Dog semen was collected in cooperation with Central Veterinary Clinic – Sofia, Bulgaria. Ejaculates from clinically healthy dogs (4 to 11 years) were collected using the manual method.

Computer-assisted sperm analysis (CASA):

The motility and velocity of the spermatozoa were assessed by CASA System Sperm Class Analyzer® (Microptic®, Spain), analytical module „Motility and concentration".

- Buffalo bulls: 16 ejaculates were thawed and spermatological parameters were analyzed at the beginning of the experiment and at every hour until the 6th hour after thawing. CASA was performed using "Leja 20" chambers with 2 μl drop volume. A minimum of 1000 spermatozoa per sample were analyzed. Based on the received data, the ejaculates were classified into 2 groups– with high cryotolerance (group A) and low cryotolerance (group B) of the gametes.
- Dogs: CASA was performed on 10 fresh semen samples using cover slides (18x18 mm) with 8 μl drop volume. A minimum of 1000 spermatozoa per sample were analyzed. Based on the received data, the samples were distributed into 2 groups – with good (group 1) and poor (group 2) quality of the sperm.

Microscopic sperm motility evaluation:

- Boars: 10 ejaculates were thawed and divided into two groups: high cryotolerance (HCT) group with more than 40% motility after thawing and low cryotolerance (LCT) group with less than 5-10% motility after thawing.

Seminal plasma (SP) isolation:

- Buffalo Bulls: SP was isolated by centrifugation at 4ºC, 2000rpm for 10min and again at 12000rpm for 5min, where after it was filtrated through 0.22µm filter membrane Millipore®.
- Boars: SP was isolated from all samples by double centrifugation at room temperature at 3000x g for 5 min and again at 10000x g for 5 min.
- Dogs: SP was isolated from all samples by double centrifugation at 2500 rpm, 4°C, and 5 min and followed by 10000 rpm, 4°C, and 10 min.

High-Performance Liquid Chromatography (HPLC):

SPP separation was performed by High Performance Liquid Chromatography on Binary HPLC Pump 1525 with UV/Visible Detector 2489 (Waters Company®), with semi-preparative size exclusion chromatographic column TSK gel G3000SW, 21mm x 300mm, 10 to 500 kDa (Tosoh Bioscience®). Gel Filtration Markers Kit for Protein Molecular Weights 12,000-200,000 Da™ (Sigma-Aldrich®) was used for MW determination.
The distinct SPP fractions were collected for further analysis.

- Buffalo Bulls: Sample volume of 1000 µl was applied, at 20 min run time and 6 ml/min flow rate.
- Dogs: Sample volume of 150 µl was applied, at 20 min run time and 6 ml/min flow rate.

Fast Protein Liquid Chromatography (FPLC):

- Boars: Chromatography separation of SP from 5 boars with pre-researched HCT and 5 boars with LCT was performed. FPLC was performed using a Ceramic Hydroxyapatite Column type II (CHT) (Bio-Rad®) at 1 mL/min flow rate, 1000 µl sample volume and 5 mg/ml quantity of proteins.

Spectrophotometric analysis of protein concentration:

After the chromatography each collected protein fraction was analyzed spectrophotometrically for determination of protein concentration (Ultrospec 2000 UV/VIS Spectrophotometer, Pharmacia Biotech®).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

- Buffalo Bulls: The separated SPP fractions from group A were further characterized by 12% SDS-PAGE (TV100 Bio-Rad®). Standard protein marker (SigmaMarker™ wide range, 6.500-200.000 Da, Sigma-Aldrich®) was used for MW determination. Visualization of the protein bands was made by Coomassie Brilliant Blue staining.
- Boars: All obtained protein fractions were characterized by 15% SDS PAGE (Bio-Rad® Mini Protean tetra system, at 150 V DC). The gels visualization was done via the Coomassie brilliant blue (0.05%) method. SERVA® Protein Marker was used as standard.

Protein Identification by Mass spectrometry (MS):

- Boars: The proteins, characteristic only to ejaculates with HCT or LCT, were identified via MS. The protein bands of interest from the two different groups (LCT and HCT), were cut out from the SDS-PAGE gels and prepared for MS (Bruker-autoflex III smartbeam®) and identification. The results were compared against a database of protein sequences in the MASCOT application.

Results and Discussion

Buffalo bulls

Results from the HPLC analysis:

The results from the HPLC analysis show specific differences in the chromatographic profiles of the studied samples. Pronounced peaks are observed in all chromatograms that are more distinct at 280 nm wavelength. The proteins in those peaks vary from 5 to 500 kDa. The comparison of the results between ejaculates from group A and group B demonstrates differences in the chromatograms, which correspond to different quantitative and qualitative composition of proteins in the SP (figure 1). Groups of SPPs with pronounced peaks on 12 min (about 30 kDa) and 14 min (about 12 kDa) are found in ejaculates with proven high cryotolerance of the sperm. The same peaks, but with low light adsorption, are found in ejaculates with low cryotolerance of the gametes. This result speaks of a lower concentration of these proteins. Also, in group A there is a well pronounced peak between 16 and 18 min (molecular weight (MW) between 6 and 14 kDa), which is almost absent in group B.

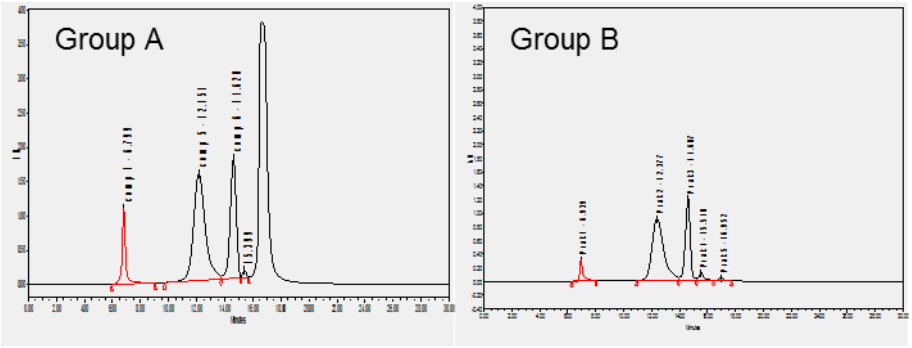

Figure 1. HPLC profile (λ=280 nm) of SPPs from Buffalo bull ejaculates with high sperm cryotolerance (group A) and ejaculates with low sperm cryotolerance (group B).

Results from the spectrophotometric analysis for protein concentration:

8 protein fractions were collected from the ejaculates with proven high cryotolerance the spermatozoa, which manifest into 8 well-defined peaks. The concentration of proteins varies from 1,076 mg/ml in fraction 4 to mg/ml 0,069 in fraction 8 (Table 2).

Table 2. Protein concentration in SPP fractions from Buffalo bull ejaculates with high cryotolerance of the spermatozoa

Quantity of proteins in SP from ejaculates with high cryotolerance			
Fraction 1	0,333 mg/ml	Fraction 5	0,465 mg/ml
Fraction 2	0,379 mg/ml	Fraction 6	0,223 mg/ml
Fraction 3	0,217 mg/ml	Fraction 7	0,183 mg/ml
Fraction 4	1,076 mg/ml	Fraction 8	0,069 mg/ml

Results from the SDS-PAGE of the SPPs from ejaculates with high cryotolerance of the gametes:

Proteins with high MW (200-150 kDa) are predominant in fraction 1. Low MW proteins (20-12 kDa) are predominant in fraction 4. It is noteworthy that lower concentrations of proteins are available in fractions 5 and 6. Also in fraction 1 protein bands with MW about 200 kDa are observed, as well as a small amount of proteins with lower MW, below 20 kDa. In faction 2 a protein band with MW about 90 kDa is seen. In faction 4 a large amount of proteins with low MW from 20 to 12 kDa and below 12 kDa can be seen. In fractions 5 and 6 protein bands with MW below 14 kDa are observable (figure 2).

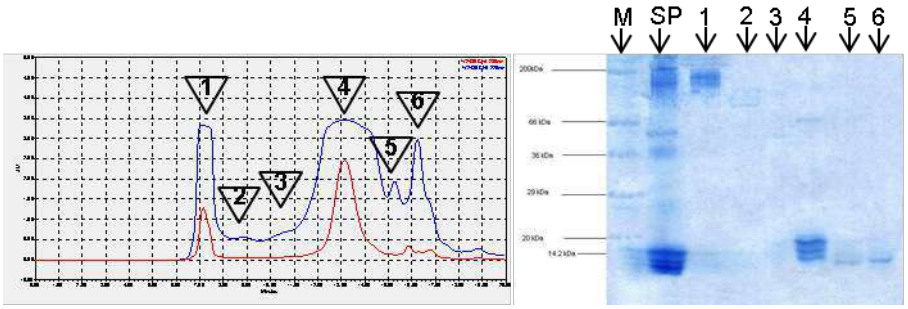

Figure 2. SDS-PAGE of the SPPs from Buffalo bull ejaculates after HPLC separation. M-Marker; SP- Seminal plasma; 1-6 – SPP fractions.

Boars

Results from the FPLC analysis:

The results obtained demonstrate differences in the protein profile of the SP from boar ejaculates with LCT and HCT of the spermatozoa (figure 3).

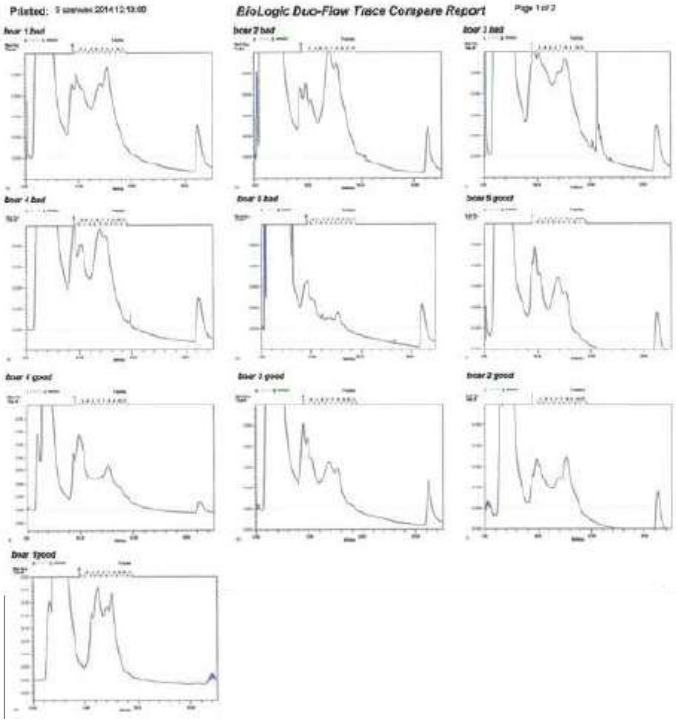

Figure 3. Chromatographic profiles of SP from 5 boars with LCT (bad) and 5 boars with HCT (good) of the gametes.

9 SPP fractions were collected from each SP after FPLC. The analysis of the chromatograms demonstrates significant differences between the chromatographic profiles of ejaculates with HCT and those with LCT.

Results from the spectrophotometric analysis of protein concentration:

5 HCT ejaculates and 5 LCT ejaculates were analyzed. The concentration of proteins varies from 5 mg/ml to 12.5 mg/ml (table 3).

Table 3. Protein concentration in SP from ejaculates with high and low cryotolerance of the gametes

Quantity of proteins in SP- HCT group:		Quantity of proteins in SP- LCT Group	
boar 1	5 mg/ml	boar 1	8.5 mg/ml
boar 2	7.75 mg/ml	boar 2	12.7 mg/ml
boar 3	11.5 mg/ml	boar 3	7.5 mg/ml
boar 4	5 mg/ml	boar 4	10.3 mg/ml
boar 5	12.5mg/ml	boar 5	10 mg/ml

Results from the SDS-PAGE of the SPPs with HCT of gametes:

All separated protein fractions were characterized by 15% SDS-PAGE. The gels of all tested animals were compared. The most significant differences were found between boars 1, 2 and 5 of the HCT group and boars 2, 3 and 5 of the LCT group.

The presence of protein bands specific to LCT and HCT was proven (figure 4). On gel 1(left) proteins identified solely in the SP from boars with LCT are framed in black, while on gel 2(right) are demonstrated protein bands established only in boar ejaculates with HCT of the spermatozoa.

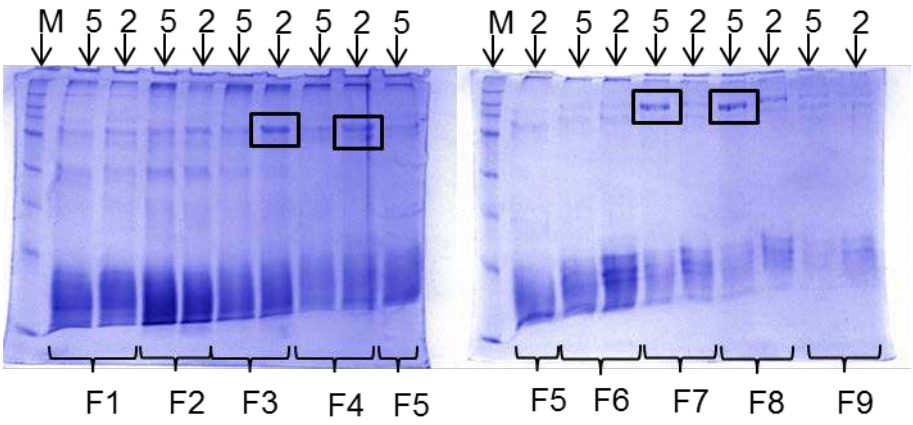

Figure 4. SDS-PAGE after FPLC separation of the SPPs from boar ejaculates with HCT (boar 5 good) and with LCT (boar 2 bad). M- Marker; F – SPPs fraction.

MS analysis for protein identification:

The proteins established in both groups were cut off from the gels and prepared for MS analysis. MS identified the protein found only in ejaculates with LCT as hexosaminidase B (HEXB) (score: 156 for GI/262072808, SUS SCROFA). A correlation was found between high levels of this protein in ejaculates with LCT and low motility of boar spermatozoa.

MS analysis showed that boar ejaculates with HCT have high levels of the protein Lactoferrin (LF) (score: 96 for GI/116488296, SUS SCROFA).

Dogs

Results from CASA:

Group 1 demonstrates significantly lower percentage of static spermatozoa, higher percentage of spermatozoa with progressive motility and significantly higher percentage of spermatozoa with rapid motility, when compared to group 2 (figure 5).

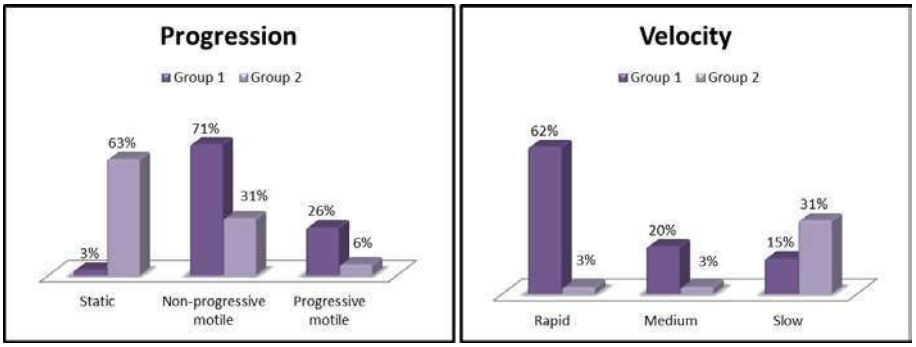

Figure 5. Comparative analysis of the motility and velocity of dog ejaculates with good and poor quality of the sperm

Results from the HPLC analysis:

Comparative HPLC analysis of the SPPs between the two groups establishes differences in the quantity of proteins contained in the separated fractions (figures 6-7).

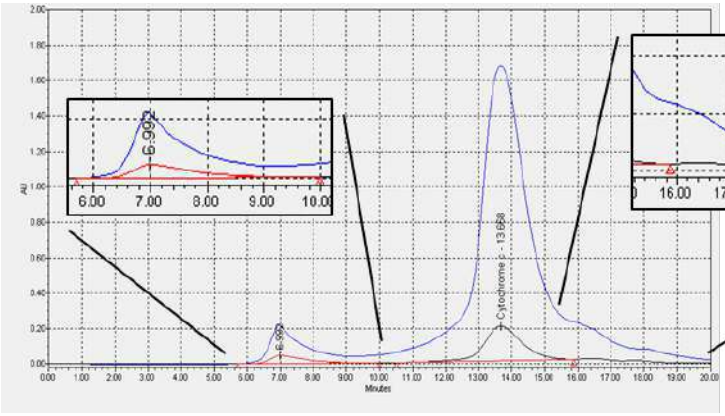

Figure 6. HPLC protein profile of SP from dogs with good quality parameters of the sperm (Group 1)

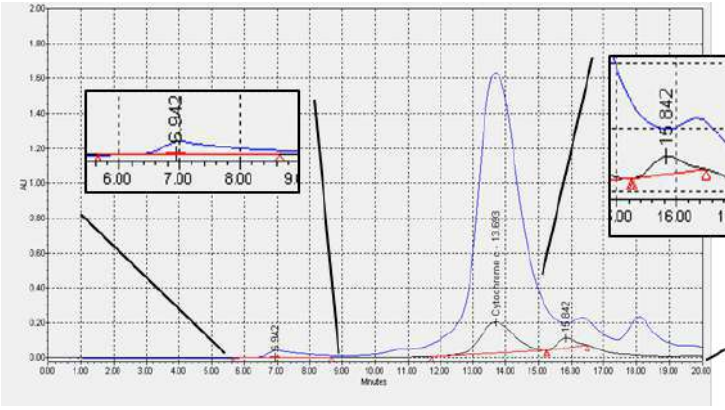

Figure 7. HPLC protein profile of SP from dogs with poor quality parameters of the sperm (Group 2)

The SPPs profile of group 1 demonstrates a well pronounced peak on 7th minute, which is almost absent in group 2. This peak contains proteins with MW over 200 kDa. It can be assumed that they belong to the group of zinc-binding proteins, which have an affinity for binding to the acrosomal region of the spermatozoa and exhibit a protective effect on the sperm plasma membrane.

Group 2 demonstrates a pronounced peak on 16th minute (below 12 kDa), which is less defined in group 1. Also there is a peak on 18th minute (below 10kDa), which is nearly absent in group 1.

Conclusion

Finding new phenotypic traits for gamete cryotolerance is an innovation that can be applied as a prognostic test in future practical use. A preliminary prognosis for ejaculates with high or low cryotolerance, related to specific SPPs, may be used in reproductive biotechnologies in animals.

The presence of the protein hexosaminidase B in boar SP is a phenotypic trait for spermatozoa with low cryotolerance. Lactoferrin is a phenotypic trait for high cryotolerance of boar semen.

The established specific chromatographic profile of SPPs from buffalo bull ejaculates with high cryotolerance of the spermatozoa can be applied in practice when assessing the quality of the semen in breeding animals.

The presence of proteins with MW over 200 kDa in dog SP is related to good motility and velocity parameters of canine spermatozoa. In ejaculates with poor quality parameters the presence of SPPs with low MW (under 12 kDa) is noticeable. The results obtained can be used in the field of reproductive biotechnology as a biological criterion for the quality of the semen.

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Reproduktivna biotehnologija u stočarstvu - trenutni status i budući izgledi

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Rezime

Do danas još uvek ne postoji optimalna biotehnologija koja osigurava maksimalno očuvanje funkcionalnih parametara spermatozoida bivola, svinja i pasa. Cilj ovog istraživanja je da se ispita biološki potencijal proteina semene plazme, koji su specifični samo za ejakulate sa visokim kriotolerancijom, i parametara kvaliteta spermatozoida. Parametri pokretljivosti i brzine spermatozoida su ocenjeni uz pomoć kompjuterske analize sperme. Proteini plazme sperme su razdvojeni ekskluzijom po veličini, tečnom hromatografijom i karakterisani poliakrilamidnom gel elektroforezom i masenom spektrometrijom.

Na osnovu dobijenih rezultata, stvoreni su diluenti sperme i metode za biološku evaluaciju potencijala oplodnje spermatozoida bivola, svinje i pasa i predloženi za praktičnu primenu.

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THE EFFECT OF INJECTIVE APPLICATION OF SELENOPYRAN ON THE PROLONGED INCREASE OF THE SELENIUM CONTENT IN BLOOD AND SPERM OF RAMS[1]

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Abstract: Selenium is a microelement of big importance for male reproduction. As a part of the antioxidative enzyme gluthationperoxidase and structural Se proteins, it plays pivotal role in the defense of spermatozoa against generated ROS and in ensuring of its motility. During the last years the interests to the organic forms of selenium was enhanced because of its better biological utilization. The present work aimed to study the effect of injective application of organic compound selenopyran in rams on the distribution of selenium content in blood and sperm and on the changes in sperm quality. The experiment was conducted with 5 rams from the Synthetic Population Bulgarian Milk breed at the age between 3-7 years and live weight 85-90 kg. The animals were injected once with an oil solution of the selenopyran in dose of 0.1mg/kg live weight (selenium content 24%) 45 days before starting the breeding season. The blood was collected before treatment and 45 days thereafter. At the same time the first and second ejaculates of rams were collected using artificial vagina and analyzed by Sperm Class Analyzer. The selenium content was measured in plasma, blood cells and sperm by atomic absorption spectrometry using SpectrAA 55B double beam spectrometer (Varian, Inc.). The results showed that one injection of organic compound of selenium – selenopyran in dose of 0.1mg/kg live weight ensured the increase and support of high level of selenium in blood (plasma and blood cells) and in sperm during investigated period. That lead to the proper spermatogenesis in

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testis and allowed the production of qualitative ejaculates with higher number of total spermatozoa as well as of spermatozoa with progressive motility.

Key words: rams, Synthetic Population Bulgarian Milk sheep breed, selenium, spermatogenesis, selenopyran, injective application.

Introduction

During the last decades there are new evidences of selenium importance for the male reproduction, especially for the properly spermatogenesis. More than 25 selenoproteins were identified in the live organisms. Most part of them occurs in the male reproductive system at tissue (testis, epididymal epithelium), cellular (intracellular membranes) and subcellular level (sperm nucleus, mitochondrial capsule) (*Ahsan et al., 2014*). Se concentrations in rodent testes exceed that of other organs, except kidneys (*Schriever et al., 2009*). According to *Kehr et al. (2009)* the distribution of Se in midpiece and head of spermatozoa is 4:1. Spermatozoa may be more vulnerable to oxidative stress if the Se content in selenoproteins is low and likely decreases the possibility of fertilization (*Beckett and Arthur, 2005*).

Also many investigations had underlined that selenium is essential for normal spermatozoa development and function in livestock animals (*Shi et al., 2010; Kendall et al., 2000; Tareq et al., 2010*). Se is actively incorporated into the developing spermatozoa of various mammalian species, including rats *(Burk et al., 1972)*, bulls *(Bartle et al., 1980)* and rams *(Pond et al., 1983)*.

