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SHORT TANDEM REPEATS (STR) IN CATTLE GENOMICS AND BREEDING

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Review paper

Abstract: Molecular markers are essential tool for determining the specific genetic makeup of an individual and are valuable approach for genetic improvement of farm animals. In cattle breeding their application is useful for improvement of breeding programs for desired traits, better productivity and high quality products. These markers provide more accurate genetic information and better knowledge of the animal genetic resources. In this review we attempt to make a brief summary on the application of one of more advanced DNA-based molecular markers in cattle breeding, namely short tandem repeat (STR, microsatellites).

Keywords: molecular markers, STR, microsatellites, genome, polymorphism, breeding, cattle

Introduction

In the middle of the last century the use of blood groups and enzymes were beneficial for studying the animal genetics. The first molecular markers used in livestock were the protein polymorphisms. Later the proteins such as hemoglobin and transferrin were involved in all studies. Most of the conducted studies for genetic variation were based on allozyme protein markers. During the 1970's a large number of studies have been documented to be useful tool in characterization of blood group and allozyme systems in livestock (*Hanotte and Janlin, 2005*). At the University of Wisconsin, Irwin and co-workers used blood group antigens for parentage verifications in the Holstein Friesians *(Hines, 1999*). Stormont studied

the blood group systems in cattle in the 1950's (*Hines, 1999*) and concluded that the blood groups are powerful tool in the recognition of incorrect parentage (*Brenig and Schütz, 2016*). Later due to intensive inbreeding and a lot of mistakes in pedigree information and incorrect relationships between the animal blood groups and proteins become uninformative (*Adamov et al., 2011*). The errors in cattle pedigrees were different in European countries: 5 – 15% in Denmark (*Christensen et al., 1982*), 4 – 23% in Germany (*Geldermann et al., 1986*), 8 – 20% in Ireland (*Beechinor and Kelly, 1987*), 12% in Netherlands (*Bovenhuis and Van Arendonk*, 1991), 2,9 – 5,2% (*Ron et al., 1996*) or 11,7% (*Weller et al., 2004*) in Israel, 10% in dairy cattle in the United Kingdom (*Visscher et al., 2002*) and 10,7% in the Czeck Republic (*Řehout et al., 2006*). The use of these markers was limited because they are products of the gene expression (*Drinkwater and Hetzel, 1991*). The level of polymorphism observed in proteins is often low which has reduced the general application of protein-typing in the studies of diversity.

In the last decades, molecular biology created valuable new means for studying cattle livestock genetics and breeding techniques - the DNA based molecular markers that are based on the mutations of the nucleotide sequence within the individual's genome. They are the most informative markers available so far (*Yang et al., 2013*). In this way the selection according to genotype has become possible in the breeding of farm animals.

The simple technique discovered in 1993 by Kary Mullis that revolutionized the molecular biology was polymerase chain reaction (PCR) (*Nicholas, 1996; Van Marle-Köster and Nel, 2003*). PCR is a fast, sensitive and reliable method and became an essential tool in molecular biology and plays a main role in "in vitro" techniques that are now applicable to the analysis of genomes. After discovery of this major scientific development blood group typing and protein biochemical proteins in animal populations were replaced by the use of molecular DNA markers.

In this review we attempt to highlight the application of short tandem repeats (STR) or microsatellites in cattle genomics and breeding.

Molecular marker

Genetic markers are two types—protein and DNA (molecular) markers. Molecular markers can be categorized into two classes, nuclear DNA and mitochondrial DNA (mtDNA) markers, based on their transmission and evolutionary dynamics (*Hanotte et al., 2003*). Nuclear DNA markers are usually bi-parently inherited. Mitochondrial DNA markers are maternally inherited, express high rates of mutation, and are non-recombining such that they have one-quarter of the genetic effective population size (Ne) of nuclear markers (*Hanotte et al., 2003*).

Molecular marker or genetic marker is a fragment of DNA sequence that is associated to a certain region of the genome (*Wakchaure et al., 2015*). Molecular markers are classified on the basis of techniques used for discovery of polymorphism. There are several types of markers used today: hybridization-based markers such as RFLP (Restriction Fragment Length Polymorphism) and PCR-based markers e.g. Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Short Tandem Repeat (STR) or Microsatellites, Minisatellite, Single Nucleotide Polymorphism (SNP) and Single Strand Conformational Polymorphism (SSCP) (*Van Marle-Köster and Nel, 2003*).

In the animal genetic studies, the molecular markers revealing polymorphism at the DNA level play an important role. The term "Smart Breeding" is used to describe marker supported breeding strategies (*Firas et al., 2015*).

To studying the genetic variation in cattle breeds polymorphic DNA markers are usually used: D-loop and cytochrome B mitochondrial DNA (mtDNA) sequences for maternal inheritance, Y chromosome specific single nucleotide polymorphism (SNP) and STR (microsatellites) for paternal inheritance and autosomal microsatellite for bi-parental inheritance (*Avise, 1994*). DNA sequences as a new class of genetic markers were described in 1989 (*Machugh et al., 1997*). The number of repeats (Thymine, Adenine, Guanine or Cytosine) are variable in any DNA of the same population and within the alleles of every individual and can be characterized by using PCR (*Weber and May, 1989; Wang et al., 1998*).

Among the most polymorphic DNA markers that are contained in a large proportion of the eukaryotic genomes are the short tandem repeat (STR's) or microsatellites (SSR) and sequence tagged microsatellite repeats (STMR's).

STR are di-, tri-, or tetra nucleotide tandem repeats in tandemly repeated DNA sequences that are present in variable copy numbers at each locus and throughout the genome (*Ashley and Dow, 1994; Forbes et al., 1995; Bruford et al., 1996; Ellegren et al., 1997; Montaldo and Meza-Herrera, 1998; Schlötterer, 1998; Schmid et al., 1999; Toth et al., 2000; Beuzen et al., 2000; Teneva, 2009 ; Teneva and Petrovic 2010; Teneva et al., 2013; Gündüz et al., 2016*). PCR-amplified microsatellite repeats in the alleles can be detected using fragment analysis and other methods.

STR are located in the noncoding intronic regions of the bovine genome. They are most valuable and informative markers for genetic studies in cattle parentage verifications, genetic variability, genome mapping, relationships of individuals and populations, evaluation of inbreeding levels (F_{IS}), the genetic structure of subpopulations and populations, assessment of effective population size (N_e) and the gene flow between populations. They are used as markers for certain cattle disease in cattle diagnosis because several microsatellite alleles are associated with mutations in coding regions of the DNA that can cause a variety of medical disorders and variation in productive traits (*Selkoe and Toonen, 2006*).

The advantages of PCR- based microsatellite analysis for cattle studies are as follows:

- Locus-specific;
- Co-dominant (heterozygotes could be distinguished from homozygotes);
- Highly polymorphic ("hypervariable");
- Allow obtaining of rapid results in 48 hours or less;
- Useful at a range of scales from individual ID to fine-scale phylogenies;
- Easy to standardize and automate, results are very reproducible

The genotyping of microsatellite markers is performed automatically and with a low cost due to the use of multiplex technique, that allows the analysis of more microsatellites in one reaction.

Autosomal microsatellite loci in cattle are often used for genetic identification of individual and parentage analysis for the successful implementation and monitoring of ex-situ conservation programs, population diversity, differentiation of populations, genetic distances and genetic relationships. Microsatellite loci are highly sensitive to genetic bottlenecks and they are commonly used for inbreeding determination in cattle populations (*Hanotte and Janlin, 2005*). They are still the “gold standart” for many genetic population and identification purposes (*Brenig and Schütz, 2016*).

Parentage control and cattle identification

In 1993, with the development of a high density map of the bovine genome, many microsatellites became available (*Steffen et al., 1993; Fries et al., 1993*). In that year initial steps in using microsatellites in cattle identification and parentage control were performed (*Trommelen et al., 1993*). Parentage testing using DNA based markers yields much higher exclusion probability (> 90%) than the testing with blood groups (70–90%) or other biochemical markers (40–60%) (*Wakchaure et al., 2015*).

Further studies were performed to establish an internationally comparable panel of molecular markers *(Machugh et al. 1994; Glowatzki-Mullis et al., 1995; Heyen et al., 1997; Kemp et al., 1995; Peelman et al., 1998; Ma et al., 1996, Moazami-Goudarzi et al., 1997; Loftus et al., 1999; Kantanen et al., 2000; Canon et al., 2001; Hanotte et al., 2003; Beja-Perira et al., 2003; Gargani et al., 2015*). In many investigations FAO list of microsatellites in large number of cattle breeds were implemented (*Ajmone- Marsan and The GLOBALDIV Consortium, 2010*).

Microsatellite markers were widely used in cattle paternity analysis studies in different continents *(Bruford et al., 1996; Montaldo and Meza-Herrera, 1998;*

Beuzen et al., 2000; Schlötterer, 2004; Visscher et al., 2002; Hansen et al., 2002; Ibeagha-Awemu and Erhardt, 2005).

In Busha cattle in Serbia *Stevanov-Pavlović et al. (2015)* evaluated 12 microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824) recommended by International Society of Animal Genetics (ISAG) for paternity testing. The authors found high PIC (Polymorphism Information Content) values ranging from 0.513 to 0.905. The results showed that the 12 marker's set recommended by ISAG can be used with high confidence for forensic purposes in Busha cattle.

Genetic diversity analysis

The inbreeding process and various crossbreeding systems may lead to the loss of genetic variation within breeds. In this reason a lot of breeds may become extinct. The scientific community alarmed the necessity for the conservation of livestock resources. In 1992 the Food and Agricultural Organization (FAO) launched a program for the Global Management of Farm Animal Genetic Resources, with the main objective being to identify conservation activities and create an awareness of possible losses of genetic resources on an international basis (*Gandini and Oldenbroek, 1999*).

A global program was initiated directed towards genetic characterization of all farm animal species using DNA markers (*Groeneveld et al., 2010*). Microsatellite markers have been widely used for studying the genetic diversity in cattle (*MacHugh et al., 1997*). Genetic variability within and among populations is often of importance and may contribute to the selection and preservation of genetic resources (*Groeneveld et al., 2010*).

Microsatellite markers were considered as a marker of choice for diversity assessment in breeds (*FAO, 2004*). A list of microsatellite markers for genetic characterization of cattle breeds have been approved by Food and Agriculture Organization (FAO) (*Navani et al., 2002*). The 12 selected markers (BM1814, BM1818, BM2113, ETH3, ETH10, ETH225, INRA023, SPS115, TGLA53, TGLA122, TGLA126, TGLA227) were included in an International comparison test of ISAG.

Based on microsatellites as a marker of choice a lot of investigations have been performed to estimate both the relationships among the breeds and the genetic diversity within and between populations (*Ashwell et al., 2004; Sun et al., 2007*). Genotyping data of 30 microsatellite loci in 69 European breeds were used to determining the main criteria for conservation of breeds (*Lenstra et al., 2006*). The selected breeds showed high degree of molecular diversity, that is an apparent

reason for their conservation. The Busa and Anatolian breeds were considered to be valuable genetic resources on the basis of their high genetic diversity (*Medugorac et al., 2009*). Conservation priorities of Nordic cattle based on genetic diversity were outlined by *Bennewitz et al. (2006*) and *Tapio et al. (2006*).

Many other authors used common microsatellite markers to assess genetic diversity within breeds and the inbreeding in different cattle breeds (*Teneva et al., 2005; 2007; Garcia et al., 2006; Tapio et al., 2006; Ginja et al., 2009a; Li and Kantanen, 2009; Qi et al., 2009*). Several studies have been conducted in European and Eurasian cattle (*Bos taurus*) in which microsatellites were used to assess genetic variability and differentiation (*Canon et al., 2001; European Cattle Genetic Diversity Consortium, 2006; Tapio et al., 2006; Li and Kantanen, 2009*). For Creole breeds, several microsatellite-based studies were reported (*Martinez et al., 2005; Armstrong et al., 2006; Quiroz-Valiente et al., 2006; Aquino et al., 2008; Ulloa-Arvizu et al., 2008; Martinez- Correal et al , 2009*). Later, *Delgado et al. (2011)* using 19 microsatellites assessed the genetic diversity and relationships among 26 Creole cattle breeds from 10 American countries representing North, Central, South America and the Caribbean Islands. Creole cattle populations showed high level of genetic diversity comparing to the breeds subjected to intensive breeding. Regardless of the detected high genetic diversity, a significant inbreeding was also detected. Creole cattle breeds represent great reservoirs of cattle genetic diversity but measures to avoid inbreeding and uncontrolled crossbreeding is highly necessitated (*Delgado et al., 2011*).

In Indian zebu cattle (*Bos indicus*) *Chaudhari et al. (2009)* reported 25 microsatellite loci with a high PIC value (> 0.5) in 145 purebred cattle originating from unrelated Kenkatha and Gaolao cattle breeds which is an indication that these markers are highly informative and appropriate for characterization of both cattle populations. The authors estimated 21.21% and 22.48% heterozygotes in Gaolao and Kenkatha populations, respectively. However, the additional analyses based on a number of fluorescent labeled microsatellite markers used to characterize the same cattle breeds showed a little genetic differentiation between them (*Alex et al., 2013*). Numerous factors such as inbreeding, genetic hitchhiking, null alleles (non-amplified alleles) and occurrence of population substructures have been established as reasons of heterozygote deficit in the studied populations.

Several microsatellite markers have also been used in conservation studies concerning certain other important cattle breeds (*Frankham et al., 2002; Navani et al., 2002*).

Meta-analysis of different microsatellite loci revealed patterns of diversity and taurine–zebu admixture over Europe, South-West Asia and Africa (*Freeman et al., 2006*). The mixed origin of Indonesian zebus using microsatellites was confirmed in the diversity study of *Mohamad et al. (2009*). In contradiction, the microsatellite analysis showed that the Indonesian Bali cattle is a pure breed (*Bos javanicus*) (*Groeneveld et al., 2010*).

Most of the microsatellite data indicated a separate position of Mediterranean cattle, but divide the Transalpine cattle into two different clusters of breeds: Central-European and Northern European (*Lenstra et al., 2006*). Conservation priorities for Nordic cattle were reported by *Bennewitz et al. (2006)* and *Tapio et al. (2006).*

Jersey is a common and unique cattle breed originating from the UK Channel Island of Jersey. A Jersey Island cattle was isolated from other UK and European cattle populations for approximately 50 generations. The genetic diversity of this breed was described for the first time by *Chikhi et al. (2004)* on the base of 12 microsatellite markers: HAUT27, HEL5, BM1314, BM1818, BM2113, INRA005, INRA063, ILSTS006, ETH10, ETH225, TGLA122, and TGLA227. This study showed that the average number of alleles per locus and the expected heterozygosity were comparatively higher with respect to that observed in a number of continental breeds. The authors reported absence of a loss of genetic diversity and inbreeding. They concluded that it is unnecessary to import unrelated animals for management purposes despite of the fact that no imports have taken place to the island since 1789.

Egito et al. (2007) also reported a significant amount of genetic variation in Brazilian local cattle populations on the base of the observed microsatellite variation in 22 STR loci. These data showed that Brazilian Creole breed constitutes an important and diverse source of genetic diversity for bovine breeding and conservation.

Recently, *Sharma et al. (2015)* investigated genetic diversity and relationship among 11 Indian cattle breeds using 21 microsatellite markers, and concluded that the Southern breed “Ongole” is distinct from the breeds of Northern/Central India. The results provide basic information about the genetic diversity and structure of Indian cattle which should have implications in the management and conservation of cattle diversity.

Several studies have been conducted in European and Eurasian cattle (*Bos taurus*) in which microsatellites were used to assess genetic diversity and differentiation *(Canon et al., 2001; Tapio et al., 2006; European Cattle Genetic Diversity Consortium 2006; Li and Kantanen, 2009*).

Allelic variation in sixteen microsatellite loci (CSSM 66, ETH 10, ETH 152, ETH 225, ETH 3, HEL 1, HEL 5, HEL 9, ILSTS 005, INRA 023, INRA 032,INRA 035, INRA 037, INRA 005, INRA 063, and TGLA 44) was studied in 10 Spanish, 5Portugese and 3 French cattle breeds. A total of 173 alleles were detected across the 16 loci analysed (*Canon et al., 2001*). Observed and expected heterozygosities per breed ranged from 0.54 to 0.72. The level of breed differentiation was considerable indicating that 93% is due to the differences among individuals while the remaining 7% corresponds to the differences between breeds. The authors concluded that the microsatellites provides reasonable statistical power for breed

assignment and allow future management of the breeds to be based on better knowledge of their genetic structure and relationships between populations.

In Romania, the genetic diversity among Romanian Grey, Brown, Spotted and Black and White cattle breeds was evaluated at 11 microsatellite loci focusing on the endangered Romanian Grey breed (*Ilie et al., 2015*). High level of genetic diversity was established in the endangered Romanian Grey cattle population. The results confirmed that the breed's genetic diversity is preserved correctly using the current conservation program directed to reduction of the genetic loss.