The polygamy of the rams and short breeding season requires good condition for semen production. For the achievement of the optimal reproductive performances during the breeding season, where rams are used intensively, their diets require an additional feed additives, including selenium at first. However, there is a lack of information for a definition of an optimal Se status in blood and sperm of rams with regard to fertility.

Despite the many studies on the effects of selenium during spermatogenesis, more detailed investigations are required in order to provide a more clear understanding about relationship between selenium content in blood and in sperm.

The aims of the present study were to examine: a) effect of injective application of organic compound selenopyran on the Se content in blood and sperm of rams; b) the correlation between the Se content in blood and sperm and sperm quality during the preparation of rams to breeding season.

Material and methods

The experiment was carried out with 5 rams from Synthetic Population Bulgarian Milk sheep breed, housed at Animal facility of the Institute of Animal Sciences - Kostinbrod. This breed is newly created (officially acknowledged during 2005) and it is the most spread breed in Bulgaria now. Rams were at the age - between 3 and 7 years old with live weight of 80 to 95 kg. The animals were raised in pens and fed with meadow hay *ad libitum* and concentrated mix forage 500 g/head (250 g wheat and 250 g Dried Distillers Grains with Solubles). **S**alt and mineral licks were placed in pens (EuroLick MultiVit®), as the concentration of Se in licks was 10 mg per kg. 45 days before breeding season the experimental rams were injected once subcutaneously with oil solution of selenopyran (9-phenyl-ymmetrical octahydroselenoxanthene) in dose of 0.1 mg /kg live weight. The Se content in this organic source was 24%. As mentioned in previous investigations (*Abadjieva et al., 2014*), the advantages of selenopyran are the lower toxicity in comparison with sodium selenite (LD_{50}=1600 mg/kg against LD_{50}=3.25 mg/kg) and ability to slowly liberate the selenium according to the needs of the organisms (*Boryaev and Kravchenko, 2006*).

Blood collection

The blood samples were collected before treatment and 45 days thereafter. The blood was collected from *v. jagualris* in vacutainers covered with EDTA. Plasma and blood cells were separated by centrifuge at 3000 rpm for 15 min and stored at -20°C until analysis.

Semen collection and analysis

From each ram the first and second ejaculates were collected by using artificial vagina in triplication (n=57 in total) before treatment and 45 days thereafter. At the time of sampling, the ejaculates were diluted (1:3, vol/vol) with 6A ram semen extender and transported to the IBIR-BAS laboratory within 1 hour. The estimation of semen quality parameters was done by Sperm Class Analyzer (SCA, Microptic, Spain) after appropriate additional dilution of samples. The total concentration, average percentage and number of motile sperm were measured by SCA software.

Se measurement

The content of selenium was analyzed in sperm, in blood plasma and blood cells by the atomic absorption spectroscopy method in the Central Laboratory for Chemical Testing and Control –Bulgarian Food Safety Agency. All samples were digested using microwave pressure digestion system MARSXpress (CEM) with IR temperature sensor control and XP-1500 Plus fluoropolymer closed vessels. 0.5g sample with 5ml concentrate HNO_3 was placed in vessels and heated to 185°C for

15min. The used reagents were of analytical reagent grade (Merck CGaA). For analysis stock solution of Se containing 1000 mg/ L (LGC Standards) was used for daily prepared analytical calibration standard with concentration 10µg/L by serial dilutions with 0.5% (v/v) HNO_3. SpectrAA 55B double beam atomic absorption spectrometer (Varian, Inc.) was used for all determinations. Hallow cathode lamp from Varian operated at 10mA with spectral bandwidth of 1.0nm. The selected wavelength was 196.0 nm. Argon (99.996% purity) was used for carrier gas.

The statistical processing of the data was done by the STATISTIC computer programme (Stat Soft Inc., Ver.10.0). The one-way and regression analysis were done. The mean differences considered statistically significant at $P<0.05$.

Results and discussion

The distribution of Se in ram blood plasma, blood cells and sperm are presented in the Table 1.

Table 1. Content of selenium in blood and sperm of the treated rams

Parameters	**Se content, µg/L**											
Rams (n=5)	**Blood plasma**				**Blood cells**				**Sperm**			
	Mean	SEM	Min	Max	Mean	SEM	Min	Max	Mean	SEM	Min	Max
Before treatment	59.3	14.9	25.3	106.5	<20	-	-	-	143.3	20.1	124.3	174.0
45 days thereafter	2026.3	219.7	1423.2	2581.0	654.7	87.0	472.2	943.4	637.3	66.0	509.4	883.6
P	0.000020				0.001				0.000385			

The blood Se concentration measured before treatment showed that the animals should be considered as a Se deficiently because levels below 70 µg/L are subnormal (*Pavlata et al., 2000*). The injective application of selenopyran leads to significant increase of the Se level in both plasma and blood cells.

Most literature data presents either serum or whole blood Se concentration analyses. Serum Se concentration reflects more acute or recent changes in Se nutrition or injective input of Se, whereas whole blood Se reflects more chronic or "historical" Se status (*Stowe and Herdt, 1992*). In our study we investigated blood plasma and blood cells separately and established that Se content in plasma was about 3 folders higher than in blood cells in both before treatment and 45 days thereafter.

These results should be explained trough the injective application of selenopyran and its immediate introduction to blood plasma. The majority of the glutathione peroxidase contented Se is incorporated into the red blood cells at the

time of erythropoiesis and the response to a Se treatment in blood cells requires a time. That corresponds with our results: the high level of Se appears in blood cells only 45 days after treatment.

Compared to the reference range (*Stowe and Herdt, 1992*) of selenium in blood serum 120–150 μg/L for ewes, the average values in our rams, injected with 0.1mg/kg live weight selenopyran, were very high. Despite such high level of Se in blood we didn't observe any toxic effects. We suppose that the most part of Se in blood plasma is presented in the form of selenopyran and slowly released Se. Moreover, the quality of sperm after treatment with selenopyran was improved (Table 2). The level of Se in sperm significantly increased and remained high till 45-th day after treatment. Also the close correlation between Se level in blood plasma and sperm was established after treatment with selenopyran (Fig.1, r=0.84; p=0.049). These results confirmed low toxicity of preparation of organic compound selenopyran by its unique chemical structure where selenium atom is binding to the heterocyclic ring (*Boryaev and Kravchenko, 2006)*. The similar results about low toxicity of organic selenium were reported in cows: the treatment with selenium yeast lead to high concentration of Se in blood (more than 1000 μg/L) without negative effects (*Juniper et al, 2008*).

Whole blood Se is an indicator of circulating Se and it reflects Se status. After treatment we found the largest amount of Se in blood plasma, followed by blood cells and whole semen. *Cheah and Yang (2011)* underline that sperm count and concentration of selenium in semen are in direct ratio. The results of our experiment confirm this statement (Table 2): the concentration of spermatozoa increased with the increase of the Se content in semen. Also the number of spermatozoa with progressive motility was enhanced after selenopyran treatment.

Table 2. Sperm characteristic of investigated rams

Rams(n=5)	**Concentration of spermatozoa, mln/ml**		**Number of spermatozoa with progressive motility, mln/ml**		**% of progressive motility**		**Volume, ml**	
Ejaculates	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Before treatment, (n=28)	849.3	56.6	117.6	10.6	14.4	1.2	0.86	0.06
45 days thereafter, (n=29)	1423.1*	135.6	209.4*	28.3	13.7	1.1	1.0	0.08
P	0.00031		0.00407		0.68604		0.34734	

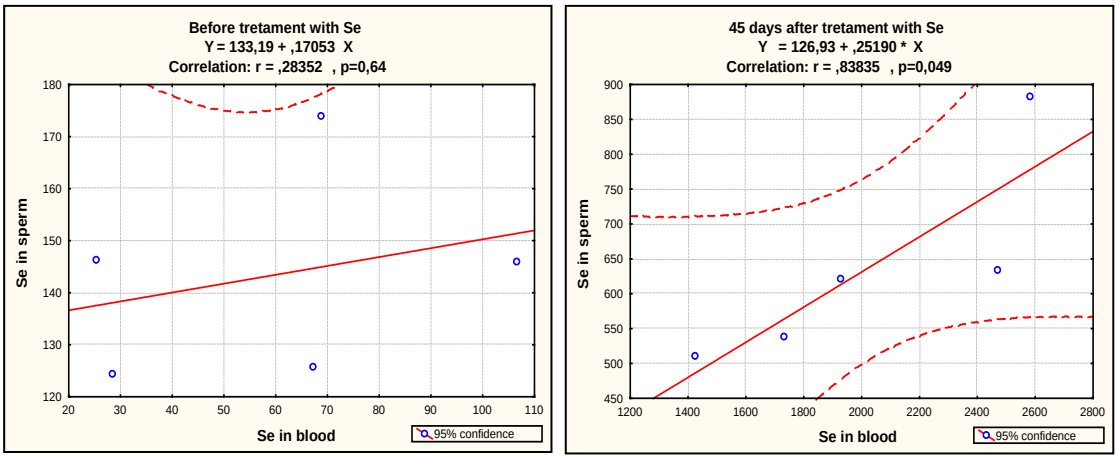

Figure 1. Correlation between selenium content in blood and sperm

The mechanism of Se metabolism regulation is strongly dependet on species. In men, *Iwanier and Zachara (1995)* found similar to ours relations in Se content in blood and sperm. In bulls, the Se content was 10 times higher in seminal plasma than in blood serum (*Saaranen et al., 1989*).

Our results demonstrate the important role of Se for ram reproduction showing significant increase of Se content in sperm before breeding season, when spermatogenesis initiates. The high Se concentration during spermatogenesis is related to its protective property and its associated enzymes, such as mitochondrial capsule protein in spermatozoa (*Alabi et al., 2000; Kehr et al., 2009*). The increase of Se content in sperm in our experiment probably ensures the sufficient amount of Se to be taken into the spermatozoa.

Conclusion

The obtained results showed that one injection of organic compound of selenium – selenopyran in dose of 0.1mg/kg live weight supports a high level of selenium in blood (plasma and blood cells) during the 45 days (spermatogenesis period in rams) and provides the sufficient amount of Se in sperm that ensures the quality ejaculates with higher number of total spermatozoa, as well as of spermatozoa with progressive motility.

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Uticaj injektivne primene selenopirana na produženo povećanje sadržaja selena u krvi i spermi ovnova

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Rezime

Selen je mikroelement od velikog značaja za mušku reprodukciju. Kao deo antioksidativnog enzima glutationperokidaze i strukturnih Se proteina, on igra ključnu ulogu u odbrani i obezbeđivanju pokretljivosti spermatozoida. Tokom poslednjih godina zainteresovanost za organske oblike selena je povećana zbog njegove bolje biološke iskoristivosti. Ovaj rad ima za cilj da prouči efekat injektivne primene organskog jedinjenja selenopiran u ovnova na distribuciju i sadržaj selena u krvi i spermi i na promene u kvalitetu sperme. Ogled je izveden sa 5 ovnova sintetičke populacije bugarske mlečne rase u uzrastu od 3-7 godina i telesne mase 85-90 kg. Životinjama je jednom ubrizgavan uljni rastvor selenopiran u dozi od 0.1 mg/kg žive težine (sadržaj selena 24%) 45 dana pre početka sezone parenja. Krv je sakupljena pre tretmana i 45 dana nakon toga. Istovremeno, prvi i drugi ejakulat ovnova je uzet pomoću veštačke vagine i analiziran pomoću Sperm Class Analyzer. Sadržaj selena je meren u plazmi, krvnim ćelijama i spermi atomskom apsorpcionom spektrometrijom korišćenjem SpectrAA 55B double beam spectrometer (Varian, Inc.) - spektrometar dvostrukog snopa.

Rezultati su pokazali da jedna injekcija organskog jedinjenja selena – selenopirana, u dozi od 0,1 mg/kg žive mase osigurava povećanje i održavanje visokog nivoa selena u krvi (plazma i krvne ćelije) i u spermi tokom perioda ispitivanja. To je dovelo do odgovarajuće spermatogeneze u testisima i omogućilo je proizvodnju kvalitetnih ejakulata sa većim brojem ukupnih spermatozoida, kao i spermatozoida sa progresivnim pokretljivošću.

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PRODUCTIVITY OF MILK AND MILK COMPOSITION OF AN INDIGENOUS SHEEP BREED IN MACEDONIA[1]

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Abstract: Several production traits have been examined in 180 Ovchepolian sheep during a four-year production period (2010–2013). The sheep ranged in age from the first to the 7th lactation and 4319 individual lactation controls were realized in total. Besides the basic statistics, all data were analysed using a multi-factorial fixed model. The influence of certain factors was studied using the F-test and the analyses were made using the SPSS set of programs. Most of the factors (year, lactation, lambing month and number of milk recording) had a highly significant influence ($P<0.001$) on daily milk production (milk from the morning, evening and the total amount of milk, % of milk fat and kg fat) in this breed of sheep. The month of milk recording also had a significant influence ($P<0.05$) on all traits. Only fertility had no impact on the variations in the tested parameters, aside from the total daily milk, on which a highly significant influence was manifested ($P<0.01$). The average milk lactation among the tested sheep population during all four years was, on average, 58 ± 0.247 l, while the production of milked milk for the same period was 37 ± 0.217 l. The length of the lactation period in these sheep for the four years studied averaged 182 ± 0.31 days. The maximal daily milk yield in this sheep population was measured in 2011 (0.302 ± 0.26 l). Regarding their age, the highest daily milk yield was determined in sheep in the third lactation (0.365 ± 0.26), while those in the 7th lactation had the lowest (0.255 ± 0.27).

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Key words: Ovchepolian sheep, daily milk production; fat %; fat kg; influencing factors

Introduction

Of the ten total genotypes (Ovchepolian, Sharplaninian, Karakachanian, Würtemberg, Würtemberg crossbreeds, Awassi, Awassi crossbreeds, East-Friesian, Sardinian crossbreeds, Pleven Blackead sheep) provided in the system for registration and identification in Macedonia, Ovchepolian sheep represent the population with the greatest significance for sheep breeding in the country. Their importance emanates both from their sheer numbers and by virtue of their possessing the largest total productivity. Besides Sharplaninian and Karakachanian sheep, the Ovchepolian sheep is one of three indigenous breeds; therefore, this breed is a target for the protection of biodiversity in sheep breeding in Macedonia. This breed has simple production parameters: 45 kg live weight rams, 36 kg adult sheep. The lactation period is 191 days and average milk lactation (annual) is 72.49 kg, with large variations from 38.74 – 91.28 kg. Fertility is at a high level (100%), while the percentage of twinning is quite low, 5–6%. It is present on about 2/3 of the territory of the country and it represents about 60% of the total sheep population in the Republic of Macedonia *(Porcu and Markovic, 2006)*.

Due to its low production traits, in the past the breed has often been the subject of crossbreeding with other imported breeds (Awassi, Würtemberger and other Merino breeds) in order to advance certain traits (milk yield, live weight, growth, wool, etc.) *(Pacinovski et al., 2006)*. An attempt was made in this work to determine the influence of certain paragenetic factors on daily milk production in this breed of sheep; in other words, an attempt to determine the level of influence of many factors such as genotype, year, lactation, lambing month, month of milk recording, number of milk recording and fertility on daily milk production and the percentage and quantity of milk fat produced.

Keeping in mind the fact that the selection of dairy sheep populations depends greatly on proper control of daily milk production, breeders work constantly on improving the methods for scoring this type of sheep, whereupon as a significant progress in this field is the usage of the so-called “test-day” model. However, according to most of them, success in this selection depends greatly on knowledge of the factors affecting milk production on the day of milk recording, and they influence milk yield throughout lactation and the milking period.

Material and Methods

The basic experimental material was the Ovchepolian sheep, located on a farm in the Shtip surrounding. The number of sheep involved in the four year

survey (2010–2013) were as follows: 167 sheep in 2010, 198 in 2011, 189 in 2012 and 170 in 2013; during four years of production 724 sheep were studied in total (Tab. 1).

Table 1. Number of tested sheep per year

Breed	Year				Total
	2010	2011	2012	2013	
Ovchepolian sheep	167	198	189	170	724

According to age group, the sheep ranged in age from the first to the 7th lactation, with most between the third and the sixth lactation (Tab. 2).

Table 2. The age range of the tested sheep per year

Year	Lactation number							Total
	I	II	III	IV	V	VI	VII	
2010	18	33	59	31	18	8	/	167
2011	30	18	33	59	31	19	8	198
2012	/	30	18	33	59	30	19	189
2013	/	/	30	18	32	59	31	170
Total	48	81	140	141	140	116	58	724

During the four years of production there were 4319 individual lactation tests realized in total, ranging according to age as follows: 287 in the first lactation, 480 in the second, 833 in the third, 839 in the fourth, 841 in the fifth, 697 in the sixth and 342 in the 7th lactation (Tab. 3).

Table 3. Lactation tests on individual sheep

Year	Lactation number							Total
	I	II	III	IV	V	VI	VII	
2010–2013	287	480	833	839	841	697	342	4319

A combined (barn-pasture) farming system that includes use of available vegetation during 7–8 months, while the rest of the year the sheep were additionally fed with meadow hay (November–February) and concentrate (November–April), has been applied on this farm.

Lambs were with their mothers until the age of 2–2.5 months, while milk production by the sheep was followed by a standard A4 method *(ICAR, 2009)* that involves measuring the daily production of milk per head at intervals of 28 to 34 days.

The milk recording started 10 days after lambing and lasted until the moment of drying (early August). During the suckling period, with reference to the moment, the milk recording was made such in a way that the lambs were separated

from their moms for the morning milk control, 12 hours before the milk recording. Afterward, the lambs were returned to their mothers for 24 hours, after which they were separated again for the morning milk control, 12 hours before the evening milk recording was made. There were 6 milk measurements realized in total, and from each of them a collective individual milk sample of 50 ml (at least 25 ml from each milking) was taken for milk fat analysis. Based on these milk yield measurements, the following was calculated:

– Total milk production of one lactation, in litres (l);
– Total milked milk of one lactation, in litres (l);
– Amount of milk consumed by lambs, in litres (l);
– Suckling period length in days;
– Length of lactation, in days.

- The lactation milk is calculated by multiplying the length of a lactation period (days) and an average daily milk production (litres) during the whole lactation, while the milk production during a suckling period is calculated by multiplying the length of a suckling period (days) and an average daily milk production (litres) during the whole lactation.

Concerning the statistical processing, the traits of daily milk production (morning, evening and total amount of milk, % of fat and kg fat) were analysed using the following model:

$$Y = \mu + G_i + Y_j + L_k + MB_l + TD_m + TM_n + F_o + eijklmno$$

where:

– Y is an individual observation of each trait during a (daily) test (milk from the morning, evening and total amount of milk, % of fat, milk fat kg);
– μ is the general common average of the tested traits;
– G_i is the effect of the i-th genotype, with (i = Ovchepolian sheep);
– Y_j is the effect of the j-th year, with (j = 2010, 2011, 2012 and 2013);
– L_k is the effect of the k-th lactation, with (k = 1, 2, 3, 4, 5, 6, 7);
– MB_l is the effect of the l-th lambing month (l = December, January, February, March);
– TD_m is the effect of the m-th recording day, with (m = 1, 2, 3, 4, 5, 6);
– TM_n is the effect of the n-th recording month, with (n = February, March, April, May, June, July);
– F_o is the effect of the o-th number of newborn lambs, with (m = 1, 2);
– eijklmno is the residual influence.

The influence of certain effects was studied using the F-test, while statistical analyses were performed with Statistical Package *SPSS (2004) version 13*.

Results and Discussion

Average values of traits: According to the data in Table 4, the average milk lactation (annual) in the Ovchepolian sheep was 58 ± 0.247 l for the four tested years, while the amount of milked milk produced for the same period was 37 ± 0.217 l. During the suckling period, which is on average 65 days, the lambs from this population had an average lactation of 182 days. The results from our surveys on annual milk production in Ovchepolian sheep are also congruent with those in the literature, with some exceptions.

A slightly higher milk lactation yield (68.31 l) in this sheep population was found by *Todorovski et al., (1988),* but at the same time they found a lower content of milk fat, 5.59%. *Tashkovski et al., (1968)* also found a higher annual milk yield (64.38 l) in Ovchepolian sheep. A similar milk yield in Ovchepolian sheep has also been reported by many other authors, with a few exceptions *(Shokarovski, 1957; Tashkovski, 1962; Tashkovski and Tokovski, 1969; Tokovski et al., 1977; Shokarovski et al., 1992).*

Compared with our results, *Shokarovski et al., (1992)* determined a significantly lower amount of milking milk in a domestic sheep population, whereby the average milked milk of sheep was 17.31 kg.

This is almost a 50% less amount of milking milk with reference to our surveys, which the authors point to as a major reason for the low profitability of sheep farms in the Republic of Macedonia, where this sheep population is farmed.

A very similar amount of milked milk in traditionally and intensively farmed sheep from a domestic population was determined by *Kozarovski et al., (1989),* according to which in the first the average milk yield of 34 l milked milk is determined and in the second it is 28.35l.

Table 4. Descriptive statistical data on the investigated Ovchepolian sheep dairy traits, LS – mean ± SE

Parameter	N	Mean	Min	Max	Std. deviation	Cv
Lactation milk (litres)	181	58 ± 0.247	27	96	10.92	20.60
Suckling milk (litres)	181	21 ± 0.130	9	41	4.69	23.45
Milking milk (litres)	181	37 ± 0.217	14	62	8.25	25.00
Length of suckling period (days)	181	65 ± 0.194	41	75	8.00	14.04
Length of lactation (days)	181	182 ± 0.310	154	204	13.51	7.51
Morning milk yield (litres)	4319	0.152 ± 0.001	0.05	0.36	42.07	27.68
Evening milk yield (litres)	4319	0.169 ± 0.001	0.05	0.40	49.09	29.05
Total daily milk yield (litres)	4319	0.321 ± 0.02	0.06	0.74	88.37	27.62
Fat (%)	4319	7.77 ± 0.010	5.2	9.89	0.70	9.01
Daily fat yield (kg)	4319	0.025 ± 0.002	0.004	0.06	0.006	24.00

The determined annual milk production in the indigenous sheep population tested is significantly less than that found in other autochthon sheep breeds in the Balkans: 100 kg in Lipe sheep in Serbia, 120 kg in Istrian sheep in Croatia, 90–120 kg in Dubian sheep in Bosnia and Hercegovina, 100–110 kg in Pivska sheep in Montenegro, and 90–130 kg in Rechka sheep in Albania etc. *(Porchu and Markovic, 2006)*.