Genetic markers with PIC values higher than 0.5 are normally considered as informative in a population (*Botstein et al., 1980*). Higher PIC values were also observed in the taurine and indicus breeds using microsatellite markers (*Bradley et al., 1994*; *Canon et al., 2001; Maudet et al., 2002; Kumar et al., 2003; Metta et al., 2004; Mukesh et al., 2004; Pandey et al., 2006; Sodhi et al., 2006; Chaudhari et al., 2009*).

Molecular characterization of Indian breed Hallikar, the native cattle breed of Karnataka was performed using 19 cattle specific microsatellite markers recommended by FAO. The study proved that the cattle specific microsatellite markers used were highly polymorphic and highly informative for genetic characterization of cattle breeds (*Kumar et al., 2003*).

In comparison with other European and Balkan countries, in Bulgaria there is a big gap in molecular characterization of cattle based on microsatellites and other molecular markers. *Teneva et al. (2005; 2007*) studied local Bulgarian Grey and Bulgarian Shorthorn cattle breeds through microsatellite markers. They established a high PIC value (>0.5) and high heterozigosity based on 11 STRs.

Genome mapping

Molecular markers provide researchers with tools to develop genetic linkage maps. The maps show the position of markers and genes on a chromosome and the distance between genes. The genetic maps have been used to select markers that are distributed across the whole genome. The markers are used in QTL mapping studies to follow the inheritance of specific regions of chromosomes through generations. Microsatellite markers are particularly appropriate for linkage mapping (*Wakchaure et al., 2015*). The efforts to map the cattle genome is progressing. The bovine genetic map contains over 2 200 microsatellites (*Van Marle-Köster and Nel, 2003).* The microsatellite-based genetic map is a fundamental tool for linkage mapping of monogenic as well as polygenic traits of interest. A high-density bovine microsatellite-based genetic map has been constructed in 2004 *by Ihara et al.* and it consists of 3960 markers including 3802 polymorphic ones (*Ihara et al., 2004*). This map is a powerful tool for mapping of

QTLs and is a genetic basis for the development of well-annotated gene maps in cattle (*Ihara et al., 2004*).

Association of microsatellites with productive traits and disease

During the past decades, the development in molecular genetics have led to the identification of multiple genes or genetic markers linked to genes that affect quantitative traits. This provided an opportunity to enhance the selection for traits that are difficult to be improved by conventional breeding due to their low heritability.

Usually, microsatellites should be neutral DNA markers maintaining their characteristics relatively constant (*Mariani and Bekkevold, 2014; Brenig and Schütz, 2016*). However, several of the microsatellites in the ISAG parentage control panel are under artificial selection and hence are not completely neutral. ETH10 on bovine chromosome 5, for example, is associated with growth and carcass traits in Angus, Brangus, and other cattle breeds *(DeAtley et al., 2011; Meirelles et al., 2011*). The ETH10 locus was also associated with coat colour in Brown Swiss cattle (*Gutierrez-Gil et al., 2007; Drogemuller et al., 2009*). BM1818 was proven to be associated with somatic cell score (SCS) and specific alleles of this locus are favorable or unfavorable for mastitis resistance (*Chu et al., 2005*). In another study, significant differences in allelic frequencies for BM1824, ETH10, INRA023, SPS115 and TGLA53 alleles were described in Japanese Black cattle depending on selection of sires for intramuscular fat (*Smith et al., 2001*).

After *Brenig and Schütz (2016*) most of the 12 microsatellite markers which were included in ISAG/FAO panel BM1814, BM1818, BM2113, ETH3, ETH10, ETH225, INRA023, SPS115, TGLA53, TGLA122, TGLA126, TGLA227 are associated with economical important traits. The authors concluded that microsatellite markers recommended for parentage control in cattle are influenced by selective breeding and are DNA markers related to adaptiveness. At least 40 different QTLs have been described flanking the microsatellite chromosomal positions and the most frequent traits included milk protein yield, milk fat yield, somatic cell score, milk fat percentage, body weight at birth and body weight at weaning (*Hu et al., 2013*).

The application of microsatellite markers in QTL analysis has been found to be prolific in determining the effect of specific molecular markers on milk quality (*Deb et al., 2013; Olsen et al., 2004*). Several microsatellite markers have been developed for identification of the specific region of BTA6 with effect on milk fat and milk protein (*Kuhn et al., 1999*).

Singh et al. (2013) reported that molecular markers have a great contribution to the better production performance and disease resistance in livestock.

Using microsatellite markers and identification of the particular biomarkers associated with various diseases and economically significant clinical conditions (such as mastitis) has helped to increase the specificity and accuracy of disease resistant breeding and to enhance productivity (*Deb et al., 2013*).

The results of *Hanotte et al. (2003*) from mapping the quantitative trait loci controlling the trypanotolerance revealed that the selection for trypanotolerance within an F_2 cross between N'Dama and Kenya Boran cattle could produce a synthetic breed with higher trypanotolerance levels than the currently existing in the parental breeds. In this QTL mapping the authors genotyped a cattle group at 477 microsatellite loci, distributed among the 29 cattle autosomes for 16 phenotypic traits.

Statistical methods used in microsatellite analysis

The average number of alleles (MNA), observed (H_o) and expected (H_e) heterozygosity and estimation of polymorphism information content (PIC), are the most commonly calculated population genetic parameters for assessing the diversity within cattle breeds (*Mburu and Hanotte, 2005; Hanotte and Janlin, 2005*). PIC values indicate the informativeness of the studied microsatellite loci. Hardy-Weinberg equilibrium test is always used to predict whether the population is stable or not. The observed genotypes are compared with the expected genotypes in a x^2- test for likeness of fit. The high heterozygosity values observed in the studies indicate the presence of large number of polymorphic loci. The most simple parameters for evaluating the distribution of diversity between breeds using genetic markers are the genetic differentiation or fixation indices e.g. F_{st}, G_{st}, R_{st}. They reveal the variation among populations. The most widely used is F_{st}, which measures the degree of genetic variation between subpopulations through the calculation of the standardized variances of allele frequencies amongst populations (*Weir and Basten, 1990; Mburu et al., 2003*). The genetic distances can also be analyzed in terms of genetic diversity and individual breed contributions to the total diversity of the breeds.

The most commonly used approach so far is the method proposed by Weitzman (*Weitzman, 1993; Hanotte and Janlin, 2005*). It involves calculation of a matrix of genetic distances and construction of dendrograms. Individual breed contributions are calculated by comparing the total length of the dendrogram including all breeds. Priority breeds for conservation would be the breeds contributing most to the diversity of the set. The Weitzman approach applied in 49 African cattle breeds (*Reist-Marti et al., 2003*) allowed their separation into two groups, the 'taurine' and 'indicine'.

The main cattle microsatellite genetic parameters like observed number of alleles, allele frequency, FIS, observed and expected heterozygosity, the presence of null alleles, the neutrality of the microsatellites, genetic distances, Analysis of molecular variance (AMOVA) usually are analysed by a number of commonly used population genetic computer programs for genetic microsatellite statistical analysis: GENEPOP, ARLEQUIN, POPGENE, MICROSAT, PHYLIP, STRUCTURE MICROSATELLITE ANALYZER (MSA*)*, MICROCHECKER *(Mburu and Hanotte, 2005*).

Conclusion

The development of polymorphic microsatellite markers in advanced genetics and biotechnology gives the opportunity for the selection, improvement of cattle health and production. The microsatellite technology with its advantages and disadvantages has a huge variety of applications in cattle breeds. Microsatellite markers for improving milk production and other main productive traits as well as their association with disease in cattle breeds are useful for breeders. They may also be efficiently applied in conservation decisions. The employment of microsatellite markers in determining the resistance to economically important diseases such as mastitis and other cattle diseases is helpful to test the leak of animals and their productivity. Consequently, this genomic technology provides a valuable information for cattle genetics and breeding today and in the future.

Kratki tandemski ponovci (Short tandem repeats - STR) u genomici i odgajivanju goveda

Atanaska Teneva, Elena Todorovska, Milan P. Petrović, Szilvia Kusza, Kathiravan Perriassamy, Violeta Caro Petrović, Dušica Ostojić Andrić, Dimitar Gadjev

Rezime

Molekularni markeri su suštinsko sredstvo za određivanje specifičnog genetičkog sastava pojedinca i predstavljaju dragoceni pristup genetičkom oplemenjivanju farmskih životinja. U stočarstvu njihova primena je korisna za poboljšanje programa odgajivanja za željene osobine, veću produktivnost i proizvode visokog kvaliteta. Ovi markeri pružaju preciznije genetske informacije i bolje poznavanje genetičkih resursa životinja. U ovom preglednom radu pokušavamo da napravimo kratak pregled o primeni jednog naprednijeg molekularnig markera zasnovanog na DNK u stočarstvu, a to su kratki tandemski ponovci (STR, mikrosateliti).

Ključne reči: molekularni markeri, STR, mikrosateliti, genom, polimorfizam, uzgoj, stoka

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EVIDENCE OF GENETIC HYBRIDIZATION OF THE WILD BOAR AND THE INDIGENOUS BLACK PIG IN NORTHERN GREECE

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Abstract: In Greece both the black indigenous pig and the wild boar are considered as species of valuable genetic diversity while their products achieve a valuable market price. However, many crop damages are recorded, with farmers to claim that wild boar hybrids are responsible. On the other hand, black pig classification is based on phenotypic characteristics, which does not ensure breed's homogeneity in case of hybridization. Using the PCR-RFLP methodology, pig samples (n=135) from different rearing situations (feral boars, semi-extensive black pigs and extensive wild boars) were examined in order to identify whether or not hybridization exists. In the examined feral population of wild boar a 26% of hybrids was noted, while in the case of the extensive farming population of wild pigs a hybridization of 11.76% was observed. Interestingly, in both cases of the examined black pigs' populations, a mentionable hybridization with wild boar was observed, reflecting probably an implemented breeding practice or uncontrolled mating with wild boars. A pivotal level (5-7%) of inbreeding rate was also noted in the examined populations. The immediate removal of hybrids from all the examined populations should be achieved, in order to prevent and eliminate further introgression, genetic depression and loss of genetic diversity for both populations of wild boar and black pig. Finally, the applied methodology may be used by state authorities or certifying organizations to test, control or inspect farms rearing wild boar or black pig populations in order to record and eliminate hybridization events between them.

Key words: *Sus scrofa*, wild boar, *Sus domestica*, hybridization, PCR-RFLPs, genes

Introduction

Over the past decades animal production has developed a strong focus on high-yielding breeds and breeds that mainly offer high economic turn over. As a consequence, highly specialized traits in domestic animal breeds often became an obstacle in high-input-based farming systems (*Mendelsohn, 2003; Tisdell, 2003*), leading to a progressive replacement of traditional multipurpose breeds with high-yielding breeds (*Ugarte et al., 2001; Zander et al., 2013*). However, nowadays due to the high concern of consumers to healthier and of better quality livestock products, animal production trends have changed from a high-input economic systems to a more sustainable base characterized by a resource-driven activity bound to local conditions and environments. Thus, the effort of the global community is targeted at preserving the natural sources' biodiversity, existing among them the animal genetic resources. Many countries have put into force measures, laws or funds in order to protect and to preserve the autochthonous local breeds. Greece is one of these countries, which runs specific measures for the preservation of indigenous breeds with a total funding of twenty five millions euro for the period 2014-2020.

In Greece apart from the industrialized breeds used in the intensive pig farms, two populations of pigs are also exist; the feral population of wild pigs (*Sus scrofa scrofa*) and the population of black pig (*Sus scrofa domestica*), an autochthonous domesticated Greek pig breed (*Laliotis, 2001, Laliotis et al., 2017*). The wild boar is considered as very popular game specie (*Acevedo et al. 2007*). In Greece, is present in almost all mainland apart from Attica, Evian and islands, as its habitat is usually oak, chestnut or coniferous forests (*Tsachalidis and Hadzisterkotis, 2009*). In addition, wild boar's meat is considered of high quality and as a result a higher price in market is achieved. During the past decades its population was under restriction. Thus, hunting has been and still remains permitted for a certain period while a specific permissible game limit per hunter is implemented. In addition, wild boar settlements were established across the mainland of Greece firstly to protect the specie and secondly to restock and re-introduce pure specimens of the wild breed in its natural habitat for hunting purposes. Nowadays, an increase of its population is observed (*Beskardes et al. 2010*). However, a lot of damages in crops caused by wild boar populations have been recorded, while many farmers claim that theses damages are a result of pig hybrids (crossbreeds between wild boar and domesticated free-ranging pigs) and not actually from wild boar. On the other hand, the Greek black pig is a product of natural selection that was able to adapt to different and harsh environmental conditions. It is usually bred under semi-intensive systems, and the breed is considered under threat, rendering it on the list of endangered autochthonous breeds (*Laliotis, 2001; Laliotis et al., 2017*).

The discrimination of Greek breeds until today is based on phenotypic characteristics (*Rogdakis, 2002*). In the meantime, due to the cross breeding that

have taken place and the lack of preservation of pedigree books, there is difficulty in the objective and unambiguous classification of any individual animal into a certain breed. Simultaneously, payments concerning the aid to farmers that rare local breeds or wild species requires the confirmation of the breed /specie of the reared animal. As a result of the aforementioned, doubts about the correct and objective control implemented by public or private sector auditors on farms breeding rare animals are being raised, when only phenotypic characteristics are included in the inspection control.

The advent of novel DNA technology assisted the association of certain genome loci or single genes with the discrimination between species. One of these genes is the gene encoding the melanocortin-1 receptor (MC1R). The MC1R regulates melanogenesis in mammals within the mammalian melanocyte and the hair follicle. Common variations (polymorphisms) in the MC1R gene are associated with normal differences in skin and hair colour. At molecular level, the MC1R gene has been well studied in many eutherian species, among them human, rat and pig (*Valverde et al. 1995; Box et al. 1997; Ollivier and Sellier 1982; Robbins et al. 1993*). According to *Kijas et al. (1998),* a unique MC1R allele (E+) has been identified in the European wild boar (*Sus scrofa scrofa*) that is not found in any of the domestic breeds (*Sus scrofa domestica*).

The aim of the present study was to implement the genotyping procedure of the E locus of the MC1R gene on different pig sampling situations and specifically samples from i) feral boars, ii) black pigs reared under an semi- extensive system and iii) from wild pigs reared under an extensive system in order to: a) genetically test if the sampled animals objectively belong to the wild specie or the domesticated that farmers claim, b) to check if wild boar hybridization exists in the examined situations and c) to provide useful information to public and private sector concerning the inspection and certification of wild pig discrimination, which in the future may serve as a tool for the undoubted audit control.

Materials and Methods

Animals-Sampling

For the purposes of the present study the following sampling procedures were implemented for further analysis:

a) During the hunting period of wild boar in Greece, (15 September – 21 January) hair samples from fifty three (53) games of wild pigs (fifty sows and three boars) were collected (hereafter Case A). Samples were taken from different locations of North-eastern Greece.

b) Blood samples from the animals of two farms rearing the indigenous black Greek pig breed were collected (hereafter Case B1 and Case B2). The farms were located in Northern Greece and implement a semi-extensive livestock production system. The farm of “Case B1” was located near a forest area and twenty eight (28)

animals (twenty six sows and two boars) were sampled, while in the "Case B2" the respective animals were thirty seven (37) animals (thirty four sows and three boars).

c) Hair samples from the seventeen (17) animals (fifteen sows and two boar) of a farm rearing, under an extensive system, a small population of wild boar in Northern Greece, which is also serves for reintroducing wild boar in its natural habitat for hunting purposes, were collected (hereafter "Case C").

DNA extraction and Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was followed for genotyping the E locus of MC1R gene according to *Kijas et al. (1998).* Briefly, DNA was extracted from blood or hair roots using the Nucleospin blood or tissue kits (Macherey-Nagel, Germany) according to manufacturer instructions and then was electrophorized to ensure the integrity of the DNA samples.

For the PCR reaction approximately 150 ng of genomic DNA was used as template and amplified in a final volume of 50 μL containing 100 nM from each primer, 2 mM dNTPs and 1 unit MyTaqTM DNA Polymerase (Bioline). The PCR amplification conditions are shown in Table 1. Then, 25 μL of each PCR product was digested in a total volume of 40 μL, containing 10 U of the appropriate restriction enzyme (Table 1), 4 μL of restriction buffer, and 10.2 μL of ddH2O for 2 hours at 37 ^{o}C. Restriction fragments were examined by electrophoresis on 2.5% agarose gel.