The length of the suckling period as a production stage in sheep breeding depends on many factors. Mainly it is the breeder's decision, on the basis of the farming system used, the breed, the intended use of the lambs (replacement, for meat) etc. Normally, breeding lambs have a longer suckling period than lambs for producing lamb meat. This period is intended to be shorter in the intensive production system in order to increase the profitability of the farm. In any case, this is a very important factor on which the production of milked milk and the growth of the lambs depend. An already proven technology for early-weaned lambs, according to which the amount of suckled milk could be reduced to a minimum and the amount of milked milk to a maximum, was not accepted by sheep breeders in Macedonia and required hiring an additional labour force. Generally, the length of the suckling period according to the traditional technology used in Macedonia is, on average, 2 to 2.5 months, and this technology is used by many sheep breeders in the country *(Shokarovski et al., 1992)*.

Most authors who have worked on establishing the length of lactation in indigenous sheep populations have determined an average length of 180 days, which is in accordance with our survey results *(Tashkovski, 1962; Tokovski et al., 1977; Tokovski, et al., 1988; Todorovski et al., 1996)*. However, there are authors who measured a significantly shorter lactation in this population. *Tashkovski et al., (1968)* reported 162 days of lactation in Ovchepolian sheep. It is hard to determine the reasons for such a brief lactation, but this usually occurs in extremely dry and meagre years when the lambing of the sheep is very late in the year (March–April), so lactation is shortened out of necessity. However, this forced shortening occurs in only a small number of sheep (generally 3–5% of the sheep in the flock) that experienced late fertilization outside of the normal season, so the first assumption remains to be proven.

Most authors who have worked on establishing the percentage of milk fat in domestic sheep populations determined a somewhat lower percentage of milk fat. *Tashkovski et al., (1968)* reported 7.28% in Ovchepolian sheep, while *Tokovski et al., (1988),* measured an average content of milk fat of 5.59 or 5.41 in Ovchepolian sheep and Ovchepolian Pramenka. *Tashkovski, (1962)* determined 5.41% milk fat in Ovchepolian Pramenka, with variations from 4.62% to 6.31%, and, according to *Todorovski et al., (1996),* the percentage of milk fat in Ovchepolian and the Sharplaninian sheep was 5.41 or 6.41%. The smaller percentage of milk yield reported by these authors compared to the higher

percentage obtained from our surveys was due to paragenetic dietary factors, which depend greatly on the content of fat.

Influence of the factors: Analysing the results in Table 5, it can be concluded that most of the factors (year, lactation, lambing month and number of milk recording) have a significant influence ($P < 0.001$) on daily milk production (morning, evening and total amount of milk, % of fat and fat kg) in this sheep breed. The month in which the milk was measured also had a significant influence ($P < 0.001$), except with no effect on the % of milk fat ($P < 0.05$).

Only fertility had no influence on the variation in the parameters examined, with the exception of the total amount of daily milk, on which a highly significant influence was manifested ($P < 0.01$).

The determination coefficients for the examined factors in these sheep ranged from 0.326 for % of milk yield to 0.689 for total daily milk. *Pacinovski et al., (2014)* determined a similar influence of almost all factors mentioned, with the exception of fertility, on daily milk production in Awassi sheep.

Table 5. Factors influencing daily milk production in Ovchepolian sheep, F-test and its significance (F-statistics)

Factor	Df	Morning	Evening	Total	Fat (%)	Fat (kg)
Year	3	3.694*	18.240***	5.525***	79.164***	13.829***
Lactation	6	98.114***	156.477***	140.096***	29.597***	116.759***
Month of lambing	4	10.361***	9.367***	10.763***	9.503***	11.123**
Month of milk recording	6	54.256***	35.890***	49.180***	1.004^{ns}	30.941***
No of milk recordings	6	52.572***	50.380***	56.046***	9.304***	33.870***
Fertility	1	0.00^{ns}	0.273^{ns}	0.078**	1.117^{ns}	0.446^{ns}
R^2 – Coef. of determination	/	0.646	0.685	0.689	0.326	0.627

ns – P > 0.05; * – P < 0.05; ** – P < 0.01; *** – P < 0.001

In Ovchepolian sheep, the year also had a highly significant influence ($P < 0.001$) on most of the daily milk production characteristics examined, with the exception of morning milk, on which a significant influence was manifested ($P < 0.05$) (Tab. 5). *Kastelic et al., (2013)* determined a highly significant influence of the year on milk production and milk yield in Istrian sheep, an indigenous breed in Slovenia and Croatia. *Pacinovski et al., (2013)* determined a similar influence of the year on lactation in East Friesian sheep in Macedonia. According to the data in Table 6, the average daily milk productivity in these sheep was the highest in 2011 (0.302 ± 0.26 l) and the lowest in 2010 (0.285 ± 0.26 l). The percentage of milk fat was also the highest in 2013 ($7.90 \pm 0.10\%$) and the lowest in 2011 and 2012 ($7.59 \pm 0.10\%$). The daily production of milk fat ranged from 0.022 kg in 2010, 2011 and 2012 to 0.024 kg in 2013.

Table 6. The influence of the year on daily milk production in Ovchepolian sheep, LS-mean ± SE

Year	N	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
2010	167	0.137 ± 0.01	0.148 ± 0.01	0.285 ± 0.26	7.82 ± 0.10	0.022 ± 0.02
2011	198	0.136 ± 0.01	0.166 ± 0.01	0.302 ± 0.26	7.59 ± 0.10	0.023 ± 0.02
2012	189	0.134 ± 0.01	0.157 ± 0.01	0.291 ± 0.26	7.59 ± 0.10	0.022 ± 0.02
2013	170	0.141 ± 0.01	0.160 ± 0.01	0.301 ± 0.27	7.90 ± 0.10	0.024 ± 0.02

Tashkovski et al., (1968) determined an average daily milk productivity in Ovchepolian sheep of 0.46 l, comparing the average daily milk production per year. *Tokovski et al., (1988)* determined similar values of milk production, according to which it was 0.39 l and 0.38 l, respectively, in Merino sheep and Ovchepolian sheep. The differences in milk production identified in all years of measurements indicates a strong influence of feeding conditions upon milk production. In our examinations, lactation influenced ($P < 0.001$) all the examined factors significantly (Tab. 5). Average daily milk production in these sheep increased with age up to the third lactation, decreasing in the fourth and then maintaining its stability until the 7th lactation (Tab. 7). The highest expected daily milk production was identified in sheep in their third lactation (0.365 ± 0.26 l) and the lowest in sheep in their 7th lactation (0.255 ± 0.27 l). The percentage of milk fat was highest in sheep in the 7th lactation (7.90 ± 0.10%) and lowest in sheep in the third lactation (7.52 ± 0.10%). Analogous to total daily lactation, the amount of milk fat produced increased from the first to the third lactation, after which it decreased slightly.

Table 7. The influence of lactation on daily milk production in Ovchepolian sheep, LS-mean ± SE

Lactation	N	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
1	287	0.126 ± 0.01	0.140 ± 0.06	0.266 ± 0.28	7.87 ± 0.11	0.021 ± 0.02
2	480	0.132 ± 0.01	0.153 ± 0.06	0.285 ± 0.70	7.64 ± 0.10	0.022 ± 0.02
3	828	0.169 ± 0.01	0.196 ± 0.05	0.365 ± 0.26	7.52 ± 0.10	0.027 ± 0.02
4	833	0.153 ± 0.01	0.180 ± 0.05	0.333 ± 0.26	7.61 ± 0.10	0.025 ± 0.02
5	835	0.137 ± 0.01	0.158 ± 0.06	0.295 ± 0.27	7.72 ± 0.10	0.023 ± 0.02
6	691	0.124 ± 0.01	0.142 ± 0.05	0.266 ± 0.26	7.81 ± 0.10	0.021 ± 0.02
7	342	0.120 ± 0.01	0.135 ± 0.06	0.255 ± 0.27	7.90 ± 0.10	0.020 ± 0.02

In other breeds of sheep farmed in the Mediterranean area such as Massese, Laxta and Comisana sheep, all indigenous breeds of sheep *(Carnicella et al., 1989; Ruiz et al., 2000; Sevi et al., 2000)* determined an increase in milk production from the first to the third or fourth lactation. In the surveys of *Sevi et al., (2000)*, an increase in the amount of milk fat produced from the first to the third lactation was determined.

The lambing month also had a highly significant influence in relation to all factors examined (P < 0.001) (Tab. 5). A highly significant influence of this factor on daily milk production was also determined in another survey in Awassi sheep *(Pacinovski et al., 2014)*.

Analysing this factor, the highest daily milk production in the examined sheep population was recorded in the sheep lambed in March (0.306 ± 0.61), and the lowest in the sheep lambed in December (0.284 ± 0.23 l) (Tab. 8). It was the same with morning and evening milk. The sheep lambed in December had the highest percentage of milk fat (7.93 ± 0.10%), and the sheep lambed in March had the lowest (7.41 ± 0.23%). The amount of milk fat produced was about 0.023 kg per day for all lambing months. In the *Kastelic et al., (2013)* survey, a significant influence of the month or the season of lambing on milk production was also determined.

The month the milk was measured showed a highly significant influence (P < 0.001) in relation to almost all traits in the sheep population examined. This factor had no effect only on the percentage of milk fat (P > 0.05) (Tab.5).

Table 8. The influence of month of lambing on daily milk production in Ovchepolian sheep, LS-mean ± SE

Month of lambing	n	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
12	582	0.134 ± 0.01	0.150 ± 0.01	0.284 ± 0.23	7.93 ± 0.10	0.023 ± 0.02
1	2879	0.135 ± 0.01	0.157 ± 0.01	0.292 ± 0.22	7.77 ± 0.10	0.023 ± 0.02
2	630	0.138 ± 0.01	0.161 ± 0.01	0.299 ± 0.23	7.79 ± 0.10	0.023 ± 0.02
3	205	0.142 ± 0.11	0.164 ± 0.01	0.306 ± 0.61	7.41 ± 0.23	0.023 ± 0.04

Analysing the period from the 2nd to the 7th month of the milk control, the amount of milk decreased continuously in both the morning and evening milking and as a result the daily milk production per sheep also decreased. On the other hand, the percentage of milk fat increased continuously from February to July. This population achieved the largest amount of milk produced per head (0.026 ± 0.11 kg) in February, and the lowest in July (0.019 ± 0.10 kg) (Tab. 9).

Table 9. The influence of the month of milk recording on daily milk production in Ovchepolian sheep, LS-mean ± SE

Month of milk measurement	n	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
2	682	0.173 ± 0.03	0.191 ± 0.03	0.364 ± 1.56	7.31 ± 0.59	0.026 ± 0.11
3	722	0.160 ± 0.02	0.175 ± 0.02	0.335 ± 1.02	7.40 ± 0.39	0.025 ± 0.07
4	723	0.146 ± 0.01	0.161 ± 0.01	0.307 ± 0.50	7.52 ± 0.19	0.023 ± 0.04
5	723	0.131 ± 0.01	0.152 ± 0.01	0.283 ± 0.31	7.80 ± 0.12	0.022 ± 0.02
6	723	0.115 ± 0.01	0.141 ± 0.02	0.256 ± 0.74	7.98 ± 0.28	0.020 ± 0.05
7	723	0.100 ± 0.02	0.126 ± 0.03	0.226 ± 1.29	8.33 ± 0.49	0.019 ± 0.10

The number of milk recording had a highly significant influence ($P < 0.001$) on all parameters examined in Ovchepolian sheep (Tab. 5). A highly significant influence was also determined on the previous two factors (month and number of milk controls) in relation to all characteristics of daily milk production in similar surveys in Awassi sheep *(Pacinovski et al., 2014)*.

As for lactation, it increased up to the third or fourth milk control in the total and morning milk, and then decreased continuously until the sixth milk control (Tab. 10). In the evening milking, lactation increased up to the second milk control, after which it decreased until the last milk control.

The percentage of milk fat was the highest in the second milk control (7.81 ± 0.29%), and lowest in the fifth one (7.40 ± 0.39%). The amount of milk fat produced ranged from 0.020 ± 0.11 kg in the sixth to 0.024 in the second and fourth milk controls (Tab. 10).

Table 10. The influence of the number of milk recording on daily milk production in Ovchepolian sheep, LS-mean ± SE

Number of milk recording	N	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
1	723	0.132 ± 0.02	0.161 ± 0.03	0.293 ± 1.28	7.71 ± 0.49	0.023 ± 0.09
2	723	0.139 ± 0.01	0.169 ± 0.02	0.308 ± 0.75	7.81 ± 0.29	0.024 ± 0.05
3	724	0.141 ± 0.01	0.167 ± 0.01	0.308 ± 0.31	7.77 ± 0.12	0.023 ± 0.02
4	723	0.143 ± 0.01	0.163 ± 0.01	0.306 ± 0.50	7.70 ± 0.19	0.024 ± 0.04
5	722	0.138 ± 0.02	0.151 ± 0.02	0.289 ± 1.01	7.40 ± 0.39	0.021 ± 0.07
6	681	0.130 ± 0.03	0.137 ± 0.03	0.267 ± 1.56	7.63 ± 0.60	0.020 ± 0.11

The fertility or the number of lambs from individual sheep had no effect on any of the traits examined, with the exception of the total amount of milk, which manifested an effect on the level of $P < 0.01$ (Tab. 5).

In these sheep, a higher daily lactation (0.318 ± 0.15 l) was identified in those that had lambed 2 lambs compared with those who had only lambed one (0.313 ± 0.15 l) (Tab. 11).

Table 11. The influence of fertility on daily milk production in Ovchepolian sheep, LS-mean ± SE

Number of lambs	N	Morning (litres)	Evening (litres)	Total (litres)	Fat (%)	Fat (kg)
1	3362	0.149 ± 0.03	0.164 ± 0.01	0.313 ± 0.15	7.76 ± 0.56	0.024 ± 0.01
2	934	0.151 ± 0.03	0.167 ± 0.01	0.318 ± 0.15	7.76 ± 0.58	0.025 ± 0.01

The situation was identical with both morning and evening milk. The percentage of milk fat was the same for both groups of sheep (7.76%). Other authors, *Ruiz et al., (2000)* in Latxa sheep and *Pollot and Gootwine, (2004)* in Assaf sheep, found a significant influence of litter size on milk production. This factor also had a substandard influence on daily milk production in the survey of

Pacinovski et al., (2014) in Awassi sheep, where in relation to certain traits (evening and total amount of milk, daily production and milk fat) it had a significant influence ($P < 0.01$), while in relation to other traits (morning and afternoon milk, percentage of milk fat) it had no influence at all ($P > 0.05$).

Conclusion

The influence of many factors on daily milk production has been researched in Ovchepolian sheep as an indigenous sheep breed in the Republic of Macedonia.

The fact that the year had a highly significant influence ($P < 0.001$) in relation to all traits of annual milk production confirms that the diet must be given a great deal of attention in order to achieve higher milk production, excepting only selection as an important method of advancing genetic milk capacity. Commenting on the influence of lactation i.e. the age of specified traits, it is recommended not to keep animals in production for more than six lactations (7 years old) in this sheep population, except certain individuals that manifest above average milk production.

These females also need additional monitoring in order to serve as prospective ram mothers in further selection.

The average lactation (58 ± 0.247 l) and the determined minimal (27 l) and maximal (96 l) milk production in controlled heads, indicate the existence of large variations and the capacity to achieve significant success in selection in advancing the genetic capacity with the aim to produce milk, by implementing continuous and permanent selection.

Compared to the dairy sheep population in Europe and the world, which produces a significantly larger amount of milk annually and is more profitable from a visionary point of view, we can say that, genetically, Ovchepolian sheep have the potential to increase their milk productivity, but this requires a precise strategy that can predict every step of its realization. Therefore, and because it is an autochthonous breed of sheep that has been farmed on these areas for centuries, the Common European Policy (Cap-Common Agriculture Policy) provides protection for these breeds of sheep.

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Rezime

Ispitivano je nekoliko proizvodnih svojstava (laktacijska proizvodnja mleka, proizvodnja mleka u dojnom periodu, proizvodnja mleka u muznom periodu, dužina dojnog perioda, dužina laktacije, proizvodnja mleka u toku jutarnje muže, proizvodnja mleka u toku večernje muže i dnevna proizvodnja mleka) kod ukupno 180 ovcepoljskih ovaca, u toku četiri proizvodne godine (2010-2013).

Uzrast ovaca bio je od prve do sedme laktacije, i kod njih je bilo realizovano ukupno 4319 individualnih laktacijskih kontrola. Osim bazične statistike, svi podaci su analizirani pomoću višefaktorijalnog fiksnog modela. Uticaj posebnih faktora ispitivan je pomocu F-testa, dok su analize uradjene pomoću programskog paketa SPSS.

Veći broj faktora (godina, laktacija, mesec jagnjenja i broj kontrole mleka), imali su visoko značajni uticaj ($P<0,001$), na dnevnu proizvodnju mleka (jutarnje, večernje i ukupno mleko, % mlečne masti i kg mlečne masti), kod ove rase ovaca. Mesec kontrole mleka u odnosu na sva svojstva imao je visoko značajni uticaj ($P<0,001$), osim na % mlečne masti ($P>0,05$). Jedino plodnost nije imala nikakav uticaj na varijacije ispitivanih parametara, osim na ukupnu dnevnu produkciju mleka, na koju je manifestovala visoko značajni uticaj ($P<0,001$).

Prosečna laktacijska mlečnost kod ispitivane populacije ovaca, sa sve četiri godine u proseku je bila 58 ± 0,247 l, dok je proizvodnja muznog mleka u istom periodu bila 37 ± 0,217 l. Dužina laktacijskog perioda u toku četiri ispitivane godine u proseku je bila 182 ± 0,31 dana.

Maksimalna dnevna mlečnost kod ove rase ovaca, izmerena je u toku 2011. godine (0,302 ± 0,26 l). U odnosu na starost, najveća dnevna mlečnost je utvrdjena kod ovaca u trećoj laktaciji (0,365 ± 0,26), a najniža u sedmoj laktaciji (0,255 ± 0,27).

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CROSSING SYSTEM APPLICATION AND ITS EFFECT ON LAMBS GROWTH TRAITS

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Original scientific paper

Abstract: Success of crossing depends on the breeds used and the genetic distance among them, as well the combining ability of maternal and individual heterosis to make the proper choice of breeds employed in a crossing system. The aim of this study is to find the effect of genotype and crossing system on early weights and daily gain of lambs. The lambs of purebred Pirot Pramenka (P) got the lowest body weight at birth, at 30 days, at 60 days and 90 days with a value of 3.65 kg, 9.48 kg, 14.99 kg and 21.96 kg while purebred Wurttemberg attained the highest body weight at birth of 4.48 kg. The lambs of three bred crossing (PxWxF) highest on BW30, BW60 and BW90 days. The results showed a highly significant difference on average daily gain (ADG) among genotypes ($P < 0.01$) except on the difference between W – PxW of which belongs to the lower border of significant level ($P < 0.05$). The highest difference on ADG was between P – PxWxF (105.950 g), wherein the lowest difference was between W– PxW (9.290 g). It can close that the attained value between pure bred Wurttemberg (W) and two-bred crossing (PxW) was almost equal. Genotype and crossing system have significant effect on body weight and average daily gain of lambs. Based on the results obtained, it shows an advantage effect of crossing system (PxWxF) when it comes on growth and daily gain of lambs.

Key words: crossing system, body weight, growth traits, daily gain, lamb

Introduction

Crossbreeding to exploit heterosis has been practiced for a long time with livestock *(Rastogi et al., 1982)* while crossbreeding systems utilize breed diversity to increase productivity comparable to purebred flocks *(Petrovic et al., 2011; Fathala et al., 2014).* A great number of different factors influence the growth of lambs while nutrition, health condition and genotype belong to the most important ones (*Kuchtík and Dobeš, 2006).* Crossbreeding is used in order to take advantage

of better combinations of the best characteristics of two or more breeds, i.e. breed complementarities and to utilize hybrid vigor, which are translated to improvement of survival, fertility, growth and disease resistance *(Mahmoud Marai et al., 2009.* An important source for increasing sheep production is crossing different breeds of sheep not only meat breeds but also with meat-fat sheep breeds which have high maturity; high feed efficiency and meat productivity *(Fathala et al., 2014).* Crossbreeding, the mating of two individuals with different breed make-up is widely used in commercial sheep production because of the benefits it has to offer to producers (*Atashi and Izadifar, 2012).*

The success of crossing depends on the breeds used and the genetic distance among them. Likewise, the combining ability, maternal and individual heterosis is needed to make the proper choice of breeds employed in a crossing system. The greatest part of sheep breeding income generation is through production and sale of lambs. The autochthonous or as they are also called local populations, that are most numerous in most countries, including ours, have the genetic potential to meet these challenges *(Skalicki et al., 2003; Petrovic et al., 2013).* The productivity of sheep can be improved with the use of prolific ewes, and the crossbred ewes derived from mating local breed to meat-type rams *(Boujenane and Kansari, 2002).* However, not every crossing is suitable for breeding to obtain the desired objective but also the application of two-breed system and three-breed crossing of selected populations of sheep.

The aim of this study is to determine the influence of genotype and crossing system on early weights and daily gain of lambs.

Material and Methods

Investigations carried out at the Stara Planina- Pirot territory and at the Institute for Animal Husbandry, Belgrade Zemun, in three years period.

The research consists of the following genotypes of sheep

a) pure breed:
   Pirot pramenka (P)
   Wuerttemberg sheep (W)

b) b) Crosses:
   Two breed F1 (Pirot pramenka x Wurttemberg sheep) (PW)
   Three breed F1 (Pirot pramenka x Wurttemberg sheep) x Ile de France (PxWxF)

Lambing of sheep took place during the winter period, after natural mating season from June to September. The animals were on pasture in summer months and at winter period remain in the stable. The lambs have a short suckling twice a

day, supplemented too with alfalfa hay meadow and the concentrate mixture for lambs with 18% protein. Feeding has been ad libitum up to the age of lambs of 90 days. From each tested genotype among the observed has taken for investigation and analysis of 200 lambs (F1 generation) per genotype.

To determine the weight of lambs was performed by portable scale (1-30 days) and scales for flocks' accuracy of 0.10 kg (90 days). In order to determine the neonatal development of all genotypes-pure breeds and crossbreeds, the control measurements included the following growth traits of lambs: The body weights of the lambs at birth (BWB), body weight at 30 days (BW30), body weight at 60 days (BW60) and body weight at 90 days (BW90) were recorded.

For consideration of the dynamics of neonatal development of lambs, were determined the values for the following characteristics:

Average Daily Gain (ADG)

- Daily gain of 1- 30 days
- Daily gain of 31- 60 days
- Daily gain of 61- 90 days
- Average daily gain 1-90 days

The average daily gain (ADG) was calculated by getting the difference between the two successive weight divided by the period days. The statistical analysis was performed by GLM procedure of SPSS software package program version 20, using the next model:

$$Y_{eijk} = \mu + G_i + S_j + e_{ijk},$$

where the symbols have the following meanings:

Y_{eijk}- the value characteristics of the j-th crossing system, the i-th genotype

μ - general population average

G_i-fixed genotype effect

S_j- fixed crossing system effect

e_{ijk}- undetermined effects;

Results and Discussion

The lambs' body weights averages according to genotype and crossing system on different ages are shown in Table 1. It can visualize that the lowest body weight at birth, at 30 days, at 60 days and 90 days was on purebred Pirot Pramenka with a value of 3.65 kg, 9.48 kg, 14.99 kg and 21.96 kg. The highest body weight at birth was 4.48 kg attained by lambs of purebred Wurttemberg (W). The lambs

of three-bred crossing (PxWxF) attained the highest on body weight at 30 days, at 60 days and at 90 days.

Table 1. Body weight averages at birth (BWB), ages 30 days (BW30), 60 days (BW60) and 90 days (BW90) of different genotypes.

Genotype/ crossing system	BWB		BW30		BW60		BW90	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
P	3.65	0.03	9.48	0.11	14.99	0.13	21.96	0.24
W	4.48	0.08	10.83	0.17	19.11	0.27	27.70	0.15
PxW	4.17	0.04	10.98	0.11	18.53	0.21	26.55	0.21
PxWxF	4.35	0.04	12.87	0.10	22.01	0.25	32.19	0.20

Zapasnikiene and Nainiene, (2012), stated that the sheep crossbreds had higher body weight at birth than purebred, was amenable in our result (table 1) with lambs of purebred Pirot Pramenka (P) in comparison with the crosses (PxW; PxWxF) except in the case of purebred Wurttemberg (W) lambs where it showed dominantly on body weight at birth, but interesting that after one month, the lambs of crosses PxW and PxWxF increased on body weight (BW30) higher than purebred W. However only the three-bred crossing (PxWxF)) exceeded purebred W on BW60 and BW90. In the study of *Ružić-Muslić et al., (2005)* that three-bred crossing (PxWxF) realized higher body weight in comparison with two-breed crossing (PxW) affirmed the result obtained in this study. In the study of *Petrović et al., (2011),* that lambs of Pirot Pramenka had the lowest body weight from birth to weaning was consistent with the results we obtained.

Table 2. Lambs average daily gain (ADG) per genotype at first month, second month and third month, (g)

Genotype/ crossing system	First month		Second month		Third month	
	Mean	SE	Mean	SE	Mean	SE
P	194.26	3.93	183.81	3.93	232.33	8.50
W	211.85	6.86	275.77	9.39	286.38	9.83
PxW	227.10	3.86	251.76	7.64	267.25	8.99
PxWxF	284.06	3.59	304.60	8.50	339.60	9.90

The lambs' of three-bred crossing (PxWxF) is dominated on the average daily (ADG) for the three entire months (table 2) with an average value of 284.06 g - first month; 304.60 g –second month; 339. 60 g – third month. The second place was the lambs of two bred crossing (PxW) in first month with an ADG of 227.100

g. In the second and third month, the lambs of purebred Wurttemberg (W) took the second place with an ADG value of 275.77 g and 286.38 g. The lambs of purebred Pirot Pramenka (P) got the least values of ADG for the entire months (first- 194.26 g, second- 183.81 g, third- 232.33 g) of observation.

Fathala et al., (2014), noted that body weight gain was significantly higher in crossbred lambs (Crossbreeding Romanov Ewes with Edilbai Rams) in the first and the second periods of their experiment compared to purebred respectively. At the same time, their results revealed significant advantage of crossbred lambs over purebred lambs in average daily gain on both first and second periods of the experiment. The result we attained in our study (table 2) on the ADG of lambs from two-bred and three-bred crossing showed dominantly over purebred on the first period. However, on the second and third period only the lambs of three-bred crossing (PxWxF) took advantage over purebreds.

The difference in ADG between genotypes is presented in table 3, it can notice that between lambs of purebred (W) and two-bred crossing (PxW) showing the least difference in first month -15.255 g (7.20%) in favor of PxW; 24.010 g (9.54%) second month and in third month 19.135 g (7.16%) in favor of W. It also displayed that the difference between lambs of purebred Pirot Pramenka and three-bred crossing (P – PxWxF) acquired the highest ADG difference in first month- 89.795 g (46.22%); second month- 120.785 g (65.71%); 107.265 g (46.17%) in third month. The difference on gain between lambs W and PxW on the first, second and third month was on the lower level of significant difference ($P < 0.05$) as well on the second month between W – PxWxF, while all others have highly significant difference ($P < 0.01$).

Table 3. Values of daily gain differences (-) between genotypes on first, second and third month, (g)

Genotype pairwise	(-) First month	Level of significant difference	(-) Second month	Level of significant difference	(-) Third month	Level of significant difference
P - W	-17.590	P <0.05	-91.955	P <0.01	-54.050	P <0.01
P - PxW	-32.835	P <0.01	-67.945	P <0.01	-34.915	P <0.01
P - PxWxF	-89.795	P <0.01	-120.785	P <0.01	-107.265	P <0.01
W - PxW	-15.255	P <0.05	24.010	P <0.05	19.135	P <0.05
W - PxWxF	-72.215	P <0.01	-28.830	P <0.05	-53.215	P <0.01
PxW - PxWxF	-56.96	P <0.01	-52.840	P <0.01	-72.350	P <0.01

Regarding the average daily gain (ADG) of lambs for three months period (first to third), it can view in table 4. The highest in ADG obtained in PxWxF with a value of 309.42 g while purebred Pirot Pramenka got the lowest ADG value of which was 203.47 g.

Table 4. Lambs daily gain per genotype from first to third month, (g)

Genotype/ crossing system	Mean daily gain First to third month	
	M	SE
P	203.47	2.67
W	258.00	2.12
PxW	248.71	2.38
PxWxF	309.42	2.20

In table 5, the differences on daily gain among genotypes for three months have demonstrated. It showed a highly significant difference on average daily gain among genotypes ($P < 0.01$) except on the difference between W – PxW of which belongs to the lower border of significant level ($P < 0.05$). As exposed the highest difference was between pairwise P – PxWxF (105.950 g), wherein the lowest difference was between pairwise W– PxW (9.290 g).The differences on daily gain in percentages among genotypes of which showed 26,80% (P – W); 22.23% (P – PxW); 52.07% (P – PxWxF); 3.74% (W – PxW); 19.93% (W – PxWxF); 24.41% (PxW – PxWxF). The differences in daily gain among the genotypes it appeared that between lambs of purebred and two-bred crossing got the minimal percentage of difference.

Table 5. Values of differences on daily gain from first to third month between genotypes, (g)

Genotype/ pairwise	difference (-) first to third month	Level of significant difference
P - W	-54.525	$P < 0.01$
P - PxW	-45.235	$P < 0.01$
P - PxWxF	-105.950	$P < 0.01$
W - PxW	9.290	$P < 0.05$
W - PxWxF	-51.425	$P < 0.01$
PxW - PxWxF	-60.715	$P < 0.01$

The results obtained in our study showed that body weight at birth (BWB), body weight at 30 days (BW30), at 60 days (BW60) and at 90 days (BW90) between purebred Wurttemberg (W) and two-bred crossing (PxW) were nearly equal. According to *Atashi and Izadifar (2012),* they informed that "crossbred lambs generally have no considerable advantage over their purebred contemporaries under same environmental conditions", partially agree with our result but only in the case of lambs of two-bred crossing (PxW) vs. lambs of purebred Wurttemberg (W). *Malik et al. (2000),* stated that the weight of lambs at birth definitely depends on the genotype, concurred with our results. The same authors point out as well that the lambs obtained by crossing have higher daily weight gain and increased body weight in relation to the pure breed. We do agree in those statements because it is true with the result attained specially in the case of lambs; P vs. PxW, P vs. PxWxF and W vs. PxWxF.

In the study of *Kuchtík and Dobeš, (2006*), (the crossing between the Improved Wallachian and East Friesian) they found that genotype have no significant effect on the majority of growth traits was not compatible with the results we acquired.

With regards to the result acquired in this study, it show how the ADG of the local breed increased if crossed with foreign breed and demonstrated the advantage effect of three bred crossing when it comes on growth and daily gain of lambs. Our result supported by *Mahmoud Marai et al., (2009); Petrović et al., (2013),* as they emphasized in their note that "crossing over breed occurs to a greater number of genes combinations and to this extent is more likely to express favorable allele carriers of economically important traits".

Other researchers suggest that crossbreds have higher body weight at birth and higher weight gain than to pure breed *(Doloksaribu et al., 2000; Freking et al., 2000; Snowder and Duckett, 2003; Fogarty, 2006, Cloete et al, 2008). Petrovic et al. (2010*), investigated the preferably crossing combinations of Pramenka breed with foreign population-Wurttemberg and Ile de France sheep whose genetic distance will allow achieving better results. They had come to the conclusion that due to the impact of positive heterosis individuals (100%) and one parent heterosis (100%), lambs realize a high daily gain and high final body weight. This is true with the result we obtained on three-bred crossing (PxWxF). In the same manner as mentioned by *Rastogi et al., (1982)* in which breeds are combined in a three-way cross is important and should be considered in the design of breeding programs.

Conclusion

The results attained on body weights between lambs' pure bred Wurttemberg (W) and two-bred crossing (PxW) has very close values, as well as their average daily gain for three months period, the difference was on the lower

border of significant level with a value of only 9.29 g (3.74%). It can close that the values attained between pure breed Wurttemberg (W) and two-bred crossing (PxW) was almost equal. Genotype and crossing system had significant effect on body weight of lambs and average daily gain. Results of the study has shown the advantage of three-breed crossing when it comes to body weight and daily gains of lambs, which is most importantly from the perspective of production economy. Likewise, the important comparison between the two-breed crossbred (PxW) and purebred Wuerttemberg (W), where the differences are small.

In the application of crossing and crossing system, it is therefore necessary to select the right population to fulfill its goal in sheep breeding.

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Primena sistema ukrštanja i njegov uticaj na osobine porasta jagnjadi

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Rezime

Uspeh ukrštanja zavisi od rasa koje se koriste i genetičke distance između njih. Takođe, efekti majke i individualnog heterozisa u kombinaciji sa izborom rasa odlučujući su faktori ukrštanja. Jagnjad Pirotske pramenke (P) imala su najnižu telesnu masu na rođenju, sa 30, 60 i 90 dana u vrednosti od 3.65 kg, 9.48 kg, 14,99 kg i 21.96 kg, dok rasa Virtemberg ima najveću telesnu masu na rođenju čija vrednost iznosi 4.48 kg. U jagnjadi trorasnog porekla (PxWxF) masa je bila najveća tokom kontrola- BV30, BV60 i BV90 dana. Rezultati su pokazali izuzetno značajnu razliku u prosečnom dnevnom prirastu (ADG) kod ispitivanih genotipova ($P<0,01$) osim razlike između W- PxW koja je na nivou granice značajnosti ($P<0,05$) . Najveća razlika prosečnog dnevnog prirasta ADG bila je između P - PxWxF (105,950 g), dok je najmanja razlika bila između W- PXW (9,290 g). Može se zaključiti da je dostignuta vrednost između jagnjadi Virtemberške rase (-W) i dvorasnih meleza (PxW) skoro jednaka. Genotip i sistem ukrštanja imaju značajan uticaj na telesne mase i prosečnan dnevni prirast jagnjadi. Rezultati ovih

istraživanja pokazali su prednost trorasnog ukrštanja kada su u pitanju masa tela i dnevni prirast jagnjadi, što je i najvažnije iz ugla ekonomike proizvodnje. Takođe je važno poređenje između dvorasnih meleza i čiste virtemberške rase, gde su razlike male.

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TICK FAUNA OF THE AUTOCHTHONOUS ZACKEL SHEEP IN SOUTH SERBIA REGION[1]

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Original Scientific Paper

Abstract: Sheep production is an example of a sustainable production fully integrated within the local rural development. One of the main threats on the outdoor breeding of sheep is parasitism. Ticks are nuisances and vectors of several diseases agents. The distribution of ticks appears to be changing, with spread to previously unaffected areas. Tick and tick-borne disease control is one of the major components of animal health program protecting livestock in the developing countries, which reflects impact on the livelihood of resource-poor farming communities. Taking into consideration the negative impact on the health status of the livestock, also the direct and indirect economic losses, it is necessary to examine the tolerance and resistance of certain species against diseases. It is one of the most important elements of the strategy of selection and screening for resistant animals. The aim of this study was to determine the tick species persisting in 45 tested autochthonous Zackel sheep flocks, and examine their seasonal occurrence from March 2010 to January 2011, in the region of South Serbia. The result showed that *Ixodes, Dermacentor, Ripicephalus* and *Haemaphysalis* were the most abundant ticks found, affecting 50.40% tested sheep. The result of this study is a survey of tick species from autochthonous Zackel sheep in Serbia and implication of possible preventions measures for diseases caused and transmitted by ticks.

Key words: tick, autochthonous Zackel sheep, South Serbia

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Introduction

Ticks are a widespread problem for livestock producers. They also spread a number of serious diseases, the most notable being anaplasmosis, babesiosis, theileriosis and babesiosis (*Dimitrić, 1999, Zintl et al., 2003, Pavlović et al., 2003; Kocan et al., 2008, Nuttall and Labuda 2008, Telford III and Goethert, 2008, Neider et al., 2013).*

The presence of specific tick species varies with agro ecological conditions, some being more widely distributed than others. As a result of global climate change, the distribution of ticks appears to be changing, with spread to previously unaffected areas. Tick and tick-borne disease control is one of the major components of animal health programs protecting livestock in the developing countries, which reflects impact on the livelihood of farming communities. Given the importance of health problems and economic losses Tick and tick-borne disease causes small ruminant production carried out a more detailed assessment of the situation and are determined by the tick species parasiting in sheep and goat breeds reared in South Serbia region (*Dimitrić, 1999; Pavlović et al., 2009*).

Material and Methods

The study about tick fauna and season distribution of tick of in South Serbia was started in March 2010 and finished in January 2011. During study there were examined a total of 248 adult Zackel sheep from 45 flocks, originating from different municipalities of South Serbia. Ticks were collected from sheep by means lightly sprung forceps. All specimens were placed into glass specimen bottles which had a piece of hard paper inserted bearing the name of locality name of host and date and hour of collection. The tick species were detected using keys given by *Pomerancev (1950*), *Kapustin (1955)* and *Kolonin (2009).*

Results

In all 45 tested autochthonous Zackel sheep flocks (100%) tick infestation was present. Ticks were found in a total of 125 sheep (50.40%). Most abudant were *Ixodes ricinus,* followed by *Dermacentor marginatus, Rhipicephalus sanguineus, R.bursa, Haemaphysalis punctata* i *Dermacentor recticulatus.* The collected tick specimens, a total of 1789 were adults, females and males belonging to the *Ixodidae* family.

The population dynamics of recorded tick species are known for their two maxima a year, in spring (April-May) and in autumn (September-October). The considerable interchange between spring and autumn tick populations can be

attributed mainly to environmental conditions. Three species *D.r marginatus*, *D.recticulatus* and *H. punctata* occurred population maximum in April. Peak for *I.ricinus* occurred in May and it was noted that this species started to decrease in abundance in June. *R. sanguineus* and *R.bursa* after reaching their maxima start decreasing gradually until August, and disappearing completely in September and October.

There was registered an autumn population peak in September and in October, mainly for the *I.ricinus*, *D. marginatus* and *H. punctata.*

Moreover, the sex ratio within individual species showed a higher number of females in four species *(I. ricinus, H. punctata, R. sanguineus* and *D. marginatus)*, a higher number of males in *R. bursa* and an equal number of ticks of the two sexes in one species, *D. recticulatus.* Ticks were found on 50.40% of examined sheep. Relative abundance analysis revealed that the species at sheep *I. ricinus* was absolutely dominant 40.32%, followed by *D. marginatus* (29.03%), *R. bursa* (18.54%), *R.sanguineus* (6.05%), *H. punctata* (1.61%) and *D.recticulatus* (1.21%) (Figure 1).

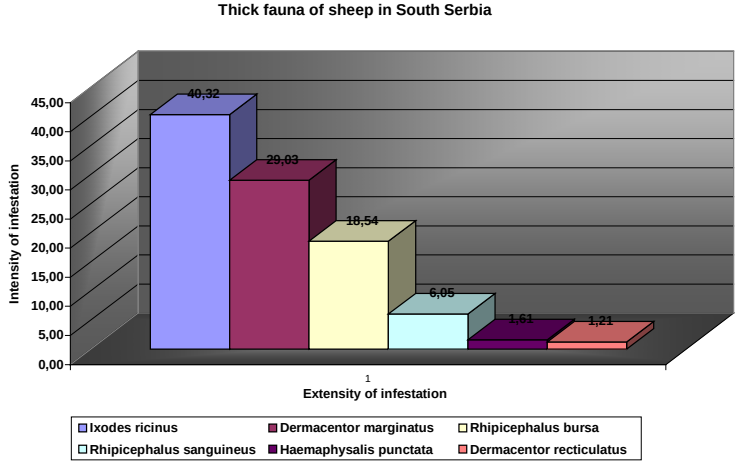

Figure 1. Detected tick fauna of autochthonous Zackel sheep in South Serbia (infestation %)

Discussion

Rearing of autochthonous Zackel sheep in South Serbia is an example of sustainable production fully integrated within the local rural development. One of the main threats on the outdoor breeding of sheep is parasitism. During study

performed from March 2010 to January 2011, in the region of South Serbia we established that *Ixodes, Dermacentor, Ripicephalus* and *Haemaphysalis* were the most abundant ticks found in all 45 tested flocks (100%), affecting 125 tested sheep (50,40%). Similar results we obtained during examination of ticks fauna in west and east part of Serbia where *I. ricinus* and *D. marginatus* were dominant tick species at sheep (*Milutinović et al., 1996, 1998*). Also, examination was done in Belgrade area by *Milutinović et al. (1997*), *Dimitrić (1999)* and later by *Pavlović et al. (1999, 2002),* and it was obtained that the most abundant tick species were *I.ricinus. R.sanguineus, D.recticulatus* and *D.marginatus*. Those tick species were also diagnosed in dog population and at foxes and badgers, hunted at spread Belgrade area (*Pavlović et al., 1997b*), also in goats and sheep flocks (*Dimitrić, 1999*). In this study, over ten years after previous investigation, it was determined that the situation has not changed in terms of ticks fauna and its number and confirmed dominate role of *I.ricinus* and *Rhipicephalus* species at Belgrade area (*Pavlović et al., 2013*). These findings are of valuable epidemiological importance because these types of ticks are vectors for a multitude vector borne diseases and zoonotises, like *Borellia burgdefori, Erlicihia spp., Anaplasma spp.,* Tick-born encephalitis, numerous haemorrhagic fewer, etc.

Taking into consideration the negative impact on the health status of the livestock, also the direct and indirect economic losses, it is necessary to examine the tolerance and resistance of sheep breeds against tick infestations. It is one of the most important element of the strategy of selection and screening for resistant animals. Existing research results necessitate further investigation of the characteristics of health, tolerance and resistance to tick infection considering breed and individual animal genetic variation of sheep in various regions of Serbia. Resistance or tolerance to ticks, and to a lesser extent to tick-borne diseases, is well documented especially in cattle breeds (*Bishop 2003; Samish, 2008; Nickolas, 2009*). Autochthonous breeds, adapted to the local fauna and flora, are much more resistant to parasites than exotic breeds. Results show higher susceptibility for parasitic infections of exotic sheep breeds compared to autochthonous Zackel sheep types (*Dimitrijević et al., 2012*).

The negative impact of widespread drug use and the related costs of treating infectious and parasitic diseases are well known. Current strategies for increasing the level of bio-security and health management in populations of domesticated animals strives for not only more rational utilization of drugs, but also towards increasingly more sophisticated use of genetic methods in disease control among farm animal species (*Gibson and Bishop, 2005*). Genetic investigations involving animal resistance to infections caused by pathogens of varying etiologies can be determined at three genetic levels: species, breed and individual animal genetic variation (*Anderson, 1979*). The impact of genetic resistance towards a causative agent of disease is greatest in cases where all levels of genetic resistance act synergistically (*Bishop, 2002, 2010*). When considering

the significance of resistance/tolerance at the breed level, the instrinsic evolutionalry advantage of breeds that are adapted to an environment should be taken into account. In tropical regions, where extreme endemic diseases are widesdpread, due to their evolutionary roots, locally adapted autonomous breeds display a far greater level of genetic resistance and adaptation, as compared to imported breeds. Individual variability and the identification of those individuals whose resistance to disease can be determined through clinical examination, or the use of genentic markers (marker assited slection), represents the first step in the formation of genetic resistance within a population (*Anderson, 1979*). Depending upon disease etiology and the availably animal genetic resources, the strategy for advancing genetic control of disease can be established through the following initiatives: the selection of locally adapted breeds, the implementation of cross-breeding methods geared at introducing genes significant in the expression of genetic resistance/tolerance towards pathogens, and the selection of individuals highly resistant to pathogens (*Bishop, 2003*).

Conclusion

During study performed from March 2010 to January 2011, in the region of South Serbia we established that *Ixodes*, *Dermacentor*, *Ripicephalus* and *Haemaphysalis* were the most abundant ticks found in all 45 tested flocks, affecting 50.40% tested sheep. Existing research results necessitate further investigation of the characteristics of health, tolerance and resistance to tick infection considering breed and individual animal genetic variation of sheep in various regions of Serbia.

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Krpeljska fauna autohtone pramenke u Istočnoj Srbiji

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Rezime

Ovčarstvo predstavlja primer održive proizvodnje koja čini sastavni deo ruralnog razvoja. Jedan od glavnih zdravstvenih problema kod ekstenzivnog načina uzgoja ovaca predstavljaju parazitske infekcije. Krpelji su vektori za uzročnike mnogobrojnih oboljenja. Rasprostranjenost krpelja se menja i u novije vreme ih nalazimo i na novim arealima. U zemljama u razvoju, borba protiv krpelja i oboljenja prenosica krpeljima predstavljaju jedan od glavnih strateških tačaka zdravstvenog nadzora nad životinjama i ljudima. Uzimajući u obzir značaj direktnih i indirektnih ekonomskih gubitaka izazvanih krpeljima i oboljenjima čiji su oni uzročnici, posebna pažnja treba da se posveti ispitivanju tolerancije i otpornosti ka parazitskim bolestima pojedinnih vrsta i rasa životinja. Potraga za otpornim jedinkama i njihova selekcija treba da bude deo strategije stočarstva. Cilj ovog rada je bio da prikaže rezultate ispitivanja o prisustvu krpeljske faune u 45 zapata ovaca autohtone pramenke. Ispitana je sezonalna distribucija pojave pojedinih krpelja u periodu između marta 2010. i januara 2011. godine, u regionu Istočne Srbije. Kod 50, 40% ispitanih ovaca ustanovljeno je prisustvo krpelja. Rezultati pokazuju da su krpelji iz rodova *Ixodes, Dermacentor, Ripicephalus* i *Haemaphysalis* najučestaliji u zapatima ovaca autohtone pramenke.