Table 1. Primers, PCR protocol, and restriction enzyme used at the present study for the molecular analysis of the MC1R gene

Gene	Primers (5′ →3′)		PCR conditions	Restriction Enzyme
	Forward	Reverse		
MC1R (AF326520) c. 914C>T	RGTGCCTGGAGGTGTCCAT	CGCCCAGATGGCCGCGATGGACCG	*94°C for 5 minutes *35 cycles: 94°C for 45 seconds 55°C for 45 seconds 72°C for 45 seconds *72°C for 50 minutes	*Bsp*HI (37^{o}C, 120 min)

Statistical Analysis

Genotype frequencies, allele frequencies and Hardy-Weinberg equilibrium estimations were calculated using PopGene Software v. 1.32 (Yeh et al., 1997). The effective number (Ne) and the inbreeding rate (ΔF) of each flock were estimated using the following equations (Falconer and Mackay, 1989):

(a) Ne= (4*males* females) / (males + females),

(b) ΔF= 1 / 2Ne.

Results

The allelic and genotypic frequencies of the examined gene are presented in Table 2. Two alleles (A and B) and three genotypes, namely E^+/E^+, E^+/E^- and E^-/E^- were identified in the examined cases. Specifically, in "Case A" 39 animals were found to carry the E^+/E^+ genotype, while 14 animals the E^+/E^- genotype. It should be noted that two of the three male samples were found to be heterozygous for the analysed gene locus.

Table 2. Genotype distribution, allele frequencies and Hardy-Weinberg status of the examined gene locus.

Genotype	Observed Genotypes				Expected Genotypes			
	Case A	Case B1	Case B2	Case C	Case A	Case B1	Case B2	Case C
E^+/E^+,	39 (73.58%)	-	1 (2.70%)	14 (88.24%)	39.92	25.08	0.68	14.06
E^+/E^-	14 (2 ♂) (26.42%)	3 (♀) (10.71%)	8 (2 ♂) (21.62%)	2 (1 ♂) (11.76%)	12.15	2.84	8.65	1.88
E^-/E^-	-	25 (89.82)	28 (75.68%)	-	0.93	0.08	27.68	0.06
Allelic Frequencies	p= 0.87 q= 0.13	p=0.05 q=0.95	p= 0.14 q=0.86	p= 0.94 q= 0.06	HWE			
					P>0.05			

In "Case B1", three animals (females) found to be heterozygotes (E^+/E^- genotype), while the rest of the animals carried the homozygote genotype E^-/E^-. In the case B2 28 animals carried the E^-/E^- genotype, 8 animals (6 females and two males) the E^+/E^- genotype and one animal (male) the E^+/E^+ genotype.

Regarding "Case C" almost all animals found to have the genotype E^+/E^+ apart from one female that found carrying the E^+/E^- genotype. The analysed gene locus wan not found consistent with the Hardy-Weinberg Equilibrium (P>0.05) in none of the examined populations.

As far as it concerns the effective number of the population, in “Case B1” found to be Ne= 7, while the inbreeding rate (ΔF) was 0.07 (7 %). The respective parameters for “Case B2” and “Case C” were estimated as Ne=11; ΔF=0.05 (5%) and Ne= 7; ΔF=0.07 (7 %), respectively.

Discussion

Both the indigenous pig breeds and the wild boar populations are considered as “pool” of valuable genetic diversity. The replacement of indigenous breeds by foreign improved breeds with greater yields led to dramatically diminish of their number, to a threat of extinction and to a loss of genetic diversity. In addition the wild boar declined significantly in Europe at the beginning of the 20th Century, rendering its population under threat (*Massei et al., 2015*). Many countries, including Greece, put into force measures and funds for the conservation of indigenous breeds, while wild life have been funded in the past as a tool for the conservation and re-colonization of the wild specie populations (i.e. wild boar).

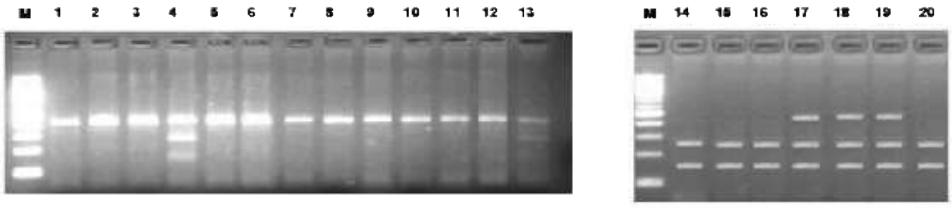

Figure 1. Genotyping analysis of the MC1R gene in the studied populations (representative samples). Wild boar (428 kb): samples: 1-3 and 6-12; Hybrids (428 kb; 256 kb; 172 kb): samples 4; 13 and 17-19; Black pig (256 kb; 172 kb): 14-16 and 20.

However, in Greece, any attempt of controlling and ensuring the rearing of a certain indigenous or wild population is accomplished through phenotypic (morphological) characteristics (i.e. coat colour, ear shape, etc.). This fact poses major risks, firstly due its subjective criteria and secondly due to the fact that many farmers cross breed their flocks with other improved (domesticated) breeds, rendering the certification and classification of the reared animal into a pure breed or population not an easy procedure. Such an example forms the breeding of the indigenous black pig and the wild boar in Greece. Herein, different rearing cases of wild boar and black pig were examined by means of their bred/population genetic purity or their hybridization using the implementation of a PCR-RFLP technique as an easy tool for checking, certifying and classifying such pig individuals.

From the observed results, none of the examined population was consistent with Hardy-Weinberg equilibrium, probably due to the small number of the observed heterozygotes. In the feral wild boar population (“Case A”) the 26% of

the examined animals found to be hybrids, meaning that hybridization of wild boar population with domesticated pig individuals had been taken place. Although two of the three analysed game males found to be hybrids, the fact itself raises serious questions at two levels; firstly at what extent these males have led to a genetic introgression of the wild population, taking into consideration the uncontrolled, and secondly suspicious are arising whether or not framers that breeding animals belonging to the wild boar or the black pig keep the genetic pure of their livestock.

In all examined cases of farmed black pigs (B1, B2) a worth noting number of hybrid animals were detected (Table 2). In the "Case B1", hybridization may be due to the implement livestock system (semi-extensive) near forest area, where wild boars may be mating more easily with the domesticated indigenous Greek breed. The cross-breeding between wild boar and free-ranging pigs or local domestic breeds (mainly Greek black pig) is a common practice in many wild boar farms in Greece (*Papatsiros et al., 2012*). The fact that in "Case B2" both wild and hybrids animals were detected may reflect an implementing breeding strategy, as firstly two mature hybrid males were detected and secondly the surrounding area of the livestock (semi-extensive fenced system) was not adjacent to any forest area. The low prolificacy performance of the black pig breed reflects to a narrow economic income. In order the farmers to cope with the aforementioned they tend to apply their own breeding strategies without any scientific assist, which in some cases may even include crossing with commercial breeds or wild boar in order to succeed higher production rates or higher value of their final meat product (*Laliotis et al., 2017).* Although crossbreeding potentially enhances production traits, it simultaneously threatens the heritage status of the indigenous breed or wild populations. As a consequence, hybrid males should not be used in mating or immediately should be removed and substituted by pure bred males in order to ensure breed's genetic purity. Otherwise, if the male hybrids will be retained, then conservation of the flock as nucleus of black pig breed is under threat.

The same breeding management should be implemented in the "Case C" where two cross bred animals between wild boar and domesticated pig was observed. The reported cross breeding animals should probably be due to the free range breeding system that is followed, where uncontrolled mating is more easily to be occurred. However, this results in a continuous cross breeding of wild boar. Besides, the genetic purity of the wild species is normally desirable per se because the mixing of gene pools of formerly distinct taxa can lead to genetic homogenization and the extinction of rarer species (*Largiadèr, 2007*). In addition, hybridization can cause problems without breeding depression and mal-adaptation to a local environment (*Rhymer and Simberloff, 1996*). As some farmers might not be aware that they maintain hybrids among their herds, the hybrids should immediately be removed in order to ensure the purity of the wild species. Moreover, the future progeny of females should be checked in order to ensure the birth of piglets belonging to the wild boar specie. Apart from the aforementioned questions are raised regarding the

financial aid that farmers receive for retaining pure bred nucleus of wild boar or black pig. A proposal may be farmers who receive any fund for this purpose should be paid accordingly to the percentage of the genetically certified pure bred animals that rare plus a penalty regarding the introgression of the populations.

In addition, inbreeding rates in the examined farms ("cases B1, B2 and C") were found to be at a small but pivotal level (5 %<ΔF<7%). However, further measures (i.e. mating with non-relatives) should be implemented in order to prevent the inbreeding depression and the occurrence of the genetic drift.

To sum up, four different wild boar or indigenous domesticated black pig populations were investigated in order to find out whether or not hybridization between species exists. Using a simple and easy implemented DNA technique we concluded that a notable percentage of specimens belonged to a cross-bred hybrids existed, rendering the conservation either of indigenous pig breed or the wild boar population under risk. Thus, the evidence of hybridization of the wild boar in the Northern Greece exists and confirms the respective reports of farmers concerning crops' damages by pig hybrids. However, a broader study recording all the areas of wild boar habitat should be undertaken in order to specify the extent of the hybridization. On the other hand, interestingly, hybridization also exists in the black pig population, rendering its homogeneity under risk. The applied method could be useful for State authorities or other certifying organizations in order to test, control or inspect farms that run under specific certification standards or specific funding aids for rearing the autochthonous Greek pig breed (black pig) or the wild boar.

Dokazi genetske hibridizacije u populacijama divlje svinje i autohtone crne svinje u severnoj Grčkoj

George P. Laliotis, Meni Avdi

Rezime

U Grčkoj, autohtona crna svinja i divlja svinja se smatraju vrstama dragocene genetičke raznovrsnosti, dok njihovi proizvodi postižu visoku tržišnu cenu. Međutim, zabeležene su brojne štete na usevima, a poljoprivrednici tvrde da su za to odgovorni hibridi divljih svinja. S druge strane, klasifikacija crne svinje bazirana je na fenotipskim karakteristikama, što ne obezbeđuje homogenost rase u slučaju hibridizacije. Koristeći metodologiju PCR-RFLP, ispitani su uzorci svinja (n = 135) iz različitih situacija u odgoju (svinje iz divljine/prirode), crne svinje iz polu-intenzivnog sistema gajenja i divlje svinje iz ekstenzivnog) kako bi se identifikovalo da li postoji hibridizacija. U ispitivanoj populaciji divlje populacije

divljih svinja zabeleženo je 26% hibrida, dok je u slučaju ekstenzivne gajene populacije divljih svinja zabeležena hibridizacija od 11,76%. Interesantno je da je u oba slučaja ispitane populacije crnih svinja posmatrana hibridizacija sa divljim svinjama, što je odraz verovatno sprovedene prakse oplemenjivanja ili nekontrolisanog parenja sa divljim svinjama. Ključni nivo (5-7%) stepena inbidinga takođe je zabeležen u ispitanim populacijama. Hibridi iz svih ispitanih populacija bi trebalo da budu odmah uklonjeni, kako bi se sprečila i eliminisala dalja introgresija, genetska depresija i gubitak genetičke raznovrsnosti za obe populacije divlje i crne svinje. Najzad, primenjenu metodologiju mogu koristiti državni organi ili organizacije za sertifikaciju, testiranje, kontrolu ili inspekciju farmi koje uzgajaju populacije divlje svinje ili crne svinje kako bi zabeležile i eliminisale događaje hibridizacije između njih.

Ključne reči: *Sus scrofa*, divlja svinja, *Sus domestica*, hibridizacija, PCR-RFLPs, geni

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ENVIRONMENTAL SOURCE OF STRESS IN LIVESTOCK PRODUCTIVITY – A STUDY OF MINNA CLIMATE DATA

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Original scientific paper

Abstract: Stress emanating from environment is a factor limiting livestock productivity in the Tropics because of elevated temperature year round; hence this study took a look at Minna climate data for evaluation of Temperature-Humidity-Index (THI) as a way of identifying climate source of stress on livestock production. Climate Normals for Minna between years 1961 and 2018 were obtained, and the data were analyzed using general formulae for calculating Temperature-Humidity-Index for livestock production. Relationships between production parameters on commercial farms and the THI data indicated that heat stress is a potential cause of oxidative stress in the area. The THI showed that the environmental conditions in the study area has potential for heat stress on animals, and that it can aggravate oxidative stress in livestock under production in the study area, hence there is need for further studies to identify the pathophysiological mechanisms of heat stress so as to develop mitigation strategies for improved animal performance and productivity. The study suggested that instead of the penchant for importing exotic breeds of livestock with the aim of upgrading the indigenous breeds, the way forward could be the utilization of genetic expression of heat and oxidative stress genes in animals as candidate markers for improvement of their productive potentials.

Keywords: Temperature-Humidity-Index, Heat Stress, Oxidative Stress and Animal Productivity

Introduction

Temperature-Humidity-Index (THI) is an indicator for determining temperature comfort zone which integrate the relative effects of temperature and humidity for optimum livestock performance and productivity. It also has to do

with environmental temperature and heat generation as factors controlling energy metabolism and exchange; hence avoidance of its extremes above and or below comfort zone, can influence animal health and production because it is also a measure of the relationship between environmental temperature and animal thermoregulatory status. The application of THI has been used for determination of comfort zones for humans and different livestock species including dairy cattle, swine, turkeys, laying hens, broilers and rabbits *(Ogunjimi et al., 2008; Joseph et al., 2014; Behura et al., 2016*). An evaluation of THI is a contribution for identification of the suitable environmental conditions under which animals will not be susceptible to oxidative stress damage which is a leading biochemical consequence of heat stress. Therefore, this study obtained climate data of Minna for evaluating THI and determine heat stress conditions under which animals can be produced in the area for prevention of oxidative stress damage and establishing basis for use of antioxidants in animal production operations in the study area.

Materials and Methods

Minna climate data for years between 1961 and 2018 were obtained from National Oceanic and Atmospheric Administration (*NOAA, 2016*). Data on parameters including mean monthly temperature, relative humidity and dew points temperature from these sources were compare with data of Nigeria Metrological Agency (NIMET) for Minna. From these data, Temperature-Humidity-Index (THI) was calculated on monthly basis for cattle and rabbits using the formulae i and ii, respectively:

$$THI = t + (0.36 \times Dt) + 41.2 \quad \text{..... i}$$

(**Source:** *Dairy Australia, 2016*)

Where t = dry bulb temperature (^{o}C), Dt = dew point temperature (^{o}C)

$$THI = t - [\left(0.31 - 0.31\left(\frac{RH}{100}\right)\right)(t - 14.40)] \quad \text{..... ii}$$

(**Source:** *Marai et al., 2001*)

Where t = dry bulb temperature (^{o}C), RH = Relative Humidity (%)

Production data for a period of 21 months were obtained from a commercial dairy farm located in Minna. The farm operate an intensive livestock production management where 250 heads of dairy cattle are being kept. The cattle include 50 Holstein Friesian – HF cows, 150 Red Holstein – RH cows, and 50 Cross Bred – CB cows (crosses of Nigeria indigenous cattle and Holstein Friesian and Red

Holstein). The data obtained were used to determine average production and performance of the cattle. Descriptive relationships were further established between the production performance and the THI determined for the study area.

Results and Discussion

From the available dataset monthly Temperature-Humidity-Index (THI) values for Minna were determined using the equations above and are presented in the Table 1 and Figure 1. Average highest monthly environmental temperature occurs annually in the month of March (33.65 ^{o}C) while the lowest environmental temperature occur annually in the month of August (27.02 ^{o}C). Highest dew point temperature occurs during the month of July (19.70 ^{o}C) while the lowest dew point temperature occurs during month of February annually. The highest Temperature-Humidity-Index occurs annually in the month of April which followed the month having the highest environmental temperature while the lowest Temperature-Humidity-Index occurs annually in the month of January.

Table 1. Monthly Temperature-Humidity-Index (THI) values of Minna (Cattle)

Months	TEMP	DP	RH	THI
Jan	31.15	5.20	24.00	74.22
Feb	33.05	4.70	21.00	75.94
Mar	33.65	10.70	30.00	78.7
Apr	32.30	15.40	44.00	79.04
May	30.05	18.30	58.00	77.83
Jun	28.35	19.20	66.00	76.46
Jul	27.30	19.70	72.00	75.59
Aug	27.02	20.00	73.00	75.42
Sep	27.45	19.10	70.00	75.52
Oct	29.15	18.40	62.00	76.97
Nov	30.65	11.80	39.00	76.09
Dec	30.70	6.70	28.00	74.31

TEMP –Temperature, **DP** – Dew Point Temperature, **RH** – Relative Humidity

The result showed that animals are always under stressful condition throughout the year in the study area going by the classification of *Kulkarni et al. (2017)* which stated that THI values in degree Fahrenheit below 70 is No stress, THI values between 70 and 75 is Mild Stress, THI values between 76 and 80 is Semi-Moderate Stress, THI values between 81 and 85 is Moderate Stress and THI values between 85 and 90 is Severe Stress. This also agrees with the THI classification reported by *Ogunjimi et al. (2008),* that THI values in degree celcius below 27.8 is no stress, 27.8 to 28.9 is moderate stress, 29 to 30 is severe stress and above 30 is very severe stress.

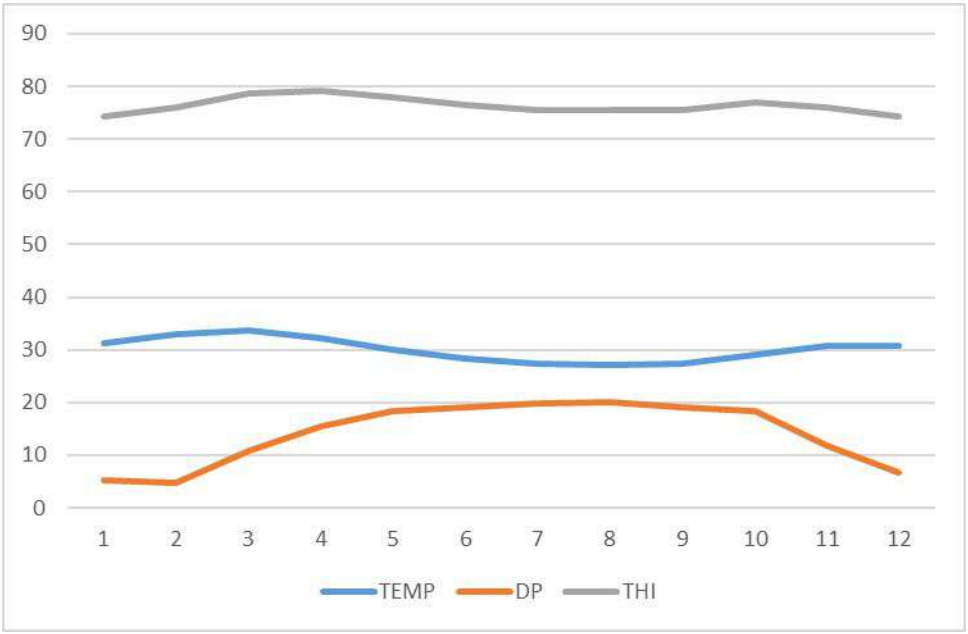

Figure 1. Monthly Temperature (TEMP), Dew Point temperature (DP) and THI (in ^{o}F)

Values of the THI as revealed in this study showed that throughout the year, heat stress is a potential threat to dairy cattle productivity because according to guidelines of heat stress management by Dairy Australia (2016), when the THI exceeds 72, cows are likely to begin experiencing heat stress and their in-calf rates will be affected. When the THI exceeds 78, milk production is seriously affected and when the THI rises above 82, very significant losses in milk production are likely, cows will show signs of severe stress and may ultimately die.

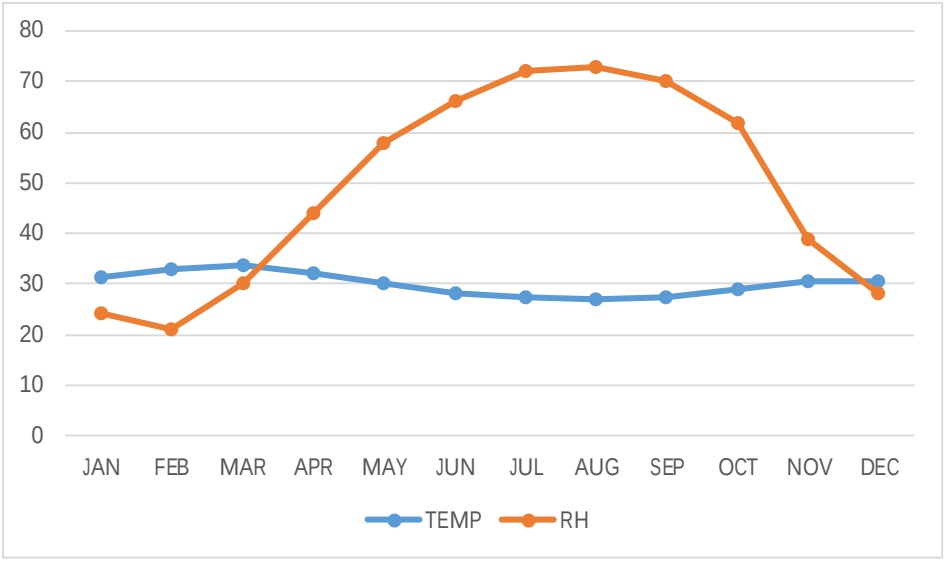

Figure 2. Monthly mean temperature and relative humidity of the study area

The relationship between temperature and relative humidity using the trends between January and March (Figure 2) annually confirmed that the period can be described as the safest time for animals in the study area. This is an indication that the THI analysis done in this study is correct and gives an understanding of heat stress condition of Minna as it affect animal performance and productivity. Although the temperature is still high, but because the relative humidity is lower than 40 % hence the THI indicated mild stress. This biologically can be confirm through performance and production capacities of animals under traditional management in the study area. Livestock in this area including cattle, sheep and goats mostly give birth to younger ones during this period which is a natural biological response of the animals to environmental challenge of heat stress.

Production and performance data obtained from the commercial dairy farm located in the study area gave an average monthly milk production for Holstein Friesian (HF) as 240.50 litres, Red Holstein (RH) as 454.80 litres and Crossbred (CB) as 123.50 litres. Gestation length was 9 months for all the breeds while conception rates upon artificial insemination were 50 % for HF, 70 % for RH and 50 % for CB; culling rates were 90 %, 35 % and 10 % for the HF, RF and CB respectively as well as other performance and production parameters as presented in Table 2.

Table 2.Monthly production and performance of dairy cattle on commercial dairy farms in the study area

Breeds	Average monthly milk production (Litre)	Conception rates (%)	Calving rate (%)	Culling rate (%)
Holstein Friesian	240.50	50.00	45.00	90.00
Red Friesian	454.80	70.00	65.00	35.00
Crossbred	123.50	50.00	70.00	10.00

From the analysis of the THI for the study area, it showed that there exist a direct positive relationships between monthly mean temperatures and the monthly THI. The higher the monthly mean temperature, the higher the THI as presented in Figure 3 and Figure 4. A similar relationship also exist between dew point temperature and the THI; as the dew point temperature increases, so the THI increases. Biological confirmation of these relationships can be deduced from performance of the dairy cattle on a commercial dairy farm located in Minna as presented in Figure 3. The figure showed that the THI is high and constant throughout the year and this can be linked to the reasons why milk production of the cattle was low.

From 21 months lactation records obtained from the commercial dairy farm; it is possible to deduce that production and performance parameters of the exotic cattle were poor which can be linked to environmental stressors such as oxidative stress on the animals. Holsteins are high performing dairy animal worldwide but their reproductive and productive performance in Tropical countries are always different from their achievable performance in Europe. What was obtained as mean daily milk production for the cattle as presented in Table 2 is similar to daily milk production of 8.38 litres reported by *Haftu (2015)* for Holstein cattle in Ethiopia. It is also similar with what was reported for Holsteins in Sudan as reported by Abdel *Rahman and Alemam (2008).*

However, these breeds are reported as excellent dairy cattle because of their superior genetic composition which is a reason why their choice for upgrading of indigenous cattle is a common livestock improvement practices (*Ogundipe and Adeoye, 2013).* Therefore, harsh environmental conditions such as high and unbearable THI and other different climatic conditions rather than poor management systems could be responsible for the poor production as revealed in this study. Confirmation of this observation is higher milk yield recorded (35 litres per day) in an experimental unit on the commercial farm where temperature control was carried out (18 ^{o}C – 20 ^{o}C); it is however unsustainable for the farm to run such facilities for full scale production considering associated cost.

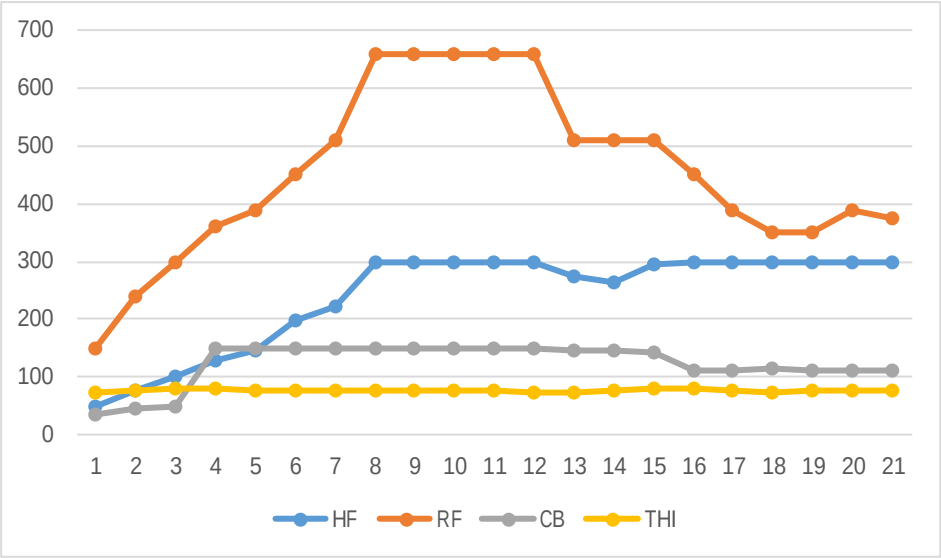

Figure 3. Monthly milk production of dairy cattle and relative THI of Minna

The relationship between monthly THI and corresponding milk production by the cattle showed that the THI negatively affected milk production capacity of the cattle. From Figure 3 above, it can be deduced that crossbred cattle from the exotic breeds and the indigenous cattle breed have highest tolerance to the effect of THI because despite the THI constantly being high, the milk production of the crossbred remained relatively constant except by the 16th month which correspond with April of the second year of production when the production level drops and remained unchanged till the 21st month. Ability of the crossbred to display this tolerance is not far-fetched from the adaptation of the local breed used for the crossing (White Fulani) to the environmental conditions obtainable in Minna. This is further expressed in the high calving rate (70 %) and low culling rate (10 %) of the crossbred. This means that in spite of the consistently high THI, there is strong capability of the crossbred to survive which confers an added advantage for it over both imported breed.

This is a pointer that with careful selective breeding, the indigenous White Fulani cattle can be made to improve on its milking capacity. If farmers must import, then it should be semen for artificial insemination as keeping imported animals has implication on production cost. This is because, there will then be the need to

engage in microclimate ameliorative practices. The high culling rate and lover calving rate when compare to the crossbred makes keeping the exotic breed *in-situ* on the farm uneconomical.

In rabbit production, elevated level of THI correspond with higher heat and moisture production especially in mature rabbits. Hence, to maintain optimum rabbit production in the study area maintenance of THI value below 27 ^{o}C will be optimum for rabbit as calculated using the Livestock Production Heat Stress Indices (*LPHSI, 1990*) formular which was reportedly modified for rabbit by *Marai et al. (2001)* is a must in order to provide comfortable environment for the animal. Following recommendations of *Ogunjimi et al. (2008),* physiological and productive conditions of rabbits are both susceptible to heat stress because under heat stress condition, there is reduced feed intake and continuous use of Metabolizable Energy (ME) for non-productive activities such as panting and faster respiration rates for the purpose of survival (*El-Raffa, 2005; Ogunjimi, 2007*) which shows that environmental conditions in the study area as presented in Table 3 is not too suitable for rabbit production without modifications.

Table 3. Monthly Temperature-Humidity-Index (THI) values of Minna (rabbit)

Months	TEMP	DP	RH	THI
Jan	31.15	5.20	24.00	27.20
Feb	33.05	4.70	21.00	28.49
Mar	33.65	10.70	30.00	29.49
Apr	32.30	15.40	44.00	29.19
May	30.05	18.30	58.00	28.00
Jun	28.35	19.20	66.00	26.87
Jul	27.30	19.70	72.00	26.17
Aug	27.02	20.00	73.00	26.00
Sep	27.45	19.10	70.00	26.23
Oct	29.15	18.40	62.00	27.61
Nov	30.65	11.80	39.00	27.80
Dec	30.70	6.70	28.00	27.05

TEMP –Temperature, **DP** – Dew Point Temperature, **RH** – Relative Humidity

In Figure 6 below, it showed that only four months of the year is when the THI was below 27.00 (June – September) which is an indication that for excellent performance of rabbit in the study area, antioxidative management practices such as the use of antioxidant diets and drugs, modification of housing to encourage cross ventilation and care for animals to ameliorate stress conditions are highly important husbandry practices.

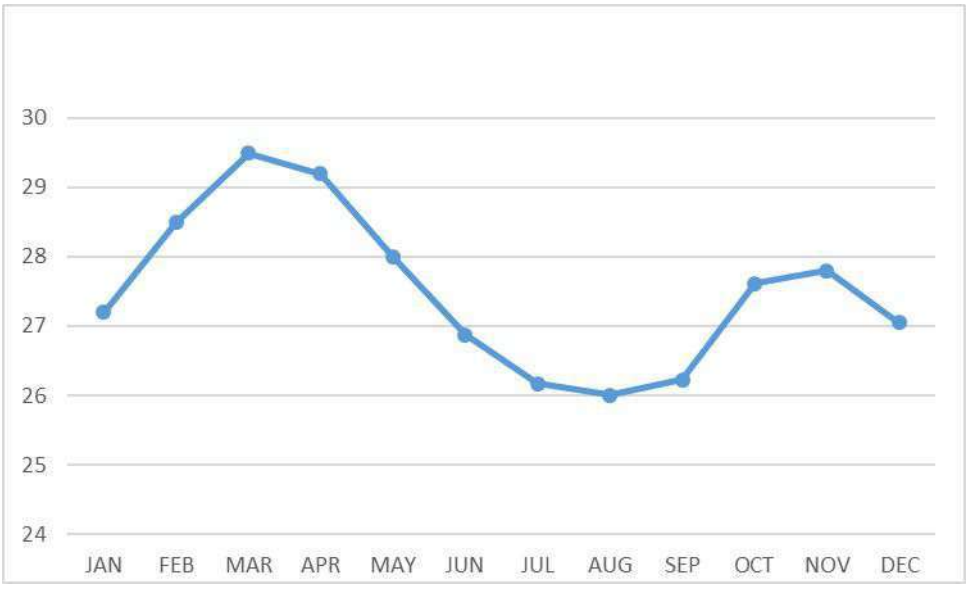

Figure 6: Monthly temperature-humidity-index for rabbits using climate data of Minna

Exposure to heat stress could lead to poor growth and reproduction because it decrease live weight gain, daily weight gain, feed intake, litter size at weaning, pre and post weaning weight gain in rabbits. In fact under heat stress, conception rates, overall nutrients digestibility as well as productivity loss is associated with incidence of heat loss in rabbit. Production loss involving conception rates, pre – weaning mortality of rabbit kits, litter weight at weaning are reported to be 70 % in a reproductive cycle of rabbit doe. This represent huge economic loss requiring multidimensional solution in housing, animal care and handling, nutrition and health management for a rabbit enterprise to be profitable (*Marai et al., 2001*).

Conclusion

Analysis of basic climate data between years 1961 and 2018 for Minna revealed that for most part of the year (nine months), animals in the study area live

under heat stress and hence their potential for food production is under threats thereby contributing to food insecurity. Plans for improving animal productive performance should form basis for livestock production research in the area. Environmental modifications including cooling systems or modifications of housing facilities may not be the only feasible approaches to heat stress management in the study area considering associated costs. As a result, nutritional manipulations that promote animal health and production capacities is suggested as additional option worthy of exploration. Instead of the penchant for importing exotic breeds of livestock with the aim of upgrading the indigenous breeds, the way forward could be the utilization of the heat shock protein of the animals as candidate markers for improvement of their productive potentials. Further research is suggested to investigate pathophysiological mechanisms of heat stress for the purpose of determining appropriate mitigation measures for management of heat stress in the study area.

Ekološki izvor stresa u stočarstvu - studija o klimatskim podacima za Minu

Sikiru Akeem Babatunde, Egena Sunday Sunday Acheneje, Alemede Iyabo Comfort, Makinde Olayinka John

Rezime

Stres koji proizilazi iz okoline je faktor koji ograničava produktivnost stoke u tropskim predelima zbog povišene temperature tokom cele godine; stoga ova studija predstavlja pregled podataka o klimatskim promenama za Minu u smislu procene indeksa temperature i vlažnosti (Temperature-Humidity-Index, THI) kao način identifikacije klimatskog stresa i njegovog uticaja na proizvodnju u stočarstvu. Dobijene su klimatske norme za Minu između godina 1961. i 2018. godine, a podaci su analizirani korišćenjem opštih formula za izračunavanje THI indeksa za stočarsku proizvodnju. Odnosi između proizvodnih parametara na komercijalnim farmama i THI podaci pokazali su da je stres toplote potencijalni uzrok oksidativnog stresa u toj oblasti. THI je pokazao da uslovi životne sredine, koji su postojali tokom istraživanja, imaju potencijal da izazovu toplotni stres kod životinja i da mogu pogoršati oksidativni stres kod stoke u proizvodnji u području istraživanja, stoga postoji potreba za daljim istraživanjima za identifikaciju patofizioloških mehanizama toplotnog stresa kako bi se razvile strategije ublažavanja radi ostvarivanja poboljšanih performansi i produktivnosti životinja. Studija je pokazala da umesto želje za uvozom egzotičnih rasa stoke u cilju oplemenjivanja autohtonih rasa, napredak bi mogao da se ostvari korišćenjem

genetske ekspresije gena za toplotni i oksidativni stres kod životinja kao marker kandidata za poboljšanje njihovog proizvodnog potencijala.