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THE POTENTIALS OF USING SELECTION INDEX IN THE ASSESSMENT OF BREEDING VALUES OF HOLSTEIN BREEDS IN SERBIA

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Original scientific paper

Abstract: The conducted research was aimed at constructing equations of selection index that would be used in the selection of the Holstein-Friesian breed animals in Serbia. The construction of the selection index includes the most important milk traits observed in standard lactation: milk yield (MY305), milk fat content (% MF305) and protein content (% MP305). The variance and covariance necessary for the construction of selection index are calculated using the mixed model by the method of least squares. The economic value of traits is expressed as a ratio of relative changes in costs per unit of traits included in the selection index. Livestock included in the research produced, in the first standard lactation, an average of 7681 kg of milk with 3.58% milk fat and 3.28% protein. The equation of the selection index presented in the paper is selected from the group of equations of selection index, as an equation with the highest correlation between the equation and the aggregate genotype, which amounted to 0.2156.

Key words: selection index, breeding value, milk traits, Holstein breed

Introduction

Breeding domestic animals is a very complex zootechnical procedure both in terms of objectives to be achieved and in the methods used. A large number of participants are involved in this business, ranging from breeders, basic, regional and national breeding organizations, centres for artificial insemination, professional and scientific organizations and universities, who define breeding objectives in accordance with the breeding program (*Stanojevic et al. 2015*).

In designing the breeding programs, selection objectives are defined which involve a larger number of traits that affect the economic efficiency of cattle

production. One of the key issues when choosing parental pairs is the identification of genetic superior livestock that possess genes whose frequency we want to raise in the next generation. One of the most efficient ways for the assessment of breeding values for a larger number of traits is the use of the selection index (*Sölkner et al., 2000*). The selection index was used for the first time in the selection of plants, whereas *Hazel and Lush (1942)* were the first to use this method in the selection of domestic animals.

The selection index combines production levels of two or more traits. The result of the selection index is the score whose basis serves for ranking and selecting livestock. In assessing the breeding value, selection index takes into account the economic value of all traits included in the index and their manifestation, heredity and connection and combines it all into one single value (selection score) that we use for ranking livestock when selecting (*Hazel and Lush, 1942; Hazel 1943*). Another advantage is its relatively simple application when the equation of selection index is determined (*Radojkovic et al., 2010; Popovac et al., 2014*). Thus obtained score is in maximum correlation with the genetic contribution of the individual unit. When assessing the breeding value by using the selection index, one can use the data on production results of the very individual unit and its relatives.

In the past, the largest number of selection indices was designed in a way to include only productive traits such as milk yield, milk fat and proteins content (*Miglior et al., 2005*). Modern breeding programs have repositioned the focus from solely milk production traits to also include functional traits, longevity and traits of the type. Thus defined breeding programs are aimed at creating a healthier and economically efficient livestock units.

The aim of this work is to design equations of selection index with differently expressed economic values of traits and their use in assessing the breeding value of cows and bulls of the Holstein breed.

Materials and methods

In the design of selection indices it is necessary to know the values of genetic and phenotypic variances and covariances, as well as the economic value for each trait involved in the design of selection index equation. For calculating the genetic and phenotypic variance and covariance, the research used production results that were achieved by 5123 primiparous in standard lactation. These livestock produced on 7 farms of the Agricultural Corporation Belgrade from 2006 to 2012. All the livestock were under milk yield control. The livestock were

offspring to 53 bull-fathers, of which each bull had at least 5 daughters, while the average number of daughters per bull was 96.7.

The breeding value rated by the selection index method can be represented by the following general equation:

$$I = b_1 (X_1 - \overline{\overline{X}}_1) + b_2 (X_2 - \overline{\overline{X}}_2) + \ldots\ldots + b_n (X_n - \overline{\overline{X}}_n)$$

where:

I – relative breeding value of the livestock unit estimated by the selection index, i.e. the value of selection index determined for the given livestock,

b_i – multiple regression coefficients for each trait included in the selection index,

$(X_i - \overline{\overline{X}}_i)$ – the difference between the phenotypic value of the trait included in the selection index for a given individual and the population average for a given trait

The construction of the selection index includes traits of primary importance regarding milk production, namely: milk yield (MY305), milk fat content (%MF305) and protein content (%MP305). All properties were observed in standard lactation.

The values of genetic and phenotypic variances and covariances were calculated using the least squares method (Harvey, 1990) and by using the following mixed model:

$$Y_{ijklm} = \mu + F_j + G_k + S_l + U_m + o_i + e_{ijklm,}$$

Where:

Y_{ijklm} – phenotypic manifestation of surveyed traits,

μ-general population average,

F_j-fixed effect of the j farm,

G_k- fixed effect of the k year of calving,

S_l- fixed effect of the l calving season,

U_m- fixed effect of the m groups based on the age at first calving (I-age at first calving less than 24 months, II-age at first calving from 24 to 29

months, III-age at first calving from 29 to 35 months, IV- age at first calving from 35 to 41 months, V-age at first calving over 41 months),

o_i- random effect of the i sire,

e_{ijklm}- random error.

In the absence of stable market relations over a longer period of milk production in our country, which are essential for determining the economic value of traits in terms of use of bio-economic model, this paper uses a methodology which is based on the use of the relationship between costs and expression of traits (*Radojkovic 2000, Vukelic et al., 2004; Popovac et al. 2014*). The economic value expressed in this way represents a relative economic indicator.

In calculating the economic value of traits included in the construction of the equation of selection index, the starting point was that all the traits included in the selection index are registered in standard lactation, and that all livestock achieved 305 feeding days during standard lactation. The main economic assumption is that the costs of one feeding day as economic size are the same throughout the surveyed period, which is not the case in practice, but higher costs at the initial phase of lactation compensate to some extent lower costs at later stages.

The economic value is expressed as the difference in costs per trait unit that appeared as a consequence of the implementation of the breeding program. Milk yield in standard lactation (MY305) served as the primary trait in the research. The breeding objective here was set as milk yield of 9000 kg of milk with 3.70% milk fat and 3.40 protein. The economic value of traits included in the construction of the selection index is obtained by comparing the relative indicators of cost reduction between the primary trait and other two traits which appears after the implementation of the breeding program. The economic values of observed traits are given in Table 1 and Table 1a:

Table 1. Relative economic value of traits (REV_1)

Trait	MY305 (kg)	%MF305 (%)	%MP305 (%)
Economic value of traits	1	980	1170

The economic value of traits included in the construction of the selection index is also calculated according to the method used by *Sharma and Basu (1986), Falconer and Mackay (1997)* and *Cameron (1997)*, where the relative economic value is expressed as $1/\sigma_p$, where σ_p is the phenotypic standard deviation of the observed trait.

Table 1a. Relative economic value of traits (REV_2)

Trait	σ_p	$1/\sigma_p$	REV_2
MY305 (kg)	1322	0,0007564	1
%MF305 (kg)	0,18	5,5556	7344,8
%MP305 (kg)	0,11	9,0909	12018,6

Results and Discussion

Table 2 shows the average values and variability of milk traits in standard lactation achieved by the livestock included in the research:

Table 2. Average values and variability of milk traits in standard lactation

Trait	n	$\bar{x}$	SD	Cv (%)
MY305 (kg)	5123	7681	1322	17,21
%MF305 (%)		3,58	0,18	5,10
%MP305 (%)		3,28	0,11	3,26

Livestock included in the research had, in standard lactation, an average production of 7681 kg of milk with 3.58% milk fat and 3.28% protein. The determined values are significantly higher than the values identified on the same population in the research by *Đedović (2000)* and *Beskorovajni (2000)*, while the given results are in accordance with the results determined by *Carlen et al. (2004)* and *Stanojević et al. (2012).*

Table 3 shows the results of examining the impact of factors on the traits included in the research.

Table 3. The values of F-tests for examined factors

Trait	F-value			
	Farm	Year	Season	Age at 1st calving
MY305	$0,581^{nz}$	$11,92^{**}$	$11,40^{**}$	$5,05^{**}$
%MF305	$0,82^{nz}$	$7,38^{**}$	$6,47^{**}$	$1,13^{nz}$
%MP305	$0,83^{nz}$	$9,40^{**}$	$4,88^{*}$	$14,98^{**}$

The farm as a factor showed no statistically significant effect on observed milk traits, while other factors showed statistically significant effects on observed traits, except in the case of age which had no statistically significant effect on milk fat content.

Table 4 shows the values of variance and heritability coefficients for observed traits.

Table 4. The values of variance and heritability coefficients of milk production traits

Traits	σ_a^2	σ_a^2	h^2	S_h^2
MY305	75299,61	1825445,1	0,165	0,036
%MF305	0,00087	0,03412	0,102	0,026
%MP305	0,00019	0,01169	0,065	0,020

The values of heritability coefficients had their values from 0,065 in terms of protein content in milk to 0,165 in terms of milk yield. The determined values of heritability coefficients are significantly lower compared to the results gained by *Carlen et al. (2004)* and *Pham Manh Hung et al. (2008).* Similar values were achieved in the research by *Stanojevic et al. (2012)* and *Đedović et al. (2013).*The determined values of heritability coefficients of milk production traits suggest the possibility of their improvement through selection, even though mainly external environmental factors influence their expression.

Table 5 shows the values of coefficients of phenotypic (above the diagonal) and genetic (below the diagonal) correlations.

Table 5. **The values of coefficients of genetic and phenotypic correlations of milk production traits**

Trait	MY305	%MF305	%MP305
MY305	-	-0,095	-0,209
%MF305	-0,1	-	0,002
%MP305	0,074	-0,265	-

The determined values of genetic correlations had their value from -0.265, in terms of milk fat content and protein content in standard lactation, to 0.074 in terms of genetic correlation between milk yield and protein content in standard lactation. The determined values of genetic correlations between milk production traits are close to the values obtained in the research by *Pantelić (2011)* and *Pham Manh Hung et al. (2008)*. The values of the phenotypic correlation are small close values to the values of genetic correlations and are consistent with the values obtained by *Nistor et al. (2009).*

After solving the system of normal equations and determining the coefficients of multiple regression, the equation of selection index was constructed with the following form:

Table 6. **The equation of selection index and the value of the coefficient of correlation index with aggregate genotype (r_{IAG})**

REV	Selection indices equations	r_{IAG}
REV_1	$I=0,046^1(X_1-7681)+28,395^2(X_2-3,58)+157,44^3(X_3-3,28)$	0,2156
REV_2	$I=0,048^1(X_1-7681)+161,635^2(X_2-3,58)+281,772(X_3-3,28)$	0,1942

[1]- the coefficient of multiple regression of coefficients for MY305, [2]- multiple regression coefficient for %MF305, [3]-multiple regression coefficient for %MP305, $X_{1,2,3}$- phenotypic value of the livestock for traits included in the SI

Presented equations of selection indices are selected from the group of equations of selection index as equations with the highest coefficient of correlation between the equation and aggregate genotype, which amounted to 0.2156 or 0.1942 for second equation of SI. Constructed equations combine, in an optimal way, phenotypic expression levels of the three traits that are included in it, where the resulting score is in maximum correlation with the breeding value of the livestock.

Conclusion

The research determined an average milk yield in the first standard lactation of 7681 kg of milk with 3.58% milk fat and 3.28% protein. The research identified a high variability of milk production traits, which is, on one hand, conditioned by hereditary factors and, on the other hand, environmental factors. The observed milk production traits were highly statistically influenced by the year of calving and calving season, as well as the age at first calving. The heritability coefficients calculated in the research indicate that the observed milk production traits in the studied population are hereditary low.

Constructed equations of the selection index should serve as a simple and fast way to rank cows in their selection as parents of the next generation. Also, the constructed equations of selection index only include milk production traits. A selection conducted in this way would be one-sided and, in the future, there should be more work on the inclusion of a certain number of traits such as reproductive traits and fitness traits in the construction of selection index. Special attention should also be given to the introduction of more complex and precise methods for assessment of breeding values such as BLUP and BLUP AM method.

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Mogućnosti upotrebe selekcijskog indeksa u proceni priplodne vrednosti krava holštajn rase u Srbiji

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Rezime

Sprovedeno istraživanje imalo je za cilj konstruisanje jednačine selekcijskog indeksa koja bi se koristila u odabiru grla holštajn-frizijske rase u Srbiji. U konstrukciju selekcijskog indeksa uključene su najvažnije osobine mlečnosti posmatrane u standardnoj laktaciji: prinos mleka (PM305), sadržaj mlečne masti (%MM305) i sadržaj proteina (%MP305).

Varijanse i kovarijanse neophodne za konstrukciju selekcijskog indeksa izračunate su primenom mešovitog modela metodom najmanjih kvadrata. Ekonomska vrednost osobina je izražena kao odnos relativne promene troškova po jedinici osobina uključenih u selekcijski indeks.

Grla obuhvaćena istraživanjem prosečno su proizvela u prvoj standardnoj laktaciji 7681 kg mleka sa 3,58 % mlečne masti i 3,28% proteina. Jednačina selekcijskog indeksa prikazana u radu odabrana je iz grupe jednačina selekcijskog indeksa, kao jednačina sa najvećom korelacijom između jednačine i agregatnog genotipa, koja je iznosila 0,2156.

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ELECTRICAL CONDUCTIVITY OF MILK AND BACTERIOLOGICAL FINDINGS IN COWS WITH SUBCLINICAL MASTITIS

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Abstract: Intramammary infections change the composition of milk and increase electrical conductivity of milk and decrease milk electrical resistance. Electrical conductivity has been used to detect mastitis during last four decades. The aim of this research was to examine the reliability of the milk electrical conductivity measuring in detection of subclinical mastitis. The experiment was conducted on a dairy farm of Holstein-Friesian breed. A total of 113 quarter milk samples were examined, 55 samples from cows in first stage of lactation and 58 from cows in third stage of lactation. Electrical conductivity (EC) of milk samples was detected by Hand-held EC meter (Draminski mastitis detector). Quarter milk samples for bacteriological analysis were taken aseptically during the morning milking in sterile test tubes. Bacteria growth was not detected in 60 quarter milk samples (53.1%), while in the other 53 samples bacteria was found (46.9%). The most common isolated bacteria in first and third stage of lactation was *Corynebacterium* spp. (38.9%) and coagulase - negative staphylococci (3.54%). High quality and healthy milk with Draminski mastitis detector was observed in 59.29% of the samples (67/113). Cows with mastitis may not always show an increased EC of milk from the infected quarter. Electrical conductivity of milk can give useful informations about udder health status, but hand-held EC meters, such as Draminski mastitis detector, cannot be used alone in diagnosis of subclinical mastitis.

Key words: electrical conductivity, Draminski mastitis detector, subclinical mastitis, cow

Introduction

In dairy industry, intramammary infections (IMI) are among the most important diseases of cows that cause great economic losses (*Boboš et al., 2013*). Mastitis is a response of udder to different internal and external factors (*Varatanović et al., 2010*), and substantially affects on the milk quality and production of dairy cow. Bacterial pathogens are major threat to mammary gland, which cause irritation and pathological changes in mammary tissue. The degree of changes depend on the pathogenicity of bacteria and the inflammatory response (*Sharif and Muhammad, 2008*). Clinical mastitis is easy to detect by clinical signs of the disease (colored and painful udder, oedema, watery appearance of milk, milk with flakes, clots or pus), but subclinical mastitis is difficult to diagnose. Subclinical mastitis is 15 to 40 times more common than the clinical form (*Jasper et al., 1982; Kelly et al., 2011*). The most important major mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* (*Katić, 2012*). In the last few years, there has been recorded an increase in udder infections with minor mastitis pathogens - *Corynebacterium* spp. and coagulase - negative staphylococci (*Indriss et al., 2013*). Mastitis caused by minor mastitis pathogens is typically a mild, subclinical reaction that is associated with increased milk somatic cell count (*Reyher et al., 2012*).

IMI changes the composition of milk, they can increase somatic cells count and electrical conductivity of the milk (*Pyörälä, 2003*; *Shahid et al., 2011*). Over fifty years, somatic cell count (SCC) is a useful indicator of the health status of mammary gland. SCC in milk from healthy quarters is less than 200 000 cells/mL. Healthy udder quarter contains only 1-11% neutrophils, but, during inflammation, the proportion of neutrophils increases over 90% (*Sharif and Muhammad, 2008*). Beside intramammary infection, the stage of lactation, age of cows, chronic diseases, mechanical and thermal irritations of udder tissue affect the somatic cell count. SCC is high immediately after parturition and increases slightly to the end of the lactation.

Electrical conductivity (EC) has been used to detect mastitis during last four decades (*Linzell and Peaker, 1975; Hamann and Zecconi, 1998*). EC is determined by the concentration of anions and cations in milk. Concentration of sodium and chloride ions increases in milk from infected quarters which leads to increased electrical conductivity of milk (*Kitchen, 1981*). As a results of the damage to the udder tissue, concentration of lactose and potassium decrease, and concentration of sodium and chloride increase. EC of milk can show substantial variation in the absence of mastitis due to factors such as lactation stage, age of the cow, milking interval and oestrus (*Biggadike et al., 2000*). Also, factors such as milk temperature, pH and fat concentration in milk have influence on the measurement of EC. Electrical conductivity of milk has a positive correlation with somatic cell count.

To the best of author's knowledge, no reports have reported the diagnostic application of measuring milk electrical conductivity in cows with subclinical mastitis in Republic of Serbia. The aim of this research was to examine the reliability of the milk electrical conductivity measuring in detection of subclinical mastitis.

Material and Methods

The experiment was conducted on a dairy farm of Holstein-Friesian breed. General condition and udder status were evaluated by clinical examination of animals. Udders of cows were examined visually and by palpating for the presence of any udder changes (redness, swelling, pain, heat). Also, milk samples from each quarters were examined for the presence of flakes and clots. Animals with visible signs of inflammation were not included in the study. Following the production cycle, milk samples were taken from cows in stage 1 - peak (0-50 d) and from the same cows in stage 3 - late lactation (121-200d) (*Novak et al., 2009*). A total of 113 quarter milk samples were examined, 55 samples from cows in stage 1 and 58 from stage 3.

Electrical resistance of milk samples was detected by Hand-held EC meter (Draminski mastitis detector, Poland). The results of milk electrical resistance measured with the Draminski mastitis detector were interpreted according to the manufacturer's instructions (Table 1). Concentration of sodium and chloride ions increases in milk from infected quarters which leads to increased electrical conductivity of milk and decreased milk electrical resistance.

Table 1. Interpretation of results obtained with Draminski mastitis detector (www.draminski.com)

Readings	Interpretation of results
Above 300 units	The milk sample is of high quality and is healthy. The incidence of subclinical mastitis is very low
Between 250 and 300 units	A progressively increasing incidence of subclinical infection as readings decrease
Below 250 units	This is an indication of a rapid increase in the severity of infection as subclinical mastitis progresses to clinical states. This is typified by somatic cells present rising from less than 1 million up to many millions

Milk samples were collected using aseptic techniques in sterile test tubes. Before sampling, cleaning and disinfection of the udder teats were done using 70% alcohol. The samples were labeled with cow's ID number and the teat from which sample was collected, and submitted to the laboratory for analysis at the temperature of refrigerator. From each sample, 0.1 mL of milk was plated on Columbia blood agar base (Oxoid, Basingstoke, UK, CM0331) with 5%

defibrinated ovine blood, MacConkey agar (Oxoid, CM0007) and Sabouraud dextrose agar (Oxoid, CM0041). Plates were incubated during 72h at 37^{o}C under aerobic conditions, and microbial growth was mentored daily. The isolates were identified by their cultural characteristics, microscopic appearance in Gram stained preparations, catalase reaction, coagulase test with rabbit plasma and CAMP test.

Results and discussion

The study included 113 quarter milk samples from cows without clinical signs of mastitis in first and third stage of lactation for bacteriological examination and determination of electrical conductivity of milk. No bacteria growth was detected in 60 quarter milk samples (53.1%), while in the other 53 samples bacteria was found (46.9%). Results of bacteriological findings are shown in Table 2.

Table 2. Bacteriological findings in milk samples in different stage of lactation

Bacteriological finding	Stage 1		Stage 3		Total	
	N	%	N	%	N	%
No bacterial growth	30	54.54	30	51.72	60	53.1
Streptococcus agalactiae	1	1.82	-	-	1	0.9
Corynebacterium spp.	22	40	22	37.93	44	38.9
Coagulase - negative staphylococci	1	1.82	3	5.17	4	3.54
Trueperella (Arcanobacterium) pyogenes	1	1.82	1	1.73	2	1.77
Other bacteria	-	-	2	3.45	2	1.77
Total samples	**55**	**100**	**58**	**100**	**113**	**100**

The most common isolated bacteria in first and third stage of lactation was *Corynebacterium* spp. (38.9%) and coagulase - negative staphylococci (3.54%). This indicates an increase of prevalence mammary gland infection with minor mastitis pathogens. These results correspond with the conclusions of *Indriss et al. (2013)* who reported an increase of intramammary infections with minor mastitis pathogenst in 106 out of 390 milk samples (27.18%).

Electrical resistance of milk from cows in different stage of lactation is given in Table 3.

Table 3. Electrical resistance of milk in different stage of lactation

Electrical resistance	Stage 1		Stage 3		Total samples	
	N	%	N	%	N	%
Above 300 units	52	94.55	15	25.86	67	59.29
Between 300 and 250 units	3	5.45	26	44.83	29	25.67
Below 250 units	-	-	17	29.31	17	15.04
Total samples	55	100	58	100	113	100

Results pointed to increased incidence of subclinical mastitis in late lactation period (74.14%). In third stage of lactation composition of milk is changing along with increasing of somatic cells number. Rapid increase of EC (resistance below 250 units) in milk from cows in first stage of lactation was not detected. High quality and healthy milk with Draminski mastitis detector was observed in 59.29% of the samples.

Electrical resistance of milk and bacteriological findings in first and third stage of lactation are presents in Table 4 and Table 5.

Table 4. Electrical resistance of milk with different bacteriological findings in first stage of lactation

Bacteriological findings	N	Electrical resistance					
		I	II	III	Min.	Max.	Mean value±SD
No bacterial growth	30	28	2	/	260	700	403±80.14
Streptococcus agalactiae	1	1	/	/	450	450	450
Corynebacterium spp.	22	21	1	/	270	470	404.55±45.33
Coagulase - negative staphylococci	1	1	/	/	360	360	360
Arcanobacterium pyogenes	1	1	/	/	330	330	330

I- Value above 300
II- Value between 300 and 250
III- Value below 250

Value of electrical resistance above 300 unites in first stage of lactation was the most detected value, in samples where bacteria were not isolated (Table 4). Draminski mastitis detector indicated lower milk electrical resistance in two bacteriologically negative samples. Only three samples had value of electrical resistance between 300 and 250 which points to the possibility of appearance subclinical mastitis. *Norberg et al. (2004)* indicates that cows with mastitis may not always show an increased electrical conductivity of milk from the infected quarter, but the variation in EC of milk from infected quarters may be larger than variation in EC of milk from healthy quarters. Bacteria were isolated in 54.54% of milk samples (30/55), while Draminski mastitis detector gave false negative results in 43.64% of samples (24/55).