Ključne reči: indeks temperature i vlažnosti, toplotni stres, oksidativni stres, produktivnost životinja

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DISTRIBUTION AND SIZE OF CORPORA LUTEA IN DAIRY COWS DURING PUERPERIUM

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Abstract: During puerperium phase in cows, uterus goes through involution process, while ovaries restore supressed cyclicity as a result of gestation. After 10-20 days postpartum (PP) luteinizing hormone (LH) levels begin to raise and renewal of cyclicity after parturition is probably most important factor for cows to successfuly conceive again. Almost 95% of dairy cows should restore ovarian cyclicity up to 50 days postpartum. LH surge is important for ovulation and luteinisation of granulosa and theca cells into luteal cells and proliferation of blood vessels. Up to 79% of newly formed *corpora lutea* have central vacuola, filled with fluid. The study involved the total of 54 Holstein-Friesian cows, during first 40 days of lactation. Examinations of the ovaries, were performed in the period from 10 to 40 days postpartum. Ovaries and *corpora lutea* were first palpated and then examined using portable diagnostic ultrasound linear scanner MyLab®30 VETGold portable ultrasound linear scanner with endorectal linear probe LV 513, 5-7.5 MHz (both Esaote SpA, Italy). The same equipment was used to monitor BCS, while lamenes was assessed using Zinpro Locomotion Score for dairy cows. The highest number of corpora lutea was observed after 20 and 30 days postpartum in experimental and control groups. More *corpora lutea* were observed in multiparous cows. Higher numbers of corpora lutea with similar average size were

observed in right ovaries of cows in both groups, while corpora lutea were bigger in multiparous cows. Numbers and sizes of corpora lutea, may give an insight in quality of restoration of ovarian cyclicity and a solid base for prediction on future reproductive performances.

Keywords: *corpora lutea*, puerperium, ovulation, dairy cows

Introduction

To be able to sustain next gestation, bovine reproductive system should normally recover in 45 days after calving (*Brick, 2011*). Uterus in cows undergoes involution process during puerperium phase, while ovaries restore suppressed cyclicity as a result of gestation. *Corpus luteum* (CL) of pregnancy is regressed due to prostaglandin (PGF) secretion from endometrium, which causes luteolysis (*Senger 2003; Silvia et al.,1991*). After 10-20 days postpartum (PP) luteinizing hormone (LH) levels begin to raise (*Saacke et al., 2000*) and commencing of cyclicity after parturition is probably the most important factor for cows to successfully conceive again (*Mather et al.,1981*). Usually 3-4 weeks pass before first postpartum ovulation in dairy cows, often without signs of estrus. Almost 95% of dairy cows should restore ovarian cyclicity up to 50 days postpartum.

Ovulation is enabled only if dominant follicle (DF) produces enough estradiol to stimulate LH surge (*Noakes,2009, Rutigliano et al.,2008*). Increase of estradiol 17β (E2) secretion is followed with follicular growth and LH surge is required for ovulation (*Kesler et al.,1979*). LH surge is important for ovulation and luteinisation of granulosa and theca cells into luteal cells and proliferation of blood vessels (*Lee et al.,1985, Mutevelić et al.,2003*). Formed CL continue to grow up to 8th day of estral cycle (*Santschi et al.,2011*). Average time to first ovulation for Holstein breed is three weeks (20.8±13.2 days) (*Benmrad et al., 1986*; *Fonseca at al.,1983*) and after that, luteal phase may begin. Up to 79% of newly formed corpora lutea have central vacuole of 2-10 mm in diameter, filled with fluid (*Fricke 2002; Ginther, 1998*).

Most important factors that affect puerperium are: age, breed, season, nutrition, milking, body condition, phase of lactation, metabolic and health status (*Vasconelos et al.,1999*) and it is well known fact that stress and pain have negative effects on reproductive hormones through hypothalamus-pituitary-ovary relationship. *Garbarino* (*2004*) observed a delayed ovarian cyclicity in cows with lameness in first 35 days in milk have delayed ovarian cyclicity, which could be prevented up to 71% if lameness was absent.

According to *Patton et al. (2006)* time to first postpartum ovulation and CL forming is the shortest when milking is done only once daily. Cows with positive energy balance (PEB) have higher concentrations of circulating IGF-I, because milking once daily increase insulin and glucose concentrations. Those cows loose less weight and and keep higher body condition scores (BCS) through entire lactation, then cows milked twice or three times daily (*Patton et al., 2006*). Absence or shortening of traditional dry period cause increase of IGF-I and insulin concentrations, which trigger more frequent LH surge and earlier cyclicity (*Feu et al., 2009; Gumen et al., 2005; Walch et al., 2008*). Due to improvement of energy status in early puerperium, ovulations and more double ovulations happens earlier and more frequent (*Gumen et al., 2005; Santschi et al., 2011*), which supports importance of nutrition for productive and reproductive efficiency (*Diskin et al., 2003*). Nutritional deficiencies and negative energy balance (NEB) early PP have a serious effect to size and future of DF, since smaller follicles secrete less estradiol, which consequently have less feedback for LH secretion (*Diskin et al., 2003*). Approxemately 80% of dairy cows after parturition enter the NEB period in early lactation and BCS drops as well, because energy requirements for milk synthesis are not supported by desirable nutrition, which later compromise reproductive performances (*Domecq et al., 1997; Feu et al., 2009;, Montiel et al., 2005*). Cows with expressed NEB in first 9 days of puerperium have more negative effects to luteal function, which is usually not observed before 50-70 days in milk, when programs of artificial insemination begins (*Pankovski et al., 1995*). *Petterson at al.* (*2006*) reported that only 70.4% of Swedish Holstein cows enter normal cyclicity up to 56 days in milk.

Comparative ovary diagnostics in cows before and after slaughter, showed that 35% of diagnosis were incorrect, while failure to detect CL in various phases was estimated at 15% (*Descoteaux et al., 2006*).

Using transrectal examination *Gumen et al.* (*2003*) observed anovulatory condition in 28% of primiparous cows in period between 47-60 days in milk, while that condition was detected in 15% of multiparous cows. Rapid restoration of ovarial cyclicity after parturition is essential to achieve the best productive capacity of dairy cows (*Holt et al., 1989*), which is challenging for dairy management since it was estimated that 50% of dairy cows have irregular ovary function after parturition (*Bisinotto et al., 2010*). Longer anovulatory condition PP may be due to retained placenta (RP), metritis, slower uterine involution, longer luteal phase, low BCS, etc. (*Holt et al., 1989*). *Garmo* (*2009*) suggested that longer luteal phase could be more uterine-related problem than an issue of ovarian pathology.

The aim of this research was to explore number, size and distribution of corpora lutea in ovaries of cows with normal and abnormal puerperium status by use of transrectal palpation and ultrasonography with regard to cows' BCS and lameness status.

Material and methods

The study was conducted during winter and spring seasons of 2013. In total, the study involved 54 Holstein-Friesian cows during first 40 days of lactation, which were selected for the study based on their previous parturition dynamics data and randomly assigned to experimental (EG) (n=28) and control (CG) (n=26) group. Cows in EG received one injection of 86 μg of GnRH (Fertagyl®, Gonadorelin, Merck) at 15 days postpartum to stimulate ovarian function. All the cows were kept under the same nutritive regime, milked three times daily, and housed in a tie-stall system with separated bearings. Also, all the cows were regularly vaccinated against coronavirus and rotavirus infections and tested on brucellosis, leucosis and tuberculosis. Cows in experimental group have received one injection of 86 μg GnRH (Fertagyl®, Gonadorelin, Merck) at 15 days postpartum to stimulate ovarian function.

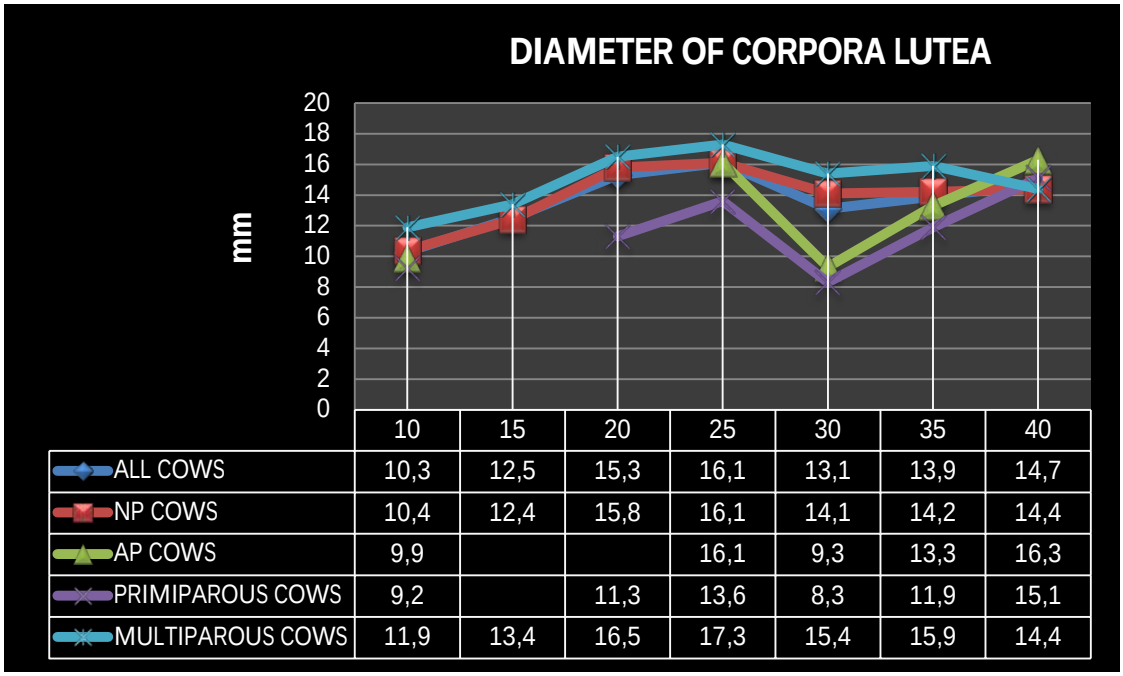
According to previous anamnestic data, EG and CG cows were further divided in subgroups: normal puerperium, abnormal puerperium, primiparous and multiparous cows. BCS and lameness score were estimated prior to parturition. Later BCS was monitored throughout puerperium period. Lameness was assessed using „Zinpro Locomotion Scoring“ (ZLS) for dairy cattle, while BCS was estimated by ultrasound measurement of subcutaneous adipose backfat thickness in sacro-gluteal area.

Ovary examinations were carried out every 5 days during the period from 10 to 40 days postpartum, every 5 days, which has included 378 transrectal examinations.

Ovaries and *corpora lutea* were first palpated and then observed using MyLab®30 VETGold portable ultrasound linear scanner with endorectal linear probe LV 513, 5-7.5 MHz (both Esaote SpA, Italy) and same equipment was used for monitoring of BCS.

Statistical analyses and graphical presentations of results were done by Excel® 2010 (Microsoft Inc., USA). Average observed values of CL diameter and adipose tissue thickness used to estimate BSC between the experimental cow groups and subgroups were compared using two-way Student’s t-test. The observed differences were significant if p-value was less than 5% ($p<0.05$).

Results

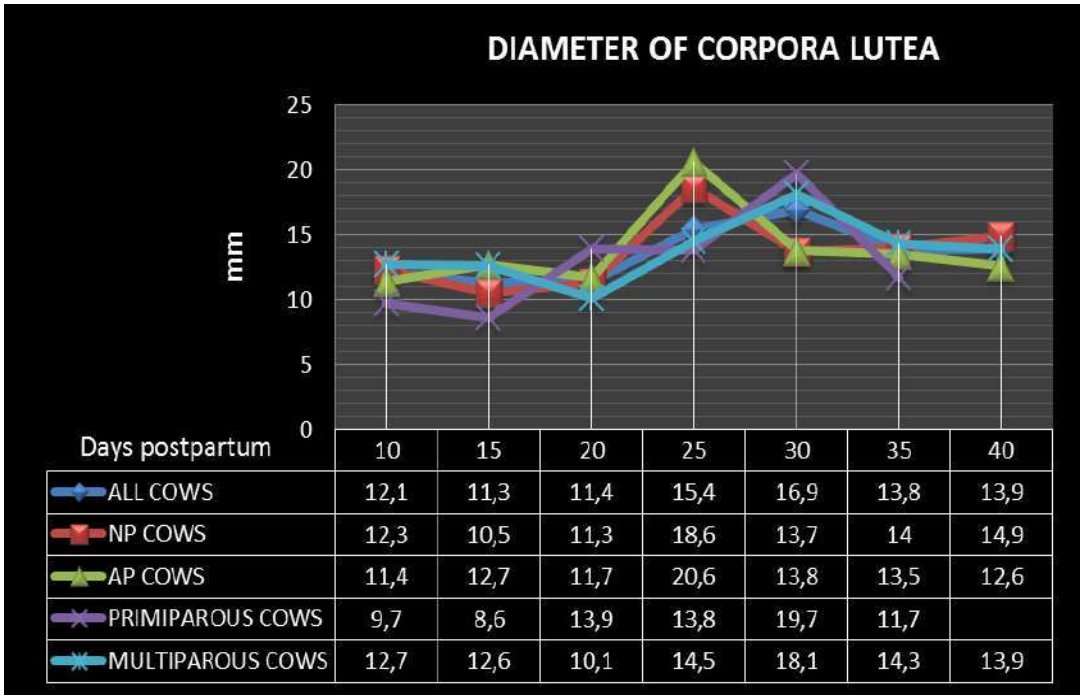


	10	15	20	25	30	35	40
ALL COWS	10,3	12,5	15,3	16,1	13,1	13,9	14,7
NP COWS	10,4	12,4	15,8	16,1	14,1	14,2	14,4
AP COWS	9,9			16,1	9,3	13,3	16,3
PRIMIPAROUS COWS	9,2		11,3	13,6	8,3	11,9	15,1
MULTIPAROUS COWS	11,9	13,4	16,5	17,3	15,4	15,9	14,4

Chart 1. Average diameter (mm) of corpora lutea in experimental cow group during first 40 days postpartum. Measurements at 10 days postpartum represent remainings of gestation CL and were not included in later analysis. Statistically significant differences ($p<0.05$) between primiparous and multiparous cows were observed at 20 and 30 days postpartum

Table 1. Total number of corpora lutea in experimental group according to period and subgroups.

DAYS PP	NP COWS	AP COWS	PRIMIPAROUS COWS	MULTIPAROUS COWS
10				
15	2			2
20	9		2	9
25	7	2	2	5
30	8	3	4	7
35	7	3	4	7
40	6	3	3	6



Days postpartum	10	15	20	25	30	35	40
ALL COWS	12,1	11,3	11,4	15,4	16,9	13,8	13,9
NP COWS	12,3	10,5	11,3	18,6	13,7	14	14,9
AP COWS	11,4	12,7	11,7	20,6	13,8	13,5	12,6
PRIMIPAROUS COWS	9,7	8,6	13,9	13,8	19,7	11,7	
MULTIPAROUS COWS	12,7	12,6	10,1	14,5	18,1	14,3	13,9

Chart 2. Average diameter (mm) of corpora lutea in control cow group during first 40 days postpartum. Measurements at 10 days postpartum represents remainings of gestation CL and were not included in later analysis. Statistically significant difference ($p<0,05$) between NP and AP cows was visible only at 40 days postpartum.

Table 2. Total number of corpora lutea in control group according to period and subgroups.

DAYS PP	NP COWS	AP COWS	PRIMIPAROUS COWS	MULTIPAROUS COWS
10				
15	8			8
20	7			7
25	8	5	4	8
30	8	5	5	9
35	7	6	4	9
40	4	5		9

Table 3. Total number and diameter of corpora lutea detected by transrectal palpation and ultrasonography.

GROUP	Transrectal palpation	Transrectal ultrasonography	CL diameter (mean±st.dev)
Experimental	38	51	15.05±0.4 mm
Control	46	63	15.52±0.5 mm

Table 4. Maximal diameter values (mm) of *corpora lutea* measured by transrectal ultrasonography from 10 to 40 days postpartum (PP) in experimental cow group.

EXAMINATION DAY	NP COWS	AP COWS	PRIMIPAROUS COWS	MULTIPAROUS COWS
10				
15	11	14.1	14.1	11
20	22	12.1	12.1	22
25	24	16.1	16.1	24
30	25	12.6	12.6	25
35	19.6	15.4	15.4	19.6
40	22	16.3	20	22

Table 5. Maximal diameter values (mm) of *corpora lutea* measured by transrectal ultrasonography from 10 to 40 days postpartum (PP) in control cow group.