Table 5. Electrical resistance of milk with different bacteriological findings in third stage of lactation

Bacteriological findings	N	Electrical resistance					
		I	II	III	Min.	Max.	Mean value±SD
No bacterial growth	30	7	16	7	190	520	277±60.3
Corynebacterium spp.	22	6	6	10	190	400	264.55±56.12
Coagulase - negative staphylococci	3	2	1	/	300	320	310±10
Arcanobacterium pyogenes	1	/	1	/	290	290	290
Other bacteria	2	/	2	/	260	290	275±21.21

I- Value above 300
II- Value between 300 and 250
III- Value below 250

In third stage of lactation, no bacteria growth was noticed in 51.72% of milk samples (30/58), but hand-held meter gave false positive results in 76.67% of these samples (23/30). These findings correspond with results of other authors (*Musser et al., 1998; Ruegg and Reinemann, 2002; Pyörälä, 2003*). False negative results were detected in 28.57% of milk samples (8/28) where minor mastitis pathogens (*Corynebacterium* spp. and coagulase - negative staphylococci) were isolated.

Measuring EC of milk in infected quarters with minor mastitis pathogens sometimes is difficult. *Woolford et al. (1998)* were more readily detected infections of udder with major mastitis pathogens than infections with coagulase - negative staphylococci. The most likely cause of this is less damage and inflammation by minor mastitis pathogens and the possibility that such infections were localized in the teat canal and teat sinus.

Stage of lactation has great influence on the electrical conductivity of milk. Concentration of chloride ions in milk increases physiologically as lactation progresses what affects on EC of milk in all four udder quarters. Higher values of electrical conductivity of milk in infected quarters can be noticed only in that quarter (*Sheldrake et al., 1983*).

Conclusion

Electrical conductivity/resistance of milk can give useful informations about udder health status, but hand-held EC meters, such as Draminski mastitis detector, cannot be used alone in diagnosis of subclinical mastitis. This conclusion correspond with findings of other autors (*Hillerton and Walton, 1991*). Results of electrical conductivity of milk should be supplemented with bacteriological findings in milk or somatic cell count.

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Električna provodljivost mleka i bakteriološki nalaz kod krava sa subkliničkim mastitisom

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Rezime

Intramamarne infekcije utiču na hemijski sastav mleka dovodeći do povećanja električne provodljivosti, odnosno smanjenja električne otpornosti mleka. Električna provodljivost mleka se koristi u detekciji subkliničkih mastitisa tokom poslednjih četiri decenije. Cilj istraživanja je da se ispita pouzdanost merenja električne provodljivosti mleka u otkrivanju krava sa subkliničkim mastitisom. Istraživanje je sprovedeno na farmi visokomlečnih krava holštajn frizijske rase. Ukupno je pregledano 113 pojedinačnih uzoraka mleka krava, odnosno 55 uzoraka od krava u prvoj fazi laktacije i 58 uzoraka od krava u trećoj fazi laktacije. Električna provodljivost mleka određena je Draminski mastitis detektorom. Pojedinačni uzorci mleka za bakteriološku analizu uzeti su tokom jutarnje muže, aseptičnom tehnikom u sterilne epruvete. Bakterije su izolovane iz 53 uzorka mleka (46,9%), dok je 60 uzoraka mleka (53,1%) bakteriološki bilo negativno. Najčešće izolovane bakterije tokom prve i treće faze laktacije bile su *Corynebacterium* spp. (38,9%) i koagulaza - negativne stafilokoke (3,54%). Prema vrednostima električne provodljivosti dobijenim Draminski mastitis detektorom, 59,29% uzoraka mleka (67/113) pokazalo je higijensku ispravnost visokog kvaliteta. Električna provodljivost mleka ne mora uvek biti povećana u inficiranim četvrtima vimena krava. Merenje električne provodljivosti mleka može da pruži značajne informacije o zdravstvenom statusu vimena, ali Draminski mastitis detektor se ne može koristiti sam u otkrivanju subkliničkih mastitisa.

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FATTY ACID COMPOSITION OF SUBCUTANEOUS AND INTRAMUSCULAR ADIPOSE TISSUE IN EAST BALKAN PIGS

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Original scientific paper

Abstract: The aim of this study was to provide information on the fatty acid profile of different adipose depots – subcutaneous (upper and inner backfat layers) and intramuscular (*m. Longissimus dorsi*) in East Balkan pigs. The animals were reared in free-range conditions and slaughtered at an average live weight of 107±1.65kg. The results of the study showed that the various adipose tissues in pigs have different lipid metabolism and hence differ in their fatty acid composition. Intramuscular fat had significantly higher content of the saturated C16:0 and C18:0 ($P<0.001$), as well as the C16:1 ($P<0.001$) than the subcutaneous fat. In regards to the content of the polyunsaturated fatty acids, the latter displayed considerably higher content of both C18:2 and C18:3 ($P<0.001$) in comparison to the intramuscular fat in *m. Longissimus dorsi*. The differences between the subcutaneous and intramuscular adipose tissue in the individual fatty acids determined the similar trend of change in the total content of saturated and polyunsaturated fatty acids. Significant differences between the backfat layers were detected for C16:1, C18:0 and C18:3 ($P<0.001$). Stearic acid (C18:0) displayed higher content of the inner, while both C16:1 and C18:3 had higher proportion in the outer backfat layer in the East Balkan pigs. Except for C20:2, the long chain polyunsaturated n-6 and n-3 fatty acids had significantly higher proportions in the intramuscular fat, however no differences were determined between the two backfat layers.

Key words: East Balkan pigs, adipose tissue, fatty acid profile

Introduction

Adipose tissue in pigs is one of the main organs involved in the regulation of lipid metabolism, particularly in the overall fatty acid synthesis with consequences in other lipid-target organs such as muscles and the liver *(Corominas et al., 2013).*

Porcine carcass fat is deposited in four depots, with different anatomical locations: visceral, subcutaneous, inter- and intramuscular. A number of studies have reported considerable anatomical variation in fatty acid composition in the pig adipose tissues, since they are not similar but each shows specific development and metabolism *(Mourot et al., 1995, 1996; Monziols et al., 2007; Daza et al., 2007).* Most of these experiments have been undertaken on the subcutaneous adipose tissue because a high proportion of fat is subcutaneous and it is very obvious at retail or in cuts. East Balkan pig is an indigenous slow-growing breed mainly in the eastern parts of Bulgaria. The animals are exclusively reared in free-range conditions, have considerably higher body fat content than other conventional lean pig breeds, possess unique sensory characteristics of meat and are used for manufacturing of high quality meat products. The extensive conditions of rearing of the pigs of the East Balkan breed suggest higher deposition of polyunsaturated fatty acids in their adipose tissues, however profound studies on the lipid profile of this breed have been scarce in Bulgaria. Hence the aim of this study is to provide information on the fatty acid profile of different adipose tissues in East Balkan pigs.

Materials and Methods

The study was carried out with 10 (5 male + 5 female) pigs of East Balkan breed. The animals were slaughtered at an average live weight of 107±1.65kg in a standard slaughterhouse. The carcasses were split in half, kept for 24 h at 4°C, after which samples of the inner and outer backfat layer as well as *m. Longissimus dorsi* were taken at the last rib.

Total lipids of the tissues were extracted according to the method of *Bligh and Dyer (1959).* Methyl esters of the total lipids, isolated by preparative TLC were obtained using 0.01 % solution of sulphuric acid in dry methanol for 14 h, as described by *Christie (1973).* The fatty acid composition of total lipids was determined by GLC analysis using chromatograph C Si 200 equipped with capillary column (TR-FAME - 60 m x 0.25 mm x 0.25 μm) and hydrogen as a carrier gas. The oven temperature was first set at 160 °C for 0.2 min, then raised until 220 °C at a rate of 5 °C/min and hold for 5 minutes. The temperatures of the detector and injector were 230 °C. Methyl esters are identified comparing to the retention times of the standards. Fatty acids are presented as percentages of the total amount of the methyl esters identified *(Christie, 1973).*

Data were statistically analysed by one way ANOVA procedure using JMP v.7 software package. Protected Fisher LSD test was performed as post-hoc test to compare means, as differences with a level of significance below 0.05 were considered significant.

Results and Discussion

The type of the adipose tissue - subcutaneous or intramuscular induced significant differences in the contents of the individual fatty acids of the studied depots (Table1). The content of C16:0 was lower in the backfat than in *m. Longissimus dorsi* ($P<0.001$) but no differences were observed between the inner and outer backfat layer. Subcutaneous adipose tissue displayed considerably lower content of C18:0 when compared to intramuscular fat ($P<0.001$) however further reduction of this fatty acid was observed in the outer layer. Significant difference between the tissues was detected in regards to the levels of C18:1 and it tended to be lower in the intramuscular fat. The differences in the content of C18:0 and C18:1 between the two backfat layers are in agreement with those reported by *Daza et al. (2007)* in Iberian pigs. On the other hand the content of C16:1 differed between the backfat layers and *m. Longissimus dorsi,* as the latter displayed the highest level of C16:1, while the lowest was observed in the inner backfat layer. Desaturation indices are indirect indicator of desaturase activity *(Attie et al., 2002).* In this study we used the product-to-precursor ratio (C16:1/C16:0 as well as C18:1/C18:0) calculated from the content of respective fatty acids in lipids extracted from the inner, outer backfat layer and *m. Longissimus dorsi*. The ratios are well related to the stearoyl-CoA (SCD) activity *(Klingenberg et al., 1995; Kouba et al., 1997).* Differences in the indices were observed between the two backfat layers, as they were lower in the inner one. This corresponded to the lower content of C16:1 on one hand and on the other- the higher content of C18:0 in this backfat layer. Surprisingly the desaturase indices determined for the intramuscular fat were not uniform, although significantly different from the ones of the adipose tissue. They suggest different activity of the enzyme toward its substrates, showing higher value of SCD16, corresponding to the considerable amount of C16:1 in *m. Longissimus dorsi* (3.58% vs.1.81 % and 1.22% for the outer and inner layers respectively). On the other hand, the highest content of C18:0 and respectively the lowest of C18:1 determined the lowest index of SCD18. This together with the considerable amount of C16:0 contributed to the higher ($P<0.001$) total saturation of the intramuscular fat, compared to the subcutaneous adipose layers. In this study we did not find changes in the saturation of the two backfat layers. This contradicted to a previous study *(Popova, 2014),* showing greater unsaturation in the outer backfat layer in Youna x Pietrain pigs. *Migdal et al. (2001)* and *Monziols et al. (2007)* reported negative gradient of the degree of unsaturation from outside inwards in selected pigs.

Pork tissues are characterised by high content of linoleic fatty acid (C18:2). The type of fat depot influenced the content of C18:2 ($P<0.001$) showing lower levels in the intramuscular fat compared to the subcutaneous adipose tissue. The same was observed in regards to the contents of the linolenic (C18:3) fatty acid.

Our study demonstrated greater deposition of these polyunsaturated fatty acids in the backfat as significant differences between layers were detected for C18:3 incorporated in the outer backfat layer. Our results contradict to those of *Monziols et al. (2007)* who reported higher levels of C18:2 in the outer layer of the subcutaneous adipose tissue, instead of C18:3 as observed in our study. However, in the experiment of *Daza et al. (2005)* no differences in the proportions of C18:2 n-6 and C18:3 n-3 between the outer and the inner layers from free-range Iberian pigs were observed. Both C18:2 and C18:3 are essential for pigs and come exclusively from feed. The preferential deposition of C18:3 in our study could be explained by the extensive rearing of the East Balkan pigs and their access to grass, which is known to have much higher content of C18:3 that the compound feeds used in pig nutrition. This is confirmed by the values of C18:3 determined in this study, being higher than the ones observed the industrially reared pigs *(Šolević Knudsen and Stanišić, 2015).*

Table. 1 Fatty acid composition in the backfat layers and intramuscular fat in East Balkan pigs

Fatty acids,%	Subcutaneous (backfat)		Intramuscular (m.LD)	SEM	Significance
	Outer layer	Inner layer			
C14:0	1.20	0.95	1.08	0.087	NS
C16:0	17.47^{a}	16.84^{a}	20.35^{b}	0.47	***
C16:1	1.81^{a}	1.22^{b}	3.58^{c}	0.17	***
C18:0	3.25^{a}	4.41^{b}	5.79^{c}	0.26	***
C18:1	52.79^{a}	53.66^{a}	49.81^{b}	0.79	**
C18:2	20.27^{a}	20.13^{a}	14.36^{b}	0.61	***
C18:3	2.28^{a}	1.84^{b}	1.14^{c}	0.08	***
C20:2	0.44^{a}	0.42^{a}	0.34^{b}	0.02	**
C20:3	0.10^{a}	0.08^{a}	0.17^{b}	0.01	***
C20:4	0.09^{a}	0.20^{a}	2.77^{b}	0.23	***
C20:5	0.07^{a}	0.07^{a}	0.23^{b}	0.02	***
C22:5	0.20^{a}	0.15^{a}	0.37^{b}	0.02	***
SFA[1]	21.93^{a}	22.20^{a}	27.22^{b}	0.55	***
MUFA[2]	54.61	54.89	53.39	0.82	NS
PUFA[3]	23.46^{a}	22.90^{a}	19.38^{b}	0.81	**
P/S	1.08^{a}	1.04^{a}	0.72^{b}	0.04	***
n-6/n-3	8.31^{a}	10.16^{b}	10.21^{b}	0.38	***
C16:1/C16:0	0.10^{a}	0.07^{b}	0.17^{c}	0.008	***
C18:1/C18:0	16.61^{a}	12.56^{b}	8.89	0.73	***

P<0.01;*P<0.001; Within a row, values connected with different letters are significant (P<0.05)
[1]SFA- Saturated fatty acids
[2]MUFA- monounsaturated fatty acids
[3]PUFA- polyunsaturated fatty acids

The contents of C20:2 displayed significantly lower content in the intramuscular fat compared to the backfat (P<0.01), while both C20:3 and C20:4

had higher proportions in the muscle (P<0.001). The same was observed for the contents of C20:5 and C22:5 (P<0.001). The levels of these fatty acids did not differ between the two backfat layers.

The ratio n-6/n-3 exceeds the recommended limit of 4 in the backfat and intramuscular fat, showing the relatively unbalanced fatty acid profile of the pork tissues. However significant difference between the backfat layers and the intramuscular fat existed corresponding to the discrepancies in the contents of C18:3.

The total contents of SFA and PUFA (Table 1) showed significant difference between the subcutaneous and intramuscular adipose tissue (P<0.001) and corresponded to the already reported changes in the content of C16:0 and C18:2. No difference was observed in the total MUFA content.

In regards to P/S ratio, in all three tissues the values were within the range 0.57-1.03, higher than the minimal recommended value of 0.4.

Significant correlation coefficients (Table 2) were found for the proportions of C16:0 and C18:1 between the inner and outer layers of the subcutaneous adipose tissue, as well as for the content of C18:2 between the inner layer and the intramuscular fat. The correlation coefficients for the levels of the other major fatty acids between the tissues were not significant. These results are indicative for the different fatty acid metabolism in the various adipose depots in pigs.

Table 2. Correlation coefficients between the major fatty acids proportion of the inner and outer layers (I/O), inner layer and intramuscular fat (I/M), and outer layer and intramuscular fat (O/M)

Fat depot	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4
I/O	0.309	0.766*	0.115	0.105	0.656*	0.583	-0.106	0.496
I/M	-0.295	-0.001	0.110	-0.144	0.262	0.719*	-0.051	0.438
O/M	0.248	0.348	0.092	0.403	0.229	0.081	-0.043	0.414

*P<0.05.

Conclusion

The data presented confirm that the various porcine adipose tissues differ in their metabolism and fatty acid profile. Intramuscular fat displayed significantly higher content of the saturated C16:0 and C18:0, as well as the C16:1 than the subcutaneous fat. In regards to the content of the polyunsaturated fatty acid, the latter had higher content of both C18:2 and C18:3 in comparison to the fat in *m. Longissimus dorsi*. The differences in these individual fatty acids between the subcutaneous and intramuscular fat contributed to the similar pattern in their total content. Significant differences between the backfat layers were detected for C16:1, C18:0, and C18:3. C18:0 displayed higher content of the inner, while both C16:1 and C18:3 had higher proportion in the outer backfat layer in the East

Balkan pigs. With exception of C20:2, the of the long chain polyunsaturated n-6 and n-3 fatty acids displayed significantly higher proportions in the intramuscular fat, however no differences were determined between the two backfat layers.

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Sastav masnih kiselina potkožnog i intramuskularnog masnog tkiva istočno-balkanske svinje

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Rezime

Cilj ove studije je da pruži informacije o profilu masnih kiselina različitih masnih depoa - potkožni (gornji i unutrašnji slojevi leđne slanine) i intramuskularni (*m. longissimus dorsi*) istočno-balkanske rase svinja. Životinje su gajene u uslovima slobodnog držanja i zaklane u proseku žive mase od 107 ± 1,65kg. Rezultati studije su pokazali da različiti depoi masnog tkiva kod svinja imaju različit metabolizam lipida i stoga se razlikuju u sastavu masnih kiselina. Intramuskularna mast je imala značajno veći sadržaj zasićenih C16: 0 i C18: 0 (P<0,001), kao i C16:1 (P<0.001) nego potkožno tkiva. Što se tiče sadržaja polinezasićenih masnih kiselina, potkožno tkivo prikazuje znatno veći sadržaj i C18:2 i C18:3 (P<0,001) u odnosu na intramuskularnu masti u *m. longissimus dorsi*. Razlike između potkožnog masnog tkiva i intramuskularne masti u pojedinim masnim kiselinama određuju i sličan trend u ukupnom sadržaju zasićenih i polinezasićenih masnih kiselina. Značajne razlike između slojeva leđne slanine su otkriveni za C16:1, C18:0 i C18:3 (P<0,001). Stearinska kiselina (C18: 0) prikazuje veći sadržaj u unutrašnjem, a C16:1 i C18:3 su imali veći udeo u spoljašnjem sloju leđne slanine istočno-balkanskih svinja. Osim C 20:2, polinezasićene masne kiselina dugog lanca n-6 i n-3 su imale značajno veće razmere u intramuskularnoj masti, međutim, statistički signifikantne razlike nisu utvrđene između dva sloja leđne slanine.

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EFFECT OF ADDITION OF EXOGENOUS ENZYMES IN HYPOCALORIC DIET IN BROILER CHICKEN ON PERFORMANCE, BIOCHEMICAL PARAMETERS AND MEAT CHARACTERISTICS

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Abstract: In developing countries, broiler farmers often use imbalanced energy diets, hence our study aims to evaluate the combined effect of addition of commercial exogenous enzymes (CEE), in low energy level corn/soybean meal based-diet on performance, serum biochemical parameters, meat characteristics in male and female of broiler chickens. A total of 120 one day old Hubbard F15 broiler chickens were divided on 2 groups (60 animals/group) with 5 replicates/group. The control group received a standard diet, while CEE group received the same diet supplemented with enzymes (250 g/ton). Addition of enzymes reduced significantly feed ($p<0.001$) and water intakes ($p<0.05$); in meantime, feed conversion ratio tended to be lower ($p=0.08$). No changes were observed in pH, protein or moisture contents of meat in both sexes broiler between CEE and control groups. No perturbation was found in all serum biochemical parameters in both sexes between CEE and control groups, except total protein and albumin levels were significantly higher in male birds fed enzymes when compared to male birds of the control group ($p<0.001$; $p<0.01$) respectively. Addition of enzymes allowed a decrease of 950 g/bird in feed intake for the total rearing period, hence save 337 €/1000 birds; thus, use of CEE in hypocaloric diet enhances broilers feed efficiency and procures an economic benefit to farmers.

Keywords: exogenous enzymes, hypocaloric diet, poultry, performance.

Introduction

Poultry production industry, mainly in developing countries, is facing a number of challenges, not the least of which are the pressures to produce more and high quality products to satisfy customer needs in a cost effective manner. As chicken meat is the most affordable in developing country, market demand is increasing rapidly since standard living and population growth continue to rise. While management of the supply chain and its contribution to agricultural production are improving, high production costs, long production cycle and market instability remain challenges of broilers meat producers. High prices of chicken meat at food retailing in developing countries like Algeria are mostly related to the excessive charges of feed, consequence of low feed efficiency and long rearing period. Broilers are usually reared during 42 to 65 days, depending mostly on the quality of feed given to birds. Although nutrients utilization of corn and soybean meal by broilers, which are the major energy and protein contributor to the diets, is generally considered to be high, their energy utilization depends on the amount of indigestible carbohydrates present, specially oligosaccharides and non-starch polysaccharides (NSP). Corn contains 9.7% NSP, whereas soybean meal contains 30.3% NSP (*Bach Knudsen, 2001*). Consequently, the presence of such anti-nutritional factors reduced the nutritional value of corn/soybean meals based diets and thus, mainly soybean is partially digested by poultry (*Pack and Bedford, 1997*). Water soluble NSP fed to broilers is not only indigestible but also interfere with the digestion and absorption of other nutrients by increasing the viscosity of digesta in the gut (*Ward and Marquardt, 1983*.). Thus, NSP are the major cause of growth depression and poor feed conversion in poultry. Exogenous enzymes have been used commercially in poultry diets for over 20 years and their addition is now a routine practice (*Amerah et al., 2011*). Addition of exogenous enzymes to corn/soybean meal based diets can overcome the anti-nutritive effect of water soluble NSP; numerous studies have reported the beneficial impact of exogenous enzymes on chick performance and nutrient digestibility (*Odetallah et al., 2002*; *Silva and Smithard, 2002; Abudabos, 2012*). Although, the value of enzymes for diets based on corn and soybeans is not well established in the scientific literature, few studies reported yet the effect of exogenous enzymes on performances, serum biochemical parameters and meat quality in male and female broiler chickens, as well as on litter moisture. Logic would suggest that if the use of enzymes can improve body weight gain and feed conversion ratio of chickens receiving isocaloric diets, the use of diets containing lower energy levels, might improve the scale and consistency of the response. In developing countries, broiler farmers often use imbalanced energy diets, and as under such us conditions, we attempted therefore in this study to investigate the effect of supplementation of commercial

exogenous enzymes (CEE) in hypocaloric corn/soybean meal based-diet on mortality, growth performance, serum biochemical parameters, meat and carcass traits, litter moisture and feed cost in broiler chickens Hubbard F15.

Materials and Methods

Experimental design

The experiment was conducted at ORAVI (Res ELMA, Setif), a public commercial poultry farm. A total of 120 one day old Hubbard F15 broiler chickens were divided randomly on 2 groups (60 animals/ group) that were replicated five times with twelve animals per replicate. The first group is a control group received a standard corn/soybean meal based-diet, while the second one is CEE group received the same diet supplemented with commercial enzyme at a concentration of 250 g/ton of feed as indicated in its commercial card. CEE is a multi-enzyme complex consisting of endo-ß-1,4-xylanase derived from *Trichoderma longibrachiatum*, endo-ß-1,4-glucanase, amylase and protease and the side activities cellulase and galactomannase..