EXAMINATION DAY	NP COWS	AP COWS	PRIMIPAROUS COWS	MULTIPAROUS COWS
10				
15	17	22	11.5	22
20	21	19	21	12.6
25	20	23	20	23
30	33	21.4	22.4	33
35	25	17.3	15.2	25
40	22.5	15.4		22.5

Table 6. Total number of *corpora lutea* in groups with appearance of central vacuola/lacuna.

Group	Total number of *corpora lutea*	Presence of *vacuola/lacuna*
Experimental	51	25.5%
Control	63	46.03%

Table 7. Frequency of detection of *corpora lutea* and respective ovulation

GROUP	LEFT OVARY CL	RIGHT OVARY CL	OVULATION
EXPERIMENTAL (n=28)	35.4%	64.6%	74.3%
CONTROL (n=26)	40.1%	59.9%	85.0%

Table 8. Assessment of lameness in all animals at dairy farm, according to ZLS.

ZINPRO LOCOMOTION SCORE					
ZLS	1	2	3	4	5
% cows	1.1	59.2	36.3	3.1	0.3

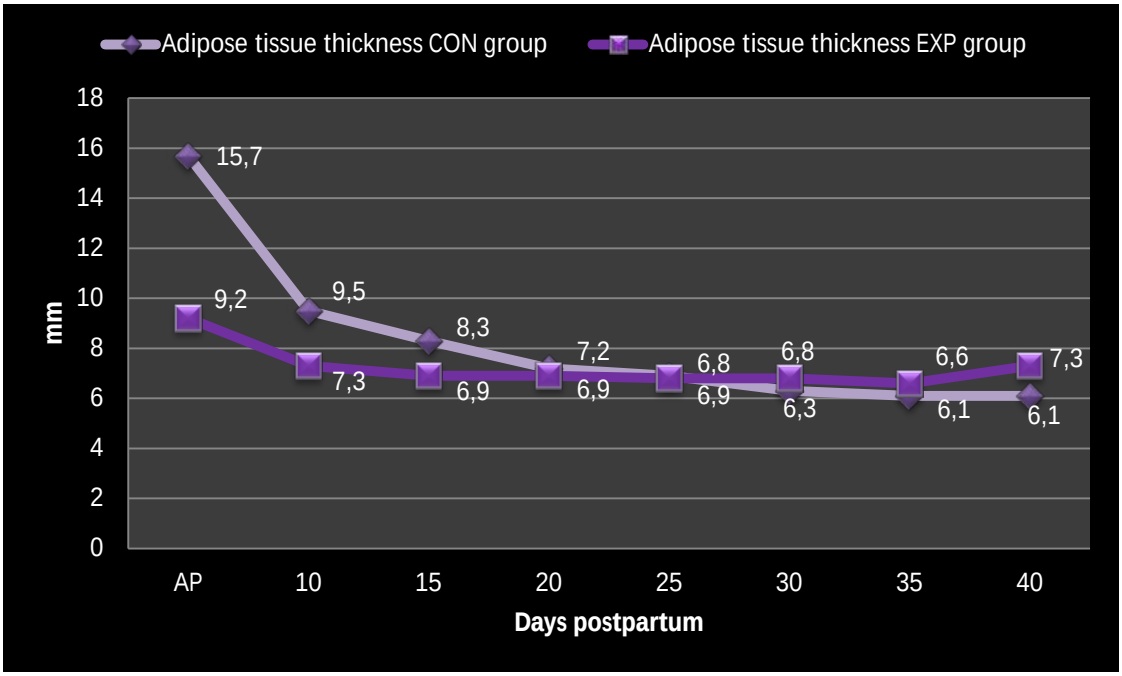


Chart 3. Average adipose backfat thickness used to assess BCS between groups during antepartum (AP) and postpartum period. Signifficant differences ($p<0.05$) between groups are visible only in antepartum period.

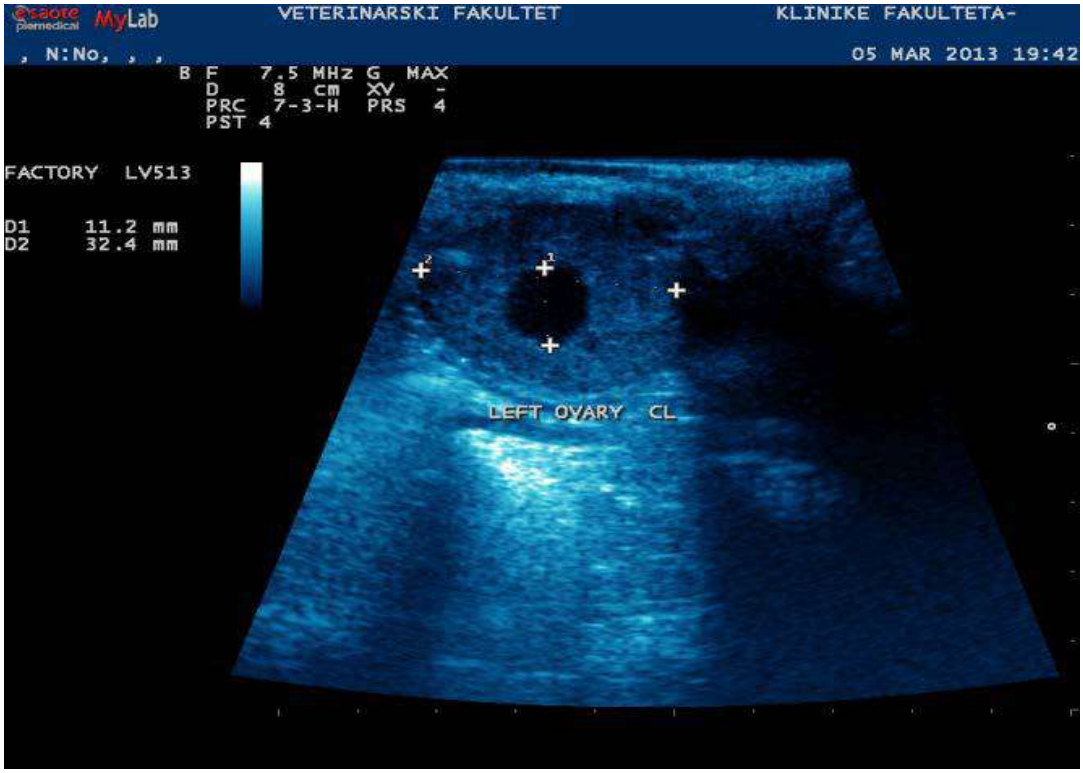


Figure 1. Sonogram: clearly visible large corpus luteum with central vacuola/lacuna 11.2 mm in diameter.

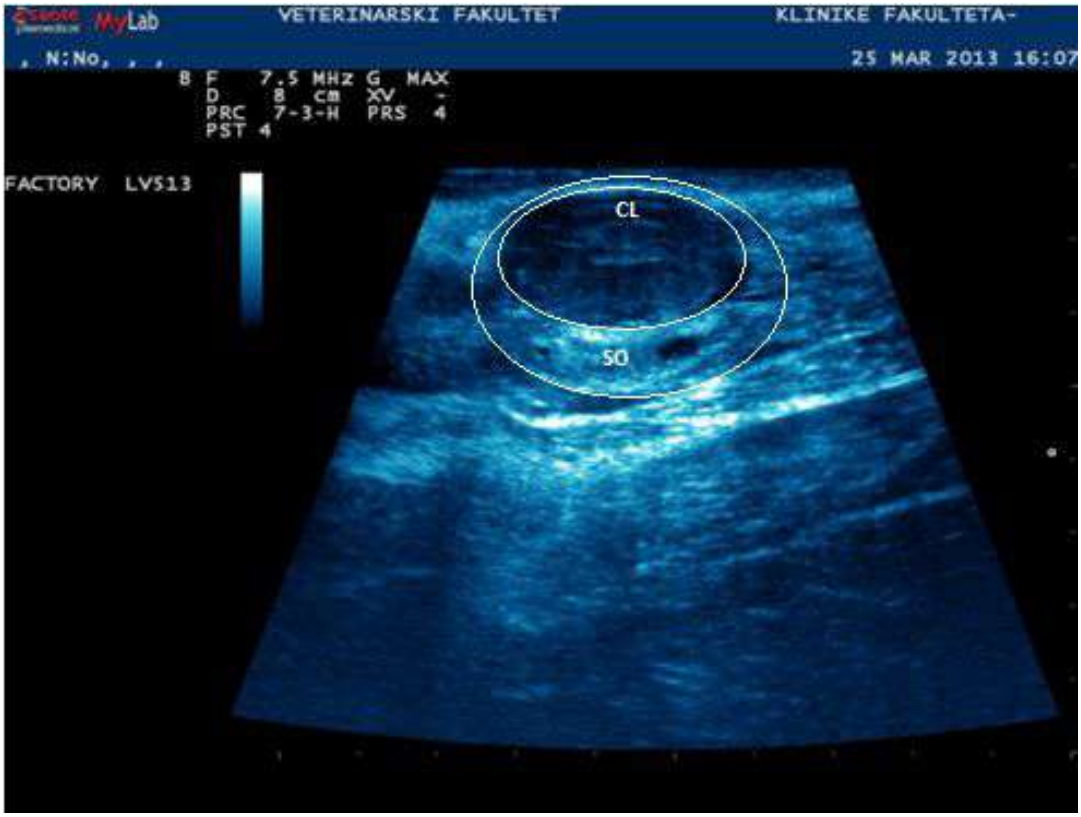


Figure 2. Sonogram: visible compact corpus luteum (CL) and ovarian stroma (SO).

Discussion

Total number of observed CL was highest in the period 20-30 days PP in EG and CG, while detection rate of central vacuola/lacuna in them was 25.5% in EG and 46.03% in CG, which fall in a range of results of other authors (*Fricke, 2002*; *Ginther, 1998*). In EG, CL number among NP cows was 2.7 times higher than in AP cows and two times higher in multiparous cows compared to primiparous cows. In CG, total CL number in NP cows was 1.6 times higher than in AP cows, and 1.7 times higher in multiparous than in primiparous cows as well. In EG and CG cows, more CL were observed in right ovaries, while differences between groups were about 5%.

Observed CL size depended on growth or regression phase. Average CL diameter in both EG and CG was estimated at approximately 1.5 cm in average, which

suggests that CL size in puerperium is not maximal, as previously described by others (*Kamimura et al., 1993*), and that CL size is larger in third than in first two postpartum ovulations as well.

Release of LH necessary for ovulation is delayed in cases of low energy intake and BCS, high milk yield and stress (*Stevenson, 2001*), which was mostly present in both cow groups, asevident by CL absence in several subgroups. Prolonged anovulatory condition PP is commonly caused by RP and metritis (*Harrison et al., 1986*), which is in line with anamnestic data in our study. According to the available dairy records, the most common puerperal disorders were RP and metritis, particularly in primiparous cows, in which anovulatory condition were most prevalent. Statistically significant differences between EG and CG were not common and observed only at 20 days PP between multiparous cows and at 30 days PP between primiparous and multiparous cows within EG.

Restoration of ovarian cyclicity largely depends on lactation number. Other authors (*Zhang et al., 2010*) reported that primiparous cows have longer acyclic periods, which corresponds with our results. In EG, 74.3% of cows displayed ovulation up to 40 days PP, while cows without ovulation were mostly primiparous with AP condition. In CG, 85% of cows showed ovulation up to 40 days PP, where cows without ovulation were again primiparous cows. Research in North American and Belgian Holstein herds (*Rhodes et al., 2003*) reported that about 23% and 38% cows, respectively manifested anestrus condition up to 60 days PP. Our explanation for such a high number of anestrus conditions could be a high number of primiparous cows in herd, especially those with AP condition, which coincide with our results. Primiparous cows have a higher incidence of delayed cyclicity than older cows, which requires more time for first luteal activity and prolongs the time from partus to first ovulatory estrus (*Petersson, 2007*). Higher risk for delayed luteolysis or cyclic abnormalities is due to low BCS up to 70 days PP, lameness, early luteal activity, metritis, RP, dystocia and abnormal vaginal discharge. Our study showed that the most of the studied cows had a low BCS during antepartum period and puerperium. Considering significant initial difference in BCS between EG and CG cows in antepartum period, our suggestion is that could be result of uneven food distribution among cows in different production phases, while additional stress to health status was present with moderate to severe lameness in 95% of all cows in a farm.

Application of GnRH is usually used to prevent delay of first ovulation in cows with AP condition (*Benmrad, 1986*), However, another opinions (*Chebel et al., 2010; Lee et al., 1985*) argue that an early restoration of cyclicity may have more positive or negative effects to fertility, because early luteal activity may have negative effects on uterine involution due to P4 secretion, which is also a risk factor for irregular luteal activity (*Garmo et al., 200;, Sakaguchi et al., 2004*). Delayed cyclicity is more common in cows with tie-stall housing system, which

usually have a longer period to first ovulation and ovulatory estrus (*Petersson, 2007*), as it coincides with the farm used in our study.
Anovulatory frequency in primiparous EG cows was 33.6% and16.6% in CG, while multiparous cows in both groups displayed ovulation before 40 days PP. *Gumen et al.* (*2005*) reported that up to 28% primiparous cows within 47-60 days in milk, may have an anovulatory condition, while it was observed in multiparous cows only at 15%, which is relatively similar to our results in primiparous, but not in multiparous cows. As explained by several other authors (*Garmo et al, 2009; Holtet al., 1989; Santos et al., 2004*), a prolonged luteal phase could be more related to uterine than ovarian disorders, what was obviously reflected in detrimental effect more in primiparous cows in EG and CG, as supported by findings of *Rhodes et al.* (*2003)* and *Santos (2009*).
Seasonality plays a significant role in restoration of cyclicity by means of athmospheric and nutritional factors (*Petersson K.J., 2007; Reksen et al., 2002*). Cows calved in winter may be in higher risk of delayed and abnormal cyclicity than those calved in summer. Having in mind that all EG and CG cows in our study were calved in February and March, such a seasonal effect could be one of the reasons for a higher number of anestrus primiparous cows. *Benmrad et al.* (*1986*) reported that approximately 24% cows showed anovulation condition at least 6 weeks PP, which is close to our results in EG, but not in CG, where anovulation condition up to 40 days in milk was present only in 15 % of cows, and such a difference could be explained by more multiparous cows in CG. In addition, more expressed anovulation in EG could be explained by a higher number of primiparous cows and more frequent puerperium abnormalities like RP, metritis and mastitis, as well as low BCS and lameness in those animals.

Conclusion

- The highest number of ovulations and CL could be expected in the period 20-30 days postpartum.
- Higher number of CL was found in cows with normal puerperium and multiparous cows.
- Average size of CL is smaller during puerperium phase.
- Bigger CL were detected after GnRh administration, but only in multiparous cows.
- Multiparous cows had a larger CL, than primiparous cows.
- Frequent appearance of central vacuole/lacuna inside CL, suggests its common formation, particularly in multiparous cows.

- Body condition score and lameness, had more effect in size and number of CL in primiparous cows.
- Number and size of CL, may give an insight in quality of restoration of ovarian cyclicity and present a solid base for predicting of future reproductive performances in dairy cows

Raspodela i veličina žutih tela u mlečnih krava tokom puerperija

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Rezime

Kod krava u toku puerperalne faze materica prolazi kroz proces involucije, dok jajnici obnavljaju svoju cikličnu aktivnost, potisnutu gravidnošću.Nakon 10-20 dana postpartum (PP), nivoi luteinizirajućeg hormona (LH) počinju rasti i obnova cikličnosti je verovatno najbitniji faktor kako bi krave mogle uspešno koncipirati nakon partusa. Do 50 dana postpartum, skoro 95% mlečnih krava bi trebalo da obnovi ovarijalnu cikličnost. LH talas je važan za ovulaciju i luteinizaciju granuloza i teka ćelija u lutealne ćelije i proliferaciju krvnih sudova. Novoformirana žuta tela i do 79% imaju centralnu šupljinu, ispunjenu tečnošću. Istraživanje je uključivalo ukupno 54 Holštajn-Frizijske krave, tokom prvih 40 dana laktacije. Pregledi jajnika su rađeni u periodu 10 do 40 dana postpartum, svakih 5 dana, do kraja prvih 40 dana puerperija. Jajnici i žuta tela su prvo palpirani a zatim posmatrani upotrebom dijagnostičkog ultrazvuka „Esaote MYLAB30 VETGOLD“ sa endorektalnom linearnom sondom LV 513, 5-7.5 MHz. Ista oprema je korišćena za posmatranje BCS, dok je šepavost procenjivana upotrebom „Zinpro Locomotion Score“ za mlečne krave. Najveći broj žutih tela su uočena sa 20 i 30 dana postpartum u eksperimentalnoj i kontrolnoj grupi. Više žutih tela je uočeno u višetelki. Desni jajnici u obe grupe su imali više žutih tela, prosečna veličina je bila slična, a veći su bili prisutni u višetelki. Brojnost i veličina žutih tela može dati uvid u kvalitet obnove cikličnosti jajnika, što može biti solidna osnova za predviđanje budućih reproduktivnih performansi.