A standard corn/soybean meal based-diet was formulated by the National Board of Livestock Feed (ONAB of El Eulma, Algeria), adapted to each phase of rearing broilers, was used in our experiment. The basal diet given in our experimental was hypocaloric, detailed formulation is shown in Table 1. Birds were fed starter diet from 1 to 9 days, grower diet from 10 to 41 days and finisher diet from 42 to 60 days. Feed and water were served *ad libitum* throughout the whole experimental period. All animals were exposed to a similar rearing management. The chicks were kept in ventilated broiler house in ten pens (1.2 m x 1.25 m) on wood dust litter within floor pens with stainless-steel shaving. Pens were gradually widened during grower and finisher periods as well as bird densities were respectively 8, 7 and 4 chicks per meter square at the end of starter, grower and finisher periods.

There were one hanging feeder and one drinker in the middle of each pen. A thermometer was put in the middle of the rearing area of each group to register daily at 12:00 pm the ambient temperature. The average mean temperature was initially 31 ± 0.75°C, then 30 ± 1.09°C during starter period, 26 ± 3.04°C during grower period and 22 ± 2.62°C during finisher period. The lighting program was as follows: 24h light/d until 3 day, 23h light/d from day 4 to 7, 18h light/d for the 2nd week, 14h light/d for the 3rd week; then light period was increased by 2 hours per week until 6th week when light was maintained at 22h/d. Chicks were vaccinated via drinking water against Gumboro and New Castle Disease (Ceva vaccines) at day 12 and 15 respectively.

Table 1. Formulation and calculated analysis of broiler chicken diet

Ingredients (g/kg)	Starter	Grower	Finisher
Corn	610.00	648.00	670.00
Soybean meal (48.6% CP)	297.00	270.00	180.00
Milling issues	50.00	50.00	120.00
Limestone	6.00	12.00	10.00
Di calcium phosphate	16.7	10.00	10.00
DL Methionine	0.30	-	-
Vitamin premix anti-stress	10.0	-	-
Mixture premix[1]	10.0	10.00	
Mixture premix [2]	-	-	10.00
Calculated analysis			
ME (Kcal/kg)	2781	2816	2826
Crude protein (%)	20.45	18.68	16.46
DL Methionine (%)	0.49	0.44	0.41
Calcium (%)	0.85	0.90	0.79
Phosphorus (%)	0.69	0.56	0.55

All premix mixtures were supplied by kg of diet.

Vitamin anti-stress premix: Retinol, 0.15 mg; Tocopherol, 1500 mg; Menadione, 250 mg ; Thiamine, 50 mg; Riboflavin, 400 mg; Pyridoxine, 400 mg; Cobalamin, 1 mg; Ascorbic acid, 15000 mg; Niacin, 2000 mg; Folic acid, 100 mg; Biotin, 10 mg.

[1]**Mixture premix:** Retinol, 306.69 mg; Cholecalciferol, 5.11 mg; Tocopherol, 1532 mg; Menadione, 152.9 mg; Thiamine, 202.92 mg; Riboflavin, 420 mg; Pyridoxine, 97.6 mg ; Cobalamin, 2 mg; Folic acid, 53.2 mg; Niacin, 2530.8 mg; Ca Panthotenat, 872 mg, Cholin Chloride, 106000 mg; Anticoccidial (Salinomycin), 6000 mg; Cu, 228 mg; Co, 22.8 mg; Fe, 1520 mg; Mg, 1216 mg; Mn, 5700 mg; Zn, 1900 mg; I, 114 mg; Se, 6.08 mg; DL Methionin, 80000 mg; Antioxidant BHT, 13000 mg.

[2]**Mixture premix:** Retinol, 306.69 mg; Cholecalciferol, 5.11 mg; Tocopherol, 1072 mg; Menadione , 121.6 mg; Riboflavin, 420 mg; Pyridoxine, 97.6 mg ; Cobalamin, 2 mg; Niacin, 1529 mg; Ca Panthotenat, 872 mg, Cholin Chlorure, 106000 mg; Anticoccidial (salinomycin), 6000 mg; Cu, 228 mg; Co, 22.8 mg; Fe, 1520 mg; Mg, 1216 mg; Mn, 5700 mg; Zn, 1900 mg; I, 114 mg; Se, 6.08 mg; DL Methionin, 80000 mg; Antioxidant BHT, 13000 mg.

Data collection and sampling

Mortality was recorded daily and expressed as frequency; birds were weighed at 21, 35, 49 and 60 days of age. However, the weight of birds was not recorded at the end of grower period (41 days). Feed and water consumptions were measured every 2 to 3 days at the beginning of experiment and daily at the end of experiment. Then, daily feed and water intakes, and ratio of water/feed were calculated obviously for alive birds and for each rearing period. Feed conversion ratio during the entire experiment was also calculated. At the end of the experimental period, 1 to 3 chickens per pen, with identical weights were selected to obtain 10 males and 10 females per group (control and CEE) to measure serum biochemical parameters, carcass weights and their different part yields, and estimate meat characteristics per treatment and sex.

Serum biochemical analysis

Blood samples were collected from wing vein after fasting animals 4 hours. Blood glucose was immediately measured using Ultra One Touch strips. However, serum was separated after centrifugation at 2500 g for 15 min and frozen

at -20°C until analysis. Serum concentrations of urea, total cholesterol (TC), triglyceride (TG), total protein (TP) and albumin were determined automatically with Technicon RA-XT Biochemistry Analyzer, while serum calcium was measured with Beckman coulter automate, using enzymatic colorimetric Cypress Reagent Kits (Belgium).

Animal sacrifice and dissection

At 60 days of age, all chickens were sacrificed by slitting their throat after 6 hours of fasting; feather, heads and shanks were removed. All carcasses were weighed, and then carcasses of 10 males and 10 females were dissected by the same butcher to different parts as: thighs (remained linked to tail), breast, back, wings, neck, liver, gizzard, spleen, heart, abdominal and neck fats. Parts were weighed and their yield expressed as percentage of carcass weight.

Meat characteristics

Meat characteristics were estimated in the thigh muscles by determination of pH, moisture and protein contents. After slaughter, the right thigh muscles were labelled and conserved about 12 hours at 4°C before evaluating pH and moisture content. All muscle thighs were stored individually at -20°C until analysis of crude proteins. Moisture content was determined after drying a sample of 5 g at 105°C for 24 hours (*AOAC, 1990*), while pH was determined using digital pH meter after homogenizing a sample of 5 g in 10 ml distilled water (*Patsias et al., 2008*). Crud proteins were measured following the Kjeldahl method of nitrogen analysis.

Litter moisture

At the end of the experiment, moisture content of the litter was measured in all pens. Litter was collected using an empty 200 ml of beaker, consisted of 12 cm core sample of litter **(***Eichner, 2007*). Samples were taken from 3 different areas located far from the drinker of each pen; two litter samples were taken from the area where chickens slept while the third sample was taken from the middle of the left side of the pen. Litter samples were mixed in a plastic bag, and then a quantity of 100 g of each mixed sample was oven-dried at 105°C for 30 hours. The dry litter was then weighed and litter moisture determined.

Feed cost

To estimate feed cost through the entire experiment period, quantity of CEE supplemented was subtracted from all amount of the diet consumed during each period to obtain the quantity of diet consumed per bird knowing that CEE was added to diet at 0.025%, then the cost of feed intake was calculated. Prices of different diets considered in our study were: starter diet: 41. €/quintal; grower and finisher diet: 39.19 /quintal; CEE: 23.52 €/kg.

Statistical analysis

All data are expressed as mean ± SE. For each group, means data of five replicates were calculated. Data means between both groups were then compared using Student test when variances were equal, or Mann–Whitney U test when variances were unequal. Khi-square test was used to compare mortality frequencies between both groups. $p<0.05$ was considered as statistically significant and trends were discussed when $p<0.10$.

All statistical analyses were performed using SPSS package program, version 17.0.

Results

Mortality, feed and water consumption, water/feed ratio and feed conversion

No difference was observed in the frequency of mortality in CEE group compared to control group (8% vs 13%). Table 2 shows that feed intake was similar between both groups during starter period, then decreased during grower and finisher periods in CEE group compared to control group ($p<0.01$; $p<0.001$) respectively. Water intake was lower during starter and grower periods in CEE group when compared to control group ($p<0.05$), while this difference was not observed during the finisher period. Water/feed ratio was lower during the starter period in CEE group compared to control group ($p<0.05$), then increased during the grower period ($p<0.05$) and tended to be higher during the finisher period ($p=0.07$) (Table 2). Concerning the entire experimental period, animals fed CEE consumed less feed ($p<0.001$) and water ($p<0.05$) when compared to control animals. Total feed conversion ratio tended also to be significantly reduced in CEE group compared to control ($p=0.08$). However, water/feed ratio was significantly higher in CEE group ($p<0.05$) when compared to control group (Table 2).

Table 2. Feed and water intakes, water/feed ratio and total feed conversion ratio during experimental period

Periods	Groups	Mean ± S.E.	Signification
Starter (1-9 days)			
- Feed intake (g/bird)	Control	170.67 ± 3.04	NS
	CEE	173.13 ± 4.17	
- Water intake (ml/bird)	Control	293.15 ± 2.16	p<0.05
	CEE	264.17 ± 8.54	
- Water/feed ratio	Control	1.72 ± 0.02	p<0.05
	CEE	1.53 ± 0.06	
Grower (10-41days)			
- Feed intake (g/bird)	Control	3360.34 ± 52.33	p<0.01
	CEE	2890.52 ± 76.33	
- Water intake (ml/bird)	Control	4232.93 ± 71.82	p<0.05
	CEE	3945.58 ± 65.46	
- Water/feed ratio	Control	1.26 ± 0.03	p<0.05
	CEE	1.37 ± 0.03	
Finisher (42-60 days)			
- Feed intake (g/bird)	Control	3398.80 ± 65.64	p<0.001
	CEE	2917.48 ± 53.80	
- Water intake (ml/bird)	Control	5325.51 ± 149.76	NS
	CEE	5088.90 ± 125.46	
- Water/feed ratio	Control	1.57 ± 0.04	p =0.07
	CEE	1.75 ± 0.08	
All experiment period			
-Total feed intake (g/bird)	Control	6929.80 ± 97.96	p<0.001
	CEE	5981.13 ± 87.95	
-Total water intake (ml/bird)	Control	9851.60 ± 126.60	p<0.05
- Total feed conversion	CEE	9298.75 ± 185.93	
Ratio (g:g)	Control	3.07 ± 0.11	p=0.08
- Total water/feed ratio	CEE	2.67 ± 0.15	
	Control	1.42 ± 0.03	p<0.05
	CEE	1.56 ± 0.04	

NS: not significant. CEE: commercial exogenous enzymes

Live weight and carcass characteristics

There were no significant differences in carcass or live weights recorded for any rearing period between CEE and control groups (Table 3).

Table 3. Live and carcass weights of broiler chickens

Weights	Groups	Mean ± S.E.	Signification
Live weights (g)			
21 days	Control	408.84 ± 8.65	NS
	CEE	388.92 ± 7.67	
35 days	Control	972.54 ± 35.42	NS
	CEE	965.66 ± 18.71	
49 days	Control	1547.80 ± 63.17	NS
	CEE	1599.56 ± 71.58	
Final live weights (g)	Control	2269.00 ± 103.47	NS
(60 days)	CEE	2252.80 ± 109.82	
Carcass weights (g)	Control	1847.71 ± 52.87	NS
	CEE	1741.95 ± 46.50	

NS: not significant. CEE: commercial exogenous enzymes

Likewise, supplementation of diet with exogenous enzymes did not influence the carcass weights and parts yields of male or female broiler chickens (Table 4).

Table 4. Carcass weights and relative weight of different parts of carcass in male and female broiler

		Male Birds			Female Birds		
		n	Mean ± SE	P	N	Mean ± SE	P
Carcass (g)	Control	10	1926.56 ± 80.13	NS	10	1818.30 ± 59.40	NS
	CEE	10	1807.10 ± 59.67		10	1704.00 ± 71.24	
Thighs (%)	Control	10	43.55 ± 0.40	NS	10	41.30 ± 0.44	NS
	CEE	10	44.20 ± 0.46		10	41.44 ± 0.36	
Breast (%)	Control	10	29.69 ± 0.49	NS	10	31.68 ± 0.56	NS
	CEE	10	29.29 ± 0.45		10	31.72 ± 0.60	
Back (%)	Control	10	8.60 ± 0.34	NS	10	8.93 ± 0.31	NS
	CEE	10	8.67 ± 0.27		10	8.85 ± 0.36	
Wings (%)	Control	10	11.84 ± 0.23	NS	10	12.30 ± 0.31	NS
	CEE	10	11.61 ± 0.54		10	11.94 ± 0.31	
Neck (%)	Control	10	3.35 ± 0.15	NS	10	3.00 ± 0.13	NS
	CEE	10	3.35 ± 0.24		10	3.00 ± 0.12	
Liver (%)	Control	10	2.75 ± 0.09	NS	9	2.74 ± 0.12	NS
	CEE	10	2.69 ± 0.11		10	2.91 ± 0.11	
Gizzard (%)	Control	9	1.89 ± 0.11	NS	9	1.70 ± 0.09	NS
	CEE	10	1.79 ± 0.10		10	1.70 ± 0.05	
Spleen (%)	Control	8	0.16 ± 0.01	NS	6	0.20 ± 0.01	NS
	CEE	9	0.17 ± 0.01		7	0.22 ± 0.02	
Heart (%)	Control	10	0.54 ± 0.02	NS	10	0.57 ± 0.03	NS
	CEE	10	0.53 ± 0.02		10	0.53 ± 0.02	
Abdominal fat (%)	Control	10	0.79 ± 0.10	NS	10	1.50 ± 0.22	NS
	CEE	8	1.11 ± 0.17		9	0.98 ± 0.19	
Neck fat (%)	Control	10	2.87 ± 0.20	NS	9	3.11 ± 0.22	NS
	CEE	10	2.88 ± 0.12		10	3.05 ± 0.18	

NS: not significant. n: number of birds. CEE: commercial exogenous enzymes

Serum biochemical parameters levels and meat characteristics in male or female broilers

Levels of serum TP and albumin were higher in male birds of CEE group compared to male birds of the control group ($p<0.01$; $p<0.001$) respectively, while all other serum parameters were not influenced neither in male nor in female birds by CEE addition to diet (Table 5). There is no modification in pH, protein and moisture contents in thigh muscles of CEE group compared to control group (Table 6).

Table 5. Concentration of serum biochemical parameters in male and female broiler chickens

	Groups	Male Birds			Female Birds		
		n	Mean ± SE	P	n	Mean ± SE	P
Urea (g/l)	Control	10	0.06 ± 0.01	NS	7	0.05 ± 0.01	NS
	CEE	9	0.06 ± 0.01		9	0.06 ± 0.02	
TP (g/l)	Control	10	32.90 ± 1.07	0.01	8	42.75 ± 2.38	NS
	CEE	9	43.00 ± 2.89		9	37.89 ± 2.29	
Albumin	Control	10	10.70 ± 0.21	0.001	8	15.25 ± 0.62	NS
(g/l)	CEE	8	14.25 ± 0.65		9	14.67 ± 0.67	
TC (g/l)	Control	10	0.96 ± 0.04	NS	8	1.16 ± 0.07	NS
	CEE	9	0.99 ± 0.05		9	1.05 ± 0.08	
TG (g/l)	Control	10	0.61 ± 0.05	NS	8	0.62 ± 0.07	NS
	CEE	9	0.66 ± 0.07		9	0.58 ± 0.04	
Calcium	Control	9	67.89 ± 3.14	NS	8	68.50 ± 3.96	NS
(g/l)	CEE	9	71.67 ± 1.93		9	69.22 ± 3.22	
Glycemia	Control	10	2.37 ± 0.08	NS	8	2.35 ± 0.06	NS
(g/l)	CEE	9	2.44 ± 0.15		9	2.18 ± 0.09	

Cholesterol (TC), Triglyceride (TG), Total Protein (TP). NS: not significant. n: number of birds. CEE: commercial exogenous enzymes

Table 6. Meat characteristics in male and female broiler chickens

	Groups	Male Birds			Female Birds		
		n	Mean ± SE	P	n	Mean ± SE	P
PH	Control	10	6.02 ± 0.05	NS	10	6.00± 0.05	NS
	CEE	10	6.00 ± 0.11		10	6.06 ±0.10	
Protein (%)	Control	10	21.46 ± 0.51	NS	10	19.82 ± 0.36	NS
	CEE	10	21.79 ± 1.02		10	20.72 ± 0.97	
Moisture	Control	10	78.80 ± 0.44	NS	10	79.20 ± 0.44	NS
(%)	CEE	10	78.00 ± 0.52		10	79.40 ± 0.31	

NS: not significant. n: number of birds; CEE: commercial exogenous enzymes

Litter moisture

Litter moisture of birds fed diet supplemented with CEE was increased ($p<0.01$) when compared to control group (46.20% ± 1.39 vs. 33.40% ± 3.66).

Feed cost and benefit

Addition of exogenous enzymes did not influence the cost of feed intake during the starter period, but they reduced feed cost during grower and finisher periods ($p<0.01$; $p<0.001$) respectively, as well as during the entire experimental period ($p<0.001$) (Table 7). For the total rearing period, feed intake was lower after CEE addition, corresponding to 950 g/bird less than the control group; hence, use of CEE save 337.2 €/1000 reared birds (Table 7).

Table 7. Cost of total feed intake with or without addition of exogenous enzymes through all period of experiment

Periods	Net diet intake (g/bird)	Net CEE intake (10^{-2} g/bird)	Cost of net diet intake (10^{-2} €)	Cost of net CEE intake (10^{-2} €)	Cost of total diet intake (10^{-2} €)
Starter					
Control	170.67± 3.04	/	7.02±0.13	/	7.02±0.13
CEE	173.09 ± 4.17	4.32±0.12	7.12±0.17	0.10±0.00	7.22±0.17
Signification	NS		NS		NS
Grower					
Control	3360.34±52.33	/	131.70±2.05	/	131.70±2.05
CEE	2889.79±76.32	72.28±1.91	113.26±2.99	1.70±0.04	114.96±3.04
Signification	0.01		0.01		0.01
Finisher					
Control	3398.80±65.64	/	133.21±2.57	/	133.21±2.57
CEE	2916.75±53.78	72.96±1.34	114.31±2.11	1.72±0.03	116.03±2.14
Signification	0.001		0.001		0.001
All period					
Control	6929.80±97.96	/	271.59±3.84	/	271.59±3.84
CEE	5979.63±87.93	149.54±2.20	234.35±3.45	3.52±0.05	237.87±3.50
Signification	0.001		0.001		0.001
Benefit feed (g/bird)	950.17				
Benefit (€/bird)	0.3372				
Benefit (€)/ 1000 birds	337.2				

NS: not significant. CEE: commercial exogenous enzymes

Discussion

Obviously the live weight of birds in our research, fed a low energy level corn/soybean meal based-diet, was too below to the target weight performed under commercial conditions by Hubbard F15 birds. Interestingly, addition of CEE enzymes improve feed efficiency by reducing feed intake, 950 g per bird, so birds receiving enzymes consumed 13.68 % less of feed compared to other birds who did not feed exogenous enzymes; in the meantime, feed conversion tended to be lower in CEE group compared to the control group. It was demonstrated that feed intake reduced as the energy content of feed decreased in diet having low energy and high protein diet content (*Dairo et al., 2010*). Indeed, reduced feed intake could be fully compensated by the effect of enzyme supplementation on feed efficiency so birds fulfil their nutrient requirement by taking fewer amounts of feed. The improvement of feed conversion efficiency of diets supplemented with enzymes was the result of increased metabolizable energy, consequence of higher digestibility of crude protein and NSP of diet. Our results agree with findings obtained by previous studies (*Zhou, et al., 2009; Aok, 2012*), when addition of exogenous enzymes

improved energy digestibility in diets with lower levels of metabolizable energy, it was effective in overcoming anti-nutritive effects of NSP on broiler performance only at low energy levels. However, improved feed efficiency and body weight with no change in feed consumption were observed when CEE was added to isocaloric corn/soybean meal diets (*Abudabo, 2010*). Contrary, *Zakaria et al.*, (*2008*) reported no effect of CEE on body weight, feed consumption or feed conversion ratio. The heterogeneity in the findings on the effect of CEE on performance of broiler chickens could be explained by the different diets quality and chicken breed used in each experiment. Hence, use of CEE improves the nutritional value of a hypocaloric corn/soybean meal based-diet in birds. Until now, no study reported the effect addition of enzymes in this kind of diet on broiler chicken performance.

Our data suggest that enzyme supplemented diet did not interact with sex to influence organs weight or meat yield. Also, it is important to underline that the weight of fats (abdominal or neck) were not increased to the detriment on weight of different meat parts which is beneficial in animal production. Our findings is in agreement with work of *Alam, et al.,* (*2003*) who reported that dietary enzyme did not interact with sex to influence any meat yield characteristic. On other hand, *Abudabo, A.* (*2010*) reported, an increase in breast yield after addition of CEE but not in other meat part yields in Cobb chickens. In the current study, no perturbation of serum biochemical parameters concentrations was observed in both sexes of broilers between CEE and control groups, except for serum albumin and TP levels, which were higher in male birds fed CEE-supplemented diet. Our results are consistent with those reported by a recent study (*Abudabo, 2010*); it was shown that animals that have low feed intake require higher dietary with protein content (*Larbier and Leclercq, 1992*). These suggest that addition of enzymes increases protein digestibility, consequently decreased feed intake as discussed previously. Thus, energy and amino acid values of maize based-diets for broilers can be enhanced by supplementation with an enzyme cocktail of xylanase, amylase and protease (*Zanella et al., 1999; Cowieson and Ravindran, 2008*), which are components present in our CEE. Nevertheless, increase in serum albumin, and therefore total serum protein were found when the dietary protein level was increased beyond the requirement for growth; this reflects the ability of the chicks to store "reserve" protein even after the animal has reached its maximum capacity for depositing tissue or less "labile" protein (*Leveille, and Sauberlich, 1961*). Previous studies showed an increase in protein digestibility in bird fed corn/soy bean meal diet supplemented with CEE or with other commercial enzyme as Avizyme® 1500 containing some identical enzymes present in our CEE such as xylanase, protease and amylase (*Zanella et al., 1999; Abudabo, 2010*).

Our results revealed that frequencies of mortality are similar between birds fed or no CEE. Also, addition of exogenous enzymes did not influence pH,

moisture and protein contents of thigh meat neither in male nor in female broiler chickens.

Our findings indicate that meat pH and protein content were close to those found in thigh muscle of male Hubbard F15 chickens aged 65 days and receiving a isocaloric diet (*Mikulski et al., 2011*). No study until now reported effect of CEE on meat quality in both sexes broiler chickens. Thus, we can suggest that meat of broiler chickens fed diet supplemented with CEE conserved its characteristics although feed intake reduction.