Ključne reči: žuta tela, puerperium, ovulacija, mlečne krave

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INCIDENCE OF DEFORMATIONS OF THE EXTREMITIES OF SIMMENTAL COWS IN DIFFERENT TYPES OF STALLS

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Original scientific paper

Abstract: Problems with legs and various forms of lameness of cows, in intensive milk production, are the third significant problem occuring in this production, after mastitis and reproductive disorders, both globally and in our country. The paper analyzes the incidence of the deformation of legs of 145 cows of the Simmental breed in the Kolubara region, and the influence of paragenetic factors (housing/holding and type of stall/bedding and lactation) on the incidence of deformations. The obtained results show that, of the total number of cows assessed, 3.45% had "X" position of the front legs, 14.8% had a "X" position of the hind legs. The convergent position of the front legs was recorded in 35.86%, and divergent in 8.28% of animals. The convergent position of the hind legs was observed in 16.55% of cows, and divergent in 2.76%. The outward position of the front legs was observed in 4.14% of cows, inward position in 11.03%, and broad position in 4.14% of studied cows. Also, 17.24% of the cows had a so called sable like position of hind legs, and 7.59% showed steap angle of hind legs. The pronounced soft front leg pasterns were observed in 7.59% of the cows, and the soft pasterns of the hind legs in 33.79% of the total number of observed cows. The observed changes in the ankles in the shape of swelling were recorded in 1.38% of cattle on the carpal joint and 2.76% on the tarsal ankle. The damaged shoulder and body joint (scabbed shoulder) was observed in 43.45% of the total number of cows evaluated. Scores for the front and hind legs front, back and side views, varied at different levels of significance under the influence of the type of stall/bedding, while the scores for the condition of the hind leg pasterns varied highly significantly ($p<0.001$) under the influence of the type of stall/bedding. The scores for the front leg pasterns and scores for shoulder and body joint were not significant ($p> 0.05$) depending on the type of stall/bedding and the method of housing/holding of cows.

Key words: Simmental breed, extremities, stall/bedding

Introduction

According to the literature data, leg problems and various forms of lameness of cows, in intensive milk production, are the third significant problem occuring in this production, after mastitis and reproductive disorders, both globally and in our country. Significant economic losses occur caused by the expense of treatment, reduction of milk production, deterioration of fertility and increase in the repair rate. Leg problems with cows are continuously have been increasing on dairy farms in the world over the last 20 years, so that in some farms, lameness occurs in more than half of the animals (*Enting et al., 1997*). Leg problems and various forms of lameness can contribute to reducing the amount of milk, according to individual authors, up to 30% (*Bicalho et al., 2008; Ettema et al., 2007)*. Therefore, research in this field is considered very significant in economic terms. The increase in the incidence of hoof lesions and the occurrence of infectious causes of laminitis in cows is more striking after calving (*Blowey, 1993*). Particular importance is given to the consideration of all possible factors, the consideration of their individual and complex action on the cause and severity of lameness. As major environmental impacts of lameness, the following are mentioned in the literature: the type and characteristics of the floors, the type and quantity of the bedding/litter, the possibility for use of free range, the hygienic conditions in the stalls, etc. (*Dippel et al., 2009*).

Significant economic losses are due to the costs of treatment, reduction of milk production, deterioration of fertility and increase in the repair rate. Lameness considerably reduce milk production, and is usually the result of a combination of different factors that persist for a long period of time. These factors include: heredity, nutrition, housing and environment. The lameness of cattle is a major health problem for the dairy industry. In herds where lameness is strongly expressed, there are great economic losses. Regardless of the cause, early detection and rapid treatment reduce losses, reduce animal suffering, and raise important issues for animal welfare (*Shearer, 2000*).

Lameness is a consequence of the disturbed morphologically-functional integrity of the muscular skeletal system. The causes of lameness are many mechanical or predisposing factors (housing, unhygienic conditions, humidity, environment, nutrition, heredity) that have long-term impact on animals (*Tadić and Milosavljević, 1991*).

Today laminitis is the main cause of lameness in cattle. The risk increases when cattle stand for a long time on wet and sharp edges of concrete where the softening and mechanical damage to the hoofs occur. The outer part of the hoof is most often

affected by laminitis (*Rogers, 2001*). Regardless of the way the cows are housed, the main cause of lameness is the use of a solid floors, usually concrete used in the construction of the flooring of the stables (*Boon, 2009*).
The heredity is one of the factors that influence the condition of the hoofs. It is not yet sufficiently scientifically proven, but it is known to breeders that animals that have irregular standing position often transfer this property to their offspring. This only implies that in the selection of cattle it is necessary to pay attention to the fact that, in addition to certain production performance traits, adequate attention should be focused on the choice of animals with healthy feet and hoofs. This is particularly true in the selection of bulls used for artificial insemination, given the many offspring they give. A cow that has become clinically lame exhibits estrus less often and the period from calving to subsequent fertilization and successful conception is extended (*Sprecher et al., 1996*).
Based on the research carried out by *Perišić (2007),* the problem with the hoofs was observed and stated as the third reason for excluding the animals from the production on individual farms. Of the total number of animals, 10% presented with hoof problems as the primary cause for culling, resulting in their poor consumption of food, poor reproductive abilities and pronounced leg problems.

Material and methods

The study of the frequency of the incidence of leg deformation in Simmental cows, in the tied system, was carried out on private farms in the municipalities of Lajkovac and Mionica, on a total of 145 cows.
The studied population of the Simmental cows included cows of different age-lactation number (cows were from 1st to 10th lactation), but the groups were formed so that the cows in the first 6 lactation comprised 6 groups, and the older cows 7th to 10th lactation) together formed one group.
Based on the differences that existed in the housing/holding and the type of stall/bedding, all examined cows were classified into three groups:

- Stall/bedding type and method of housing/holding 1: cows were kept on farms, which had stables with tied system, full-concrete stalls (190 centimeters long), with manure removal canal, and free housing (free housing during dry period, or daily free housing in free range areas over the day).
- Stall/bedding type and method of housing/holding 2: cows were kept in stables with a tied system throughout the year. The stalls were made of full concrete (190 centimeters long), with a manure removal canal.

- Stall/bedding type and method of housing/holding 3: cows were kept in stables with a tied system throughout the year. The stalls were made of full concrete (160 centimeters long), with a manure removal canal, with gutters placed over the canal, of tubular type, in the direction of manure removal canal.

In determining the occurrence of deformation of the extremities and their frequency, the leg positions were scored as follows:

- front legs: (the disturbed leg position, front and side view) "X" position, "O" position, convergent position, divergent position, outward position, inward position, broad position, narrow position, changes in the carpal, ankle, shoulder to body joint ("detached shoulder") and the frequency of "soft pasterns".
- hind legs: (the disturbed leg position side and rear view) "X" position, "O" position, convergent position, divergent position, saber-shaped position, steep angle position, changes in the tarsal ankle and the frequency of "soft pasterns"

The processing of collected data consisted in determining the parameters of descriptive statistics (average, minimum, maximum, standard deviation, variation coefficient) for the observed exterior properties: front leg score (FLFV) front view, hind leg score (HLRV) rear view, front leg score (FLSV) side view, hind leg score (HLSV) side view, front (FP) and hind leg (HP) pastern score and shoulder to body joint score (SB). In the variance analysis, the unified impact of the type of stall/bedding and housing/holding (3 classes) and the influence of animal age (observed through the lactation) on the frequency of leg deformation was studied using a single factorial analysis model.

Results and discussion

The average scores for front and hind leg positions, front and rear view, were not statistically significant different ($p > 0.05$), depending on the effect of cow age, expressed by lactation number (Table 2). The average score was 4.1 for front and 4.2 for hind legs (Table 1).

Table 1. Mean value and variability of the extremity scores of Simmental cattle

Traits	N	Average	Min	Max	SD	CV
Front leg score front view	145	4.1	3.0	5.0	0.60	14.63
Hind leg score rear view	145	4.2	3.0	5.0	0.63	15.00
Front leg score side view	145	4.4	3.0	5.0	0.53	12.05
Hind leg score side view	145	4.2	2.0	5.0	0.70	16.67
Front leg pasterns	145	4.9	2.0	5.0	0.35	7.14
Hind leg pasterns	145	4.6	3.0	5.0	0.58	12.61
Shoulder and front legs to body joint score	145	4.5	3.0	5.0	0.6	13.33

Table 2. Average scores for observed exterior properties of extremities by lactations

lactation	N	FLFV score	HLRV score	FLSV score	HLSV score	FP score	HP score	SB score
1	46	4.1	4.3	4.4	4.3	4.9	4.8	4.7
2	40	4.0	4.1	4.4	4.3	5.0	4.8	4.5
3	15	4.0	4.1	4.3	4.1	4.9	4.3	4.4
4	17	4.1	4.1	4.4	4.4	4.6	4.3	4.3
5	12	4.2	4.3	4.6	4.1	5.0	4.4	4.4
6	8	3.9	3.9	4.1	3.8	4.9	4.5	4.0
7	7	4.0	3.9	4.3	3.9	5.0	4.4	4.1
F exp.	145	0.313^{ns}	1.072^{ns}	0.669^{ns}	1.727^{ns}	3.33^{**}	3.25^{**}	2.302*

***- p ≤0,001; ** - p ≤0,01; * - p≤0,05; ns - p>0,05

In case of side view, the score for the front leg positions was 4.4, and the hind legs 4.2 (Table 1). Lactation number, did not influence statistically significantly ($p > 0.05$) the positions of the front and hind legs, in the side view (Table 2).

The scores for the front leg pasterns ranged from 2.0 to 5.0, with an average of 4.9 (Table 1). A highly significant ($p<0.01$) effect of animal age (lactation number) on the condition of the front leg pasterns was established (Table 2). Variation of the scores for the hind leg pasterns was from 3.0 to 5.0 with an average of 4.6 (Table 1). A highly significant ($p<0.01$) effect of the animal age (lactation number) on the condition of the hind leg pasterns was established (Table 2). The score for the blade and front leg joint with the body ranged from 3.0 to 5.0, with an average of

4.6 (Table 1). The age of animal (lactation number) showed significant ($p < 0.05$) impact on the attachment of the front legs to the body (Table 2).
In case of front and rear view, scores for the positions of the front and hind legs varied significantly ($p<0.05$) under the influence of the stall/bedding type (Table 3). Scores for the position of the front legs, side view (Table 3), varied statistically highly significantly ($p<0.001$) under the influence of the stall/bedding type, and in the hind legs very significantly ($p < 0.01$).
The condition of the front leg pasterns and the blade joint showed no significant variation depending on the type of stall/bedding, while scores for the condition of the hind leg pasterns varied highly significantly ($p < 0.001$) under the influence of the stall/bedding type (Table 3).

Table 3. Average scores for observed exterior properties of the limbs by type of stall/bedding

Stall/bedding	N	FLFV score	HLRV score	FLSV score	HLSV score	FP score	HP score	SB score
1	89	4.1	4.3	4.5	4.4	4.9	4.8	4.5
2	28	3.8	4.0	4.1	4.1	4.9	4.5	4.3
3	28	4.1	4.0	4.2	3.9	4.8	4.3	4.5
F exp.	145	3.804^{*}	4.538^{*}	8.691^{***}	5.421^{**}	1.34^{ns}	10.202^{***}	1.129^{ns}

***- $p \leq 0{,}001$; ** - $p \leq 0{,}01$; * - $p \leq 0{,}05$; ns - $p > 0{,}05$

The analysis of the frequency of the occurrence of irregular positions, joint defects and the shoulder to body joint in cows of Simmental breed in tied holding system on a sample of 145 animals resulted in the following data: of the total scored cows, disturbed position of the front legs ("X" position) was present in 5.62% of cows kept on the first type of stall/bedding. Convergent position of the front legs was observed in 52 heads, of which 35.96% were housed in the stables with the first type, 40.74% on the second type of stall/bedding, and on the third type of stall/bedding 31.03% of the total number of evaluated cows for each type of stall/bedding. The divergent position of the front legs was observed in 8.28% of the total number of estimated cows. Outward and inward positions of the front legs were recorded in 4.14% and 13.03% of cows, respectively, relative to the total number of cows evaluated. Carpal joint changes were observed in a small number of cows, only 1.38% i.e. 2 cows had carpal joint problems. In 134 cows, the front legs showed good angle to the ground, while in 11 cows that angle was irregular. A weak joint of the shoulder blade to the body was observed in a total of 63 estimated animals or 43.45%. The correct position of the hind legs was observed in 125 cows, while the "X" position of the hind legs was observed in 20 animals depending on the type of stall/bedding. The convergent position of the hind legs

had 16.55% of the observed cows, while the divergent position was only recorded in 2.76% of the total number of cows evaluated. A saber-shaped position of the hind legs was observed with 25 cows, or 17.24%, while 7.59% of the cows showed steep angles in hind legs. Changes on the tarsal ankle, in the form of thickening, swelling of the skin, wounds, etc., was observed in 2.76% of cows. The regular hind leg pasterns were observed in 106 cows, and 49 or 33.79% had soft pasterns on the hind legs. When assessing irregular positions, a large percentage of the convergent position of the front and hind legs was observed, also the shoulder blade to body joint presented very low scores in cows. A total of 82 heads did not show a bad fit/joint of shoulder blades with the body. The incidence of poor positions is contributed by both the stall/bedding, with or without litter, and its length.

Table 4. Frequency of the incidence of the leg deformations in cows

Deformation		**Stall/bedding type**						N	Cows with deformations (%)
	Type of deformation	1		2		3			
		N	%	N	%	N	%		
Front legs	„X“ position	5	5.62	-	0.00	-	0.00	5	3.45
	convergent	32	35.96	11	40.74	9	31.03	52	35.86
	divergent	7	7.87	2	7.41	3	10.34	12	8.28
	outward	5	5.62	1	3.70	-	0.00	6	4.14
	inward	14	15.73	-	0.00	2	6.90	16	11.03
	wide	4	4.49	-	0.00	2	6.90	6	4.14
	Carpal joint swelling	2	2.25	-	0.00	-	0.00	2	1.38
	„soft pasterns“	5	5.62	3	11.11	3	10.34	11	7.59
	Poor fit of shoulder blade to body („scraped shoulder“)	34	38.20	16	59.26	13	44.83	63	43.45
Hind legs	„X“ position	6	6.74	6	22.22	8	27.59	20	14.48
	convergent	22	24.72	2	7.41	-	0.00	24	16.55
	divergent	3	3.37	-	-	1	3.35	4	2.76
	Sable shaped	12	13.48	6	22.22	7	24.14	25	17.24
	Steep angle	10	11.24	-	0.00	1	3.35	11	7.59
	Tarsal joint swelling	3	3.37	-	0.00	1	3.35	4	2.76
	„soft pasterns“	17	19.10	14	51.85	18	62.07	49	33.79
Total number of evaluated cows		89	100%	27	100%	29	100%	145	100%

Conclusion

On the basis of the obtained results, it can be concluded that the average scores for the extremitis/limbs of Simmental Cows ranged from 4.1 to 4.9. By examining their variability under the influence of lactation number and type of stall/bedding, it has been concluded that the scores for front and rear leg positions front, rear and side view, varied at different levels of significance under the influence of the type of stall/bedding, while scores for the condition of the pasterns on the hind legs varied highly significantly ($p < 0.001$) under the influence of the type of stall/bedding. Scores for front leg pasterns and scores for shoulder blade to body joint were not under significant ($p > 0.05$) influence of the type of stall/bedding and the method of housing/holding of cows.

Of the total number of cows assessed, 3.45% had "X" position of the front legs, 14.8% had "X" position of the hind legs. The convergent position of the front legs was observed in 35.86%, and divergent in 8.28% of cows. Convergent position of the hind legs was observed in 16.55% of cows, and divergent in 2.76%. The forward position of the front legs was observed in 4.14% of cows, inward position in 11.03%, and wide position in 4.14%. 17.24% of cows had sable-shaped position of hind legs, and 7.59% showed steep angles. The pronounced soft front leg pasterns were observed in 7.59% of the cows, and in hind legs 33.79% of the total number of estimated cows. The observed changes in the joints, in the form of swellings was observed in 1.38% of cattle on the carpal joint and 2.76% on the tarsal joint. The affected shoulder blade and body joint (scraped shoulder) was observed in 43.45% of the total number of cows. The body weight of Busha cows on the territory of the Pirot district amounted to 226.07 kg, the height of the ridge 104.33 cm, the height of the cross 104.12 cm, pelvis width 32.52 cm, breast depth 53.97 cm, breast circumference 130.48 cm, and body length 119.67 cm.
Problems with legs and hoofs are a common cause of culling of animals from production. Outgrowths of the hoofs lead to a reduction in the angle of the pasterns to the ground and the creation of irregular legs, which is reason why animals get up and move with increased difficulty, the food consumption is reduced, as well as the production and they become more susceptible to various diseases. Regular and correct correction of the hoofs would prevent the excessive and irregular growth of the hoof mass. Problems with extremities are more pronounced than stated in the literature, since these problems are often the primary reason for the incidence of sterility that is not recorded during the culling of cows from production, but rather its consequence, i.e. sterility is recorded as the reason.