In a well-managed broiler house, litter moisture normally averages between 25 to 35 % (*Butcher and Miles, 1996*). Our study revealed that litter moisture was higher in pens of birds fed enzymes supplemented diet compared to control birds and exceeded the recommended normal range; also higher water/feed ratio observed in chickens fed enzymes supplemented diet can be attributed to the increase in protein digestibility as discussed previously. It was demonstrated that addition of enzymes makes more protein available so diet allows high protein concentration, increases water consumption and water/feed ratio (*Larbier and Leclercq, 1992; Francesch and Brufau, 2004*). Therefore, Dietary protein in excess of requirements causes an increased heat increment and water intake which results in elevated litter moisture content (*Alleman and Leclercq, 1997*). There are not yet published studies demonstrating effect of CEE on litter moisture; however, no effect of CEE supplemented diet on clean excreta moisture was reported (*Abudabo, 2010*).

Poultry industry is the important sector that can provide animal protein in a cost effective manner, mainly for poor people. However, feed is the major component of the total cost of broiler production (*Khan, 2004*). In our study, addition of CEE in corn/soybean diet procures lower diet charge by reducing feed intake, so use of CEE save 337 €/1000 birds. However, *Abudabo, A.* (*2010*), reported that addition of CEE offers potential to reduce diet cost commensurate with enhanced production. Exogenous enzymes show up as tools on poultry diets formulation flexibilization, allowing the utilization of non-conventional ingredients without impairment to birds' performance, with consequent reduction on production costs (*Costa et al., 2008*).

Conclusions

Addition of CEE in hypocaloric corn/soybean meal based-diet reduced feed and water consumptions. Feed intake decreased with no change in carcass and life weights, meat part yields, organs, fats, meat characteristics or serum biochemical levels neither in male nor in female birds, except a high serum albumin and protein levels was observed in male birds fed CEE probably by improving nutrient digestibility, mainly protein; Albeit high litter moisture found,

addition of enzymes in low diet energy allows producers to raise birds with an economic feed benefit cost and contribute to enhance broiler chicken production.

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Efekat dodavanja egzogenih enzima u hipokaloričnoj ishrani brojlerskih pilića na performanse, biohemijske parametare i osobine mesa

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Rezime

U zemljama u razvoju, u ishrani brojlera na farmama se često koriste obroci koji nisu izbalansirani sa aspekta energetske vrednosti, pa naša studija ima za cilj da ispita kombinovani efekat dodavanja komercijalnih egzogenih enzima (CEE), u ishrani koja se zasniva na obrocima od kukuruza/sojine sačme, niskog nivoa energije, na performanse, serum biohemijske parametre, osobine mesa muških i ženskih pilića. Ukupno 120 jednodnevnih Hubbard F15 brojlera je podeljeno u 2 grupe (60 životinja/grupa) sa 5 ponavljanja/grupi. Kontrolna grupa je dobila standardi obrok, dok je CEE grupa dobila isti obrok sa dodatkom enzima (250 g/tona). Dodavanje enzima značajno smanjuje unos hrane ($p<0,001$) i vode ($p<0,05$); istovremeno, konverzija hrane ima tendenciju smanjenja ($p=0,08$). Nema promena u pH, sadržaju proteina ili vlage mesa oba pola brojlera između CEE i kontrolne grupe. Nisu utvrđene bilo kakve smetnje u biohemijskim parametrima seruma oba pola izmedju CEE i kontrolne grupe, osim ukupnih proteina i albumin nivoa koji su bili značajno veće kod muških pilića hranjenih enzimima kada se uporedi sa muškim pilićima iz kontrolne grupe ($p<0,001$; $p<0,01$) respektivno. Dodavanje enzima omogućilo je smanjenje unosa hrane od 950 g/brojleru za ukupni period odgoja, čime se ostvaruje ušteda od 337 €/1000 brojlera; dakle, upotreba CEE u hipokaloričnoj ishrani povećava efikasnost korišćenja hrane kod brojlera i osigurava se ekonomska korist za proizvođače.

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THE EFFECT OF CROP DENSITY ON YIELD OF FORAGE MAIZE

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Abstract: The aim of this investigation was to estimate the effects of crop density on the plant height (PH), stem diameter (SD), number of leaves per plant (NL), forage yield hectare^{-1} (FY), dry matter yield hectare^{-1} (DMY), stem percentage (SP), leaf percentage (LP) and ear percentage (EP) in two maize hybrids of FAO maturity group 600 (ZP 684 and NS 6010). Field trials were carried out in rainfed farming in the Srem region (location Putinci: 44° 59′ 19" North and 19° 58′ 11" East) during years 2007 and 2008. Three crop densities were compared: G1 – 51,020 plants ha^{-1}, G2 – 59,524 plants ha^{-1} and G3 – 71,429 plants ha^{-1} (corresponding to spacing of 70 × 28, 70 × 24, and 70 × 20 cm). Plots were organized as completely randomized block system design in four replications. PH (265.45 cm), SD (2.40 cm), FY (68.63 t ha^{-1}) and DMY (24.63 t ha^{-1}) were significantly higher in 2007 than in 2008 (261.78 cm, 2.32 cm, 61.17 t ha^{-1} and 21.04 t ha^{-1}, respectively). Hybrid NS 6010 had significantly higher PH (266.23 cm), SD (2.39 cm) and NL (14.75) than hybrid ZP 684 (261.0 cm, 2.33 cm and 13.99, respectively). Increasing crop density significantly increased the PH, FY, DMY and SP, and significantly decreases the SD and EP. Therefore, crop density of 71,429 plants ha^{-1} (70 x 20 cm) can be recommended for growing hybrids of FAO 600 maturity group in climatic conditions of Srem in order to achieve high yields of forage and dry matter.

Key words: crop density, dry matter yield, forage yield, maize

Introduction

Maize is multipurpose crop. It is used for human food, animal feed and as industrial raw material. In Serbia, maize is largely used for feeding livestock, an estimated 80% of the total production. In the complete forage mixtures, maize is present with 50-80%, depending on the type and categories of animals *(Randjelovic et al., 2011)*. In animal nutrition grain or silage (grain silage and forage silage) is

used. Maize is a very convenient crop for forage production due to the high production of green mass per unit area (12-25 t total dry matter per hectare), high energy content of dry matter and quality of biomass for silage *(Mandić et al. 2013)*. Selection of maize is focused on maize hybrids that produce high grain yields and good quality silage combined with agronomic traits. Silage maize hybrids are certified based on fresh and dry matter yield and the proportion of the ear *(Tóthné Zsubori, 2011)*. Hybrids are grown for the maximum amount of dry matter per hectare. In many environments in Serbia, the loss of plants from sowing to harvest is around 30% and the maize yield is decreased by 1.5 to 2.2 t ha^{-1}. In Serbia, maize is sown in 70 cm inter-row, while the distance between seeds in a row determines the number of plants per unit area *(Mandić, 2011)*. At supra-optimal crop density, maize reduces the total biomass per plant, increases barrenness, and decreases harvest index *(Boomsma et al., 2009)*. The distance between the plants should be ideal so that the plants are competing minimally for nutrients, sunlight and other factors *(Lauer 1994)*. *Çarpıcı et al. (2010)* established that dry matter yield and stem percentage increased, leaf number $plant^{-1}$, stem diameter and ear percentage decreased as crop density increased. *Karaşahin (2014)* concluded that the silage and dry matter yield increased as plant density increased, while decreased stem diameter, and fresh ear ratio. While forage yield and dry matter yield increases with increasing plant densities, stem diameter decreases *(Baghdadi et al. 2012; Moosavi et al., 2012)*.

This research is focused to find optimal crop density to enhance maize forage and dry matter yield. We examined the effect of three crop densities (51,020 plants ha^{-1}, 59,524 plants ha^{-1} and 71,429 plants ha^{-1}) on the plant height (PH), stem diameter (SD), number of leaves per plant (NL), forage yield $hectare^{-1}$ (FY), dry matter yield $hectare^{-1}$ (DMY), stem percentage (SP), leaf percentage (LP) and ear percentage (EP) in maize hybrids ZP 684 and NS 6010 in different environmental conditions.

Materials and Methods

Field trials were carried out in rainfed conditions in the Srem region (location Putinci: 44° 59′ 19" North and 19° 58′ 11" East) during years 2007 and 2008. The experiment was carried out on calcareous chernozem soil type, with pH in H_2O 7.6, pH in KCl 7.18, 12.99% CaCO3, 2.69% humus, 0.18% total N, 19.12 mg 100g $soil^{-1}$ P_2O_5 and 21.8 mg 100g $soil^{-1}$ K_2O, respectively. Two maize hybrids of FAO maturity group 600, ZP 684 and NS 6010, were used as material. Three crop densities were tested: G1 – 51,020 plants ha^{-1}, G2 – 59,524 plants ha^{-1} and G3 – 71,429 plants ha^{-1} (corresponding to spacing of 70 × 28, 70 × 24, and 70 × 20 cm). Sowing was carried out manually with 2 seeds in seedbed. After sowing, rolling was applied. After germination thinning was carried out at a steady, planned

number of plants. Maize sowing was done on in the optimal time (from 16-18 April). Sub-plot area was 16.8 m^2 having 4 rows each per hybrid with row length of 6 m. Plots were organized as completely randomized block system design in four replications. Preceding crop was winter wheat in both seasons. The N-P-K fertilizer (10-30-20) in quantity 300 kg N ha^{-1} was incorporated into the soil during primary soil tillage. In spring during additional soil tillage was applied KAN - 27% in quantity 90 kg ha^{-1}. One half of the KAN was applied at the stage of 3 leaves, the remaining half at the stage of 7-9 leaves. A standard cultivation practice was applied.

The amount of rainfall and monthly air temperature during the growing season of maize (April-August) were 265.4 mm and 19.7°C in 2007, and 236.6 mm and 19.2°C in 2008, respectively (Figure 1). Climate diagram showed that in 2007 drought period was in July at the flowering stage (anthesis and silking), and in 2008 in August at the stage of grain filling.

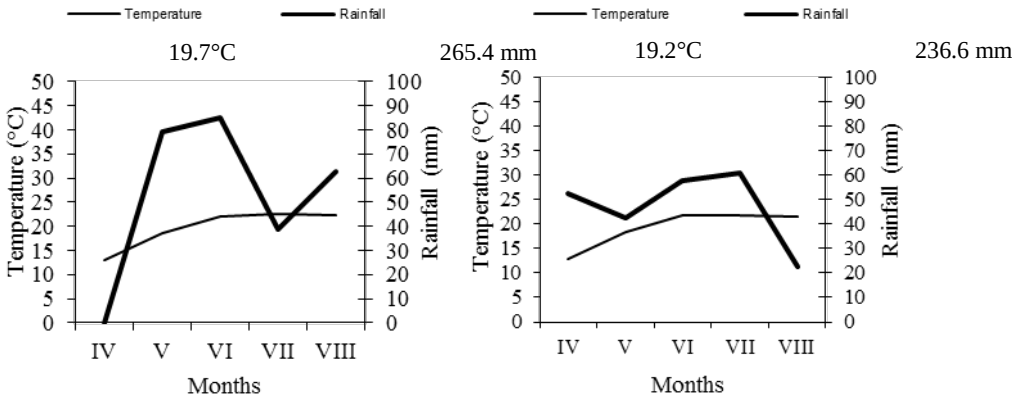

Figure 1. Climate diagram according to Walter in the 2007 and 2008 for Sremska Mitrovica, Serbia

Maize harvest was performed manually when the dry matter was 34-36% during second half of August and the beginning of September. Plants from central two rows from each sub plot were cut on height 20 cm at harvest time and forage yield was measured (FY). The yield was converted into t ha^{-1}. At maize harvest, plant height (PH), stem diameter (SD), number of leaves per plant (NL), dry matter yield $hectare^{-1}$ (DMY), stem percentage (SP), leaf percentage (LP) and ear percentage (EP) were measured from 10 random plants from each sub plot.

Data were processed using ANOVA. The statistical tests were carried out using STATISTICA (version 10; StatSoft, Tulsa, Oklahoma, USA). The significance level was set at $P \leq 0.05$ and $P \leq 0.01$. Differences between trait means were assessed using Duncan's Multiple Range Test at $P \leq 0.05$ level.

Results

Results showed that the year had highly significant effect on PH, SD, FY and DMY (Table 1). PH (265.45 cm), SD (2.40 cm), FY (68.63 t ha^{-1}) and DMY (24.63 t ha^{-1}) were higher in the 2007 than in the 2008 (261.78 cm, 2.32 cm, 61.17 t ha^{-1} and 21.04 t ha^{-1}, respectively).

Table 1. The effect of year, hybrid, and crop density on forage yield, dry matter yield and some morphological traits of maize

Factor		PH	SD	NL	FY	DMY	SP	LP	EP
Year (A)	2007	265.45^{a}	2.40^{a}	14.43	68.63^{a}	24.63^{a}	47.48	28.10	24.41
	2008	261.78^{b}	2.32^{b}	14.31	61.17^{b}	21.04^{b}	46.88	29.14	23.98
Hybrid (B)	ZP 684	261.00^{b}	2.33^{b}	13.99^{b}	65.18	22.99	46.28	28.80	24.92
	NS 6010	266.23^{a}	2.39^{a}	14.75^{a}	64.61	22.69	48.09	28.44	23.47
Crop density (C)	51020	259.52^{b}	2.43^{a}	14.39	61.90^{c}	21.80^{c}	43.28^{c}	29.76	26.97^{a}
	59524	264.21^{a}	2.36^{a}	14.38	65.28^{b}	23.04^{b}	46.61^{b}	28.05	25.34^{a}
	71429	267.12^{a}	2.29^{b}	14.34	67.51^{a}	23.67^{a}	51.65^{a}	28.06	20.29^{b}
F test	A	**	**	ns	**	**	ns	ns	ns
	B	**	*	**	ns	ns	ns	ns	ns
	C	**	**	ns	**	**	**	ns	**
	A × B	ns	ns	ns	**	**	ns	*	ns
	A × C	ns	ns	ns	ns	ns	ns	ns	ns
	B × C	*	ns	ns	ns	ns	ns	ns	ns
	A × B × C	**	ns	ns	*	ns	ns	ns	ns
M		263.62	2.36	14.37	64.9	22.84	4.18	28.62	24.20

Note: PH, plant height (cm); SD, Stem diameter (cm); NL, number of leaves per plant; FY, Forage yield hectare^{-1} (t ha^{-1}); DMY, Dry matter yield hectare^{-1} (t ha^{-1}); SP, Stem percentage (%); LP, Leaf percentage (%); EP, percentage (%); Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$); ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant

The hybrid had significant effect on PH, SD and NL. Hybrid NS 6010, in average for years and crop densities, produced significantly higher PH (266.23 cm), SD (2.39 cm) and NL (14.75) than hybrid ZP 684 (261.0 cm, 2.33 cm and 13.99, respectively).

The crop density had highly significant effect on PH, SD, FY, DMY, SP and EP. The lowest values of PH (259.52 cm), FY (61.90 t ha^{-1}), DMY (21.80 t ha^{-1}) and SP (43.28%) were measured in the lowest crop density (51,020 plants ha^{-1}), and the highest values (267.12 cm, 67.51 t ha^{-1}, 23.67 t ha^{-1} and 51.65%, respectively) in the highest crop t density (71,429 plants ha^{-1}). By contrast, the highest values of SD (2.43 cm) and EP (26.97%) were measured in the lowest p crop density (51,020 plants ha^{-1}), and the lowest values (2.29 cm and 20.29%, respectively) in the highest crop density (71,429 plants ha^{-1}).

The interaction of year and hybrid had significant effect on FY, DMY and LP. The interaction of hybrid and crop density had significant effect on PH. The interaction of year, hybrid and crop density had significant effect on PH and FY.

Discussion

PH, SD, FY and DMY were affected by year. Values for these traits were significantly higher in 2007 than in 2008. The amount of rainfall during growing period in 2007 was higher for 28.8 mm than in 2008 (236.6 mm). *Tóthné Zsubori et al. (2010)* reported that maize hybrids have the higher PH and DMY per plant in years with favorable climatic conditions. The better distribution of rainfall during vegetative stage of maize, i.e. greater amount was in May and June of 2007, especially in June, when the maize was at the stage of intensive stem growth. In 2008, the lower amount of rainfall and the higher air temperature in June reduced stem cell expansion resulting in reduced PH. *Çakir (2004)* reported that short drought stress in the vegetative stage of maize reduces PH, leaf area development and dry matter content of maize for 28-32%. High FY and DMY resulted in years with well distributed rainfall from June to August. In 2007, the amount of rainfall from June to August was higher for 44.6 mm than in 2007 (141.8 mm) which resulted in higher FY and DMY. Also, *Randjelovic et al. (2011)* stated that the amount of rainfall in this period is crucial factor for maize biomass production and grain yield. In 2007, drought period present was in July at the stage of flowering (anthesis shed and silking), and in 2008, in August at the stage of grain filling. However, results of *Mandić (2011)* showed that drought stress in July in 2007 in that location had no influence on flowering and pollination of hybrids ZP 684 and NS 6010, because there was rainfall in anthesis-silking period (lasts for five days). Also, author reported that the drought in August in 2008 reduced the grain weight per ear, 1,000 grain weight and grain yield. Different climatic conditions (temperature and quantity and rainfall distribution) significantly influence yield of maize grown under similar conditions *(Huzsvai and Nagy, 2005)*.

Hybrids significant differed for PH, SD and NL. Hybrid NS 6010 had the higher PH, SD and NL than hybrid ZP 684. The same results were obtained by *Mandić (2011)* at the same location. *Ranđelović (2009)* stated that the PH depends on the genetic basis of hybrid and growing conditions. The studied hybrids have stay green trait, i.e. leaves and stem stay green longer than the cob rapidly matures. These hybrids are suitable for the production of grain and silage. According to *Randjelovic et al. (2011)* hybrid NS 6010 produced higher DMY and grain yield than hybrid ZP 684, as well as hybrids from maturity groups FAO 400 (ZP 434 and NS 444 ultra) and FAO 700 (ZP 735 and Dunav) in region Srem at location Ruma. *Terzić et al. (2012)* reported that ZP maize hybrids (ZP 158, ZP 173/8, ZP 377, ZP

440, ZP 555 and ZP 679) of different genetic backgrounds produces DMY from 14.0 (ZP 158) to 21.3 t ha^{-1} (ZP 679).

PH, SD, FY, DMY, SP and EP were affected by crop density. PH, FY, DMY and SP were significantly increased parallel with crop density. Also, many research have showed that increasing crop density consistently increases PH *(Yilmaz et al., 2008; Moosavi et al., 2012*; *Shafi et al., 2012; Silva et al., 2014)*, FY *(Yilmaz et al., 2007; Baghdadi et al. 2012; Moosavi et al., 2012; Karaşahin, 2014)*, DMY *(Yilmaz et al., 2007; Stanton et al., 2007; Çarpıcı et al., 2010; Baghdadi et al. 2012; Moosavi et al., 2012; Ferreira et al., 2014; Karaşahin, 2014)* and SP *(Oktem and Oktem, 2005; Çarpıcı et al., 2010)*. Contrary, SD and EP were significantly decreased with increasing crop density. At high crop densities, PH increased due to competition for light (longer internodes and longer stem), while SD decreased. SD is strongly influenced by climatic conditions during stem elongation. Considering that at high densities inter-plant competition for environmental parameters (light, water, and space) increased, and hence photosynthesis was reduced, it finally caused SD reduction. The increase in DMY with the increasing crop density indicates the favorable response of biomass produced per maize population. EP decreased with increasing crop density. This can be explained by the presence of high intraspecific competition between maize plants for light, water and nutrients. In the highest crop density plants form shorter ears, number of kernels per row, the number of grains per ear, grain weight per ear, 1,000 grain weight, ear diameter, cob diameter and rachis diameter *(Mandić, 2011)*. Conforming to our results, *Widdicombe et al. (2002)*, *Turgut et al. (2005)*, *İptaş and Acar (2006)*, *Yilmaz et al. (2007)*, *Çarpıcı et al. (2010)*, *Baghdadi et al. (2012)*, *Moosavi et al. (2012)* and *Karaşahin (2014)* reported that SD decrease with increasing crop density. *Baron et al. (2006)*, *Stanton et al. (2007)*, *Yilmaz et al. (2007)*, *Çarpıcı et al. (2010)* and *Karaşahin (2014)* also observed that EP decreased with increasing crop density. Crop densities had not affected NL ranging from 14.34 to 14.39. Similar results were found in research by *İptaş and Acar (2006)*, *Moosavi et al. (2012)* and *Baghdadi et al. (2012)*. Also, LP (ranging from 28.05% to 29.76%) was not affected by crop density. Similar results reported *Çarpıcı et al. (2010)*.

Conclusions

Results of the study showed that the hybrids ZP 684 and NS 6010 can be recommended for production of forage in Srem region. Maize hybrids responded positively to high crop densities with maximum forage and dry matter yields occurring at crop density 71,429 plants ha^{-1} (70x20 cm). Also, higher crop density can be recommended because of the increase in usage of solar radiation and other inputs for the production of biomass per hectare. PH, FY, DMY and SP are

increased; SD and EP are decreased, while LP did not change as crop densities increased.

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Uticaj gustine useva na prinos krme kukuruza

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Cilj ovog istraživanja bio je da se ispita uticaj gustine useva na visinu biljke (VB), prečnik stabla (PS), broj listova po biljci (BL), prinos krme po hektaru (PK), prinos suve materije po hektaru (PSM), udeo stabla (US), udeo lista (UL) i udeo klipa (UK) dva hibrida kukuruza FAO 600 grupe zrenja (ZP 684 and NS 6010). Ogledi su izvedeni u suvom ratarenju u regionu Srema (lokacija Putinci 45° 00′ SGŠ, 19° 58′ IGD) tokom 2007. i 2008. godine. Upoređivane su tri gustine biljaka: G1 – 51,020 biljaka ha^{-1}, G2 – 5,9524 biljaka ha^{-1} i G3 – 71,429 biljaka ha^{-1} (odgovara razmaku 70 × 28, 70 × 24 i 70 × 20 cm). Ogledi su postavljeni po slučajnom blok sistemu u četiri ponavljanja. VB (265.45 cm), PS (2.40 cm), PK (68.63 t ha^{-1}) i PSM (24.63 t ha^{-1}) bili su značajno veći u 2007. godini nego u 2008. (261.78 cm, 2.32 cm, 61.17 t ha^{-1} and 21.04 t ha^{-1}). Hibrid NS 6010 imao je značajno veću VB (266.23 cm), PS (2.39 cm) i BL (14.75) nego hibrid ZP 684 (261.0 cm, 2.33 cm and 13.99). Povećanje gustine biljaka značajno je povećalo VB, PK, PSM i US, i značajno smanjilo PS i UK. Gustina biljaka 71,429 biljaka ha^{-1} (70 x 20 cm) može se preporučiti za gajenje hibrida FAO 600 grupe zrenja u klimatskim uslovima Srema u cilju postizanja visokih prinosa krme i suve materije.

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