Pojava deformacije ekstremiteta krava simentalske rase u različitim tipovima ležišta

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Rezime

Problemi sa nogama i različiti oblici šepavosti krava, u intenzivnoj proizvodnji mleka su treći problem po značaju posle mastitisa i reproduktivnih poremećaja, kako u svetu tako i u našoj zemlji. U radu su analizirane pojava deformacije stavova nogu 145 krava simentalske rase na području Kolubarskog okruga, i uticaj paragenetskih faktora (način držanja i tip ležišta i laktacija po redu) na pojavu deformacija. Od ukupnog broja ocenjenih krava 3,45 % je imalo „X“ stav prednjih nogu, 14,8% je imalo „X“ stav zadnjih nogu. Konvergentan stav prednjih nogu imalo je 35,86%, a divergentan 8,28%. Konvergentan stav zadnjih nogu imalo je 16,55% krava, a divergentan 2,76%. Isturen stav prednjih nogu imalo je 4,14% krava, podvučen 11,03%, a širok stav 4,14%. Sabljast stav zadnjih nogu imalo je 17,24% krava, a stubast 7,59%. Izražene mekane kičice prednjih nogu imalo je 7,59% krava, a mekane kičice zadnjih nogu imalo je 33,79% od ukupnog broja ocenjenih krava. Uočene promene na zglobovima u vidu otoka imalo je 1,38% krava na karpalnom zglobu i 2,76% na tarzalnom zglobu. Narušen spoj lopatice i tela (odvaljena plećka) imalo je 43,45% od ukupnog broja ocenjenih krava. Ocene za stavove prednjih i zadnjih nogu posmatrano spreda, otpozadi i sa strane varirale su na različitom nivou značajnosti pod uticajem tipa ležišta, dok ocene za stanje kičica na zadnjim nogama su vrlo visoko značajno ($p<0,001$) varirale pod uticajem tipa ležišta. Ocene za kičice prednjih nogu i ocene za spoj lopatice i trupa, nisu značajno ($p>0,05$) zavisile od tipa ležišta i načina držanja krava.

Ključne reči: simentalska rasa, ekstremiteti, ležište

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ECO-FISH MEAL AS AN ALTERNATIVE TO FISH MEAL IN DIETS FOR LAMBS

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Abstract: The effect of Eco-fish meal, as an alternative to fish meal, on the production performance of the lambs of the Mis population in the intensive fattening, was investigated. The experiment was carried out on 40 lambs, the average age of 30 days, divided in 2 groups. In addition to mother's milk, the lambs were given a concentrated mixture and a lucerne hay, at will. Isoprotein forage mixtures (16% of total proteins) differed in terms of the protein component. The protein source for treatment I had fish meal, while the animals on treatment II consumed EcoFish, a herbal substitute for fish meal, which consisted of domestic foods of known origin such as genetically unmodified and thermally treated meal of decorticated soybean grains, soybean protein isolates, gluten, livestock yeast with the addition of minerals, amino acids, vitamins, enzymes and other additives. Statistical processing of the obtained data was done using the SPSS STATISTICA, Version 20.

On treatments I and II, the average daily lamb gain was 320 and 283 g, respectively, without statistical significance. The consumption of dry matter and proteins in analogue treatments was 0.819 and 0.823 kg, and 152.62 and 157.04 g, respectively. The dry matter consumption per kilogram of gain (kg/kg of gain) was 2.56 and 2.91; of energy (MJ NEM/kg): 17.65 and 20.25; of total proteins: 476.9 and 554.9 g, respectively. The protein efficiency ratio - PER (g of gain/g of consumed protein) in analogue treatments was: 2.09 and 1.80. There were no statistically significant differences between examined treatments ($P > 0.05$).

Considering that the source of protein did not significantly affect the intensity of growth and the use of food by lambs of Mis population in intensive fattening ($P>0.05$), fish meal can be replaced by Eco-fish meal - plant protein, since according to Commission Decision 9/2001 on BCE protection (OJEC, 2001), there is a distance to the use of fish meal, as a source of protein.

Keywords: fish meal, Eco fish meal, lambs, daily gain, conversion

Introduction

In ruminant nutrition, protein requirements are provided microbial and non-degradable protein at the level of the rumen, which is absorbed directly into the small intestine (*Can et al., 2005*). The microbial protein is not sufficient for the expression of the genetic potential of lambs that have an intensive gain, which implies the use of protein nutrients with a high share of non-degradable proteins.

Fish meal is an excellent source of high-quality protein that slowly degrades in the rumen (*Amos et al., 1975, ARC 1980, Adam et al., 1982, Zebrini and Polan, 1985*) and has an excellent amino acid profile. In the research of *Orskov et al. (1971),* in fattening male lambs of 15-50 kg, the increase in fish meal in rations from 1 to 6 and 12% caused an increase in daily gain: 0.191, 0.270, 0.330 kg, respectively. Also, studies of *Beermann et al. (1986)* show that the replacement of soy meal with 3% of fish meal in the diet of lambs crosses of Suffolk x Dorset breeds resulted in increased daily gain and improved feed conversion ratio: 0.441 kg and 3.52 kg relative to 0.350 kg and 3.90 kg, realized by animals on the treatment without fish meal. In contrast, in the studies of *Ponda (1984), Hussein and Jordan (1991)* and *Cana (2004),* the intensity of lamb growth was not improved by incorporating fish meal as a protein component into a diet.

According to Commission Decision 9/2001 on the protection of BCE (OJEC, 2001), food containing fish meal can not be produced in manufacturing plants that produce food for ruminants, which in some ways leads to the distance to fish meal, as a high-quality protein source.

Considering the above facts, the aim of this study was to examine the possibility of using Eco Fish Meal (high protein plant feed), as a substitute for fish meal, in rations for fattening lambs.

Material and methods

The experiment included 40 lambs of MIS population, distributed in 2 groups of 20 heads, gender-balanced (10 male and 10 female). Lambs were about 30 days old and on average 14 kg of body weight. In addition to mother's milk, lambs were given a forage mixture and an alfalfa hay, at will. The feedingstuffs contained 16% of the total protein, but differed in terms of the protein component. The lambs in treatment I received fish meal as a source of protein, while the animals in treatment II received Eco fish meal, a replacement for fish meal. This product contains domestic nutrients of known origin such as genetically unmodified and thermally processed meal of decorticated soybean grain, soybean protein isolate, gluten, livestock yeast with the addition of minerals, amino acids,

vitamins, enzymes and other additives. The comparative chemical composition of the mentioned protein components is shown in Table 1. The structure of the used feedingstuffs is shown in Table 2, while the nutritional value of the feedstuffs is shown in the Table 3. The trial lasted for 60 days. Changes in body weight, total gain, average daily gain, feed consumption and conversion were controlled at 15 day intervals, while initial and final weight were determined by measurements in three consecutive days. Statistical data processing was performed using SPSS STATISTICA, Version 20.

Table 1. Comparative chemical composition of fish meal and Eco fish meal,%

Indicator	**Fish meal**	**Eco fish meal**
Chemical composition, %		
Dry matter	92	92
Protein	62	60
Ash	20	7
Fat	7	5
Fibres	1	4
Macroelements, %		
Calcium	5.65	0.6
Phosphorus, total	3.16	0.4
Sodium	0.56	0.16
Aminoacids,%		
Lysine	4.74	4.70
Methionine	1.75	1.77
Threonine	2.51	2.39
Tryptophan	0.65	0.65

Table 2. Structure of the concentrate mixture for fattening of lambs up to 90 days of age, %

Feedstuff	I	II
Maize	70	70
Sunflower meal	20	20
Fish meal	6	-
Eko fish meal	-	8
Livestock limestone	2	2
Salt	1	1
Premix	1	1

*Calculated according to Obračević (1990)

Table 3. Nutritional value of feedstuffs used in the experiment

Feedstuffs	DM,%	Total protein,%	MJ, NEM
Forage mixture I	88.35	16.10	6.94
Forage mixture II	88.13	16.00	6.90
Hay	88.33	15.36	4.10
Milk	16.14	5.68	2.32

Results and discussion

Tables 4 and 5 show the performance of experimental lambs that were included in the treatment fish meal (I) and Eco fish meal (II). The protein source did not significantly affect the body weight, total and average daily lamb gain (P>0.05). Our results are in agreement with *Ponda (1984), Hussein and Jordan (1991)* and *Cana et. al. (2004*), who state that the lamb growth rate is not improved by the inclusion of fish meal in diets for lambs. Also, *Banskalieva et al. (2005)* has found that supplementing the fish oil in diets for goats, aged 14 days, did not induce significant changes in the average daily gain (90.33g versus 82.42g). Comparing the effect of replacing soybean meal with fish meal in rations for fattening lambs, *Dabiry and Thonney (2001)* have found that feeding treatment did not affect the intensity of lamb growth, which is consistent with our results. Thus, fish meal can be successfully replaced with plant protein sources in fattening lamb, provided they are balanced in terms of net energy and amino acid content. The explanation for this phenomenon lies in the fact that the level of total proteins of 16%, in iso-energetic diets, provides optimum conditions for the development of the microflora of rumen and the expression of the production potential, or the genetic capacity of the growth of the experimental lambs.

Table 4. Performances of the experimental lamb

Indicators	I	II	Level of significance
Initial body weight (kg)	14.55±2.61	14.57±2.93	NS
Final body weight (kg)	31.95±3.28	30.75±3.59	NS
Total gain (kg)	17.40±2.34	16.18±1.96	NS
Average daily gain, g	320±39.09	283±32.73	NS

I Fish meal (FM)
II Eko fish meal (EFM)

Table 5. Consumption and conversion of food and nutrients of experimental lamb

Indicators	I	II
DM consumption, (kg/day)	0.819	0.823
Total protein consumption, (g/day)	152.62	157.04
Energy consumption, (MJ/day)	5.65	5.73
DM conversion, (kg DM /kg of gain)	2.56	2.91
Total protein conversion, (g/kg of gain)	476.9	554.9
Energy conversion, (MJ/kg of gain)	17.65	20.25
PER, (g of gain /g cons.protein)	2.09	1.80

*PER, protein efficiency ratio
NS = not significant (P>0.05)

Contrary to this, our results do not agree with the results of some authors' examinations. By examining the effect of replacing the urea with fish meal on the production performances of Awassi lamb, *Can et al. (2005)* has found that the animals fed fish meal had an average daily gain of 0.268 kg and with urea 0.236 kg. The average consumption of dry matter was 1.148 and 1.064, while the efficiency of protein utilization (PER) was 1.67 and 1.58 (g of gain/g of consumed protein), respectively. Also, *Ponnampalam et al. (2005),* by examining the influence of different sources of protein (canola, soybean meal, fish meal) on the production performance of lambs in fattening, have found the highest average daily gain at the level of P<0.01 (163 g) in animals on the treatment with fish meal, also the most favourable food conversion, P<0.001, (6.0 kg / kg of gain) as well as the thickness of the fat tissue (10.1 mm). Given that fish meal is an excellent source of high-quality proteins, of excellent amino acid profile, part of this response can be attributed to improved fibre digestion in rumen as a result of the availability of amino acids and ammonia used for microflora growth. This results in agreement with research by *Chalupa (1975),* who has concluded that the level of non-degradable protein, fish meal being an important source of it, is the most significant in young animals with intense muscle growth especially in the first 40 days of age, in which the microbial protein is not sufficient to express the genetic potential of the lamb.

Different responses to the effects of replacing fish meal with plant protein sources are explained by variations in the degradation of animal and plant proteins. In addition, the degree of degradability depends on the nature of the nutrient itself and on the associative effect of the ingredients of the diet as well as the method of treatment. Nutrition of ruminents with a high level of grain feeds causes a decrease in the pH of the rumen, which may result in a decreased cellulolytic activity of the microflora. Higher levels of total protein meals can compensate for poor distribution of amino acids and be more economical than procuring expensive sources of non-degradable protein.

Conclusion

On the basis of the obtained results of the examination of the effects of replacing fish meal with plant proteins in diet for lambs in fattening, the following conclusions can be made:

- The average daily lamb gain on treatments I and II was 320 and 283 g, without statistical significance (P> 0.05);
- Average consumption of dry matter and total proteins in analogue treatments was 0.819 and 0.823 kg/day and 152.62 and 157.04 g/day, respectively;
- The dry matter consumption (kg/kg of gain) was 2.56 and 2.91, while the protein conversion (g/kg of gain) was 476.9 and 554.9, respectively.
- The protein efficiency ratio (PER) was: 2.09 and 1.80 (g gain/ g of protein consumed).
- The source of protein did not significantly affect the growth rate and the efficiency of food utilization in the lambs of the MIS population in fattening (P>0.05), suggesting that fish meal can be successfully replaced by Eco fish meal, considering that, based on Commission Decision 9/2001 on protection of BCE (OJEC, 2001), there is a distance to use fishmeal as a source of protein.

Eko-fiš meal kao alternativa ribljem brašnu u obrocima za jagnjad

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Rezime

Ispitivan je uticaj eko fiš meal kao alternative ribljem brašnu, na proizvodne performanse jagnjadi Mis populacije, u intenzivnom tovu. Eksperiment je izveden na 40 jagnjadi, prosečnog uzrasta 30 dana, raspoređenih u

2 grupe. Pored majčinog mleka, jagnjad su dobijala koncentrovanu smešu i lucerkino seno po volji. Izoproteinske krmne smeše (16% ukupnih proteina) su se razlikovale u pogledu proteinske komponente.Izvor proteina na tretmanu I je bilo riblje brašno, dok su grla na tretmanu II konzumirala Eko fiš meal-biljnu zamenu za riblje brašno, koja se se sastojala od domaćih hraniva poznatog porekla kao što su genetski nemodifikovano i termički obrađeno brašno oljuštenog zrna soje, izolat proteina soje, glutena, stočnog kvasca uz dodatak minerala, aminokiselina, vitamina, enzima i drugih dodataka.
Statistička obrada dobijenih podataka je izvršena korišćenjem SPSS STATISTICA, Version 20.
Na tretmanima I i II, prosečan dnevni prirast jagnjadi je iznosio: 320 i 283 g, bez statističke značajnosti. Konzumiranje suve materije i proteina na analognim tretmanima, je iznosilo: 0.819 : 0.823 kg, odnosno 152,62 : 157,04g. Utrošak suve materije po kilogramu prirasta je(kg/kg prirasta) je iznosio: 2,56 : 2,91; energije (MJ NEM/kg): 17,65 : 20,25; ukupnih proteina: 476,9 : 554,9 g. Efikasnost iskorišćavanja proteina - PER (g prirasta/g konzumiranog proteina) na analognim tretmanima je iznosila: 2,09 i 1,80. Nisu ustanovljene statistički značajne razlike između ispitivanih tretmana (P>0,05).
Obzirom da izvor proteina nije značajno uticao na intenzitet rasta i iskorišćavanje hrane kod jagnjadi Mis populacije u intenzivnom tovu (P>0,05), riblje brašno se može zameniti Eko fiš-meal- biljnim izvorom proteina, budući da prema odluci Komisije 9/2001 o zaštiti BCE (OJEC, 2001),postoji distanca prema korišćenju ribljeg brašna, kao izvoru proteina.

Ključne reči: riblje brašno, eko fish meal, jagnjad, dnevni prirast, konverzija

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CERTAIN EGG QUALITY PARAMETERS OF GRAY GUINEA FOWL IN EXTENSIVE REARING

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Abstract. This paper presents results of determination of certain quality parameters and its phenotypic correlation in eggs originated from extensively reared gray variety of Guinea fowl. A total of 150 egg collected by sampling 30 eggs in each of five analyzed laying months were used for egg quality evaluation and statistical analysis by methods of descriptive statistics and simple linear correlation. Average egg weight, shape index and shell thickness was 38.14 g, 76.03% and 0.49 mm, respectively. Average shell, yolk and albumen weight was 5.83, 12.16 and 20.23 g, respectively, and its proportion was 15.23, 32.10 and 52.69%, respectively. Average values of yolk height, diameter, index and color were 16.54 mm, 39.95 mm, 41.50%, and 13.76, whereas values for albumen diameter, index and height as well Haugh units were 59.30 mm, 9.62%, 5.67 mm, and 82.58, respectively. Majority of examined quality parameters showed significant correlation with other parameters. Egg weight was positive correlated ($p<0.01$) with egg length (0.76) and width (0.92), shape index (0.22), shell thickness (0.60), shell weight (0.81) and proportion (0.44), albumen (0.92) and yolk weight (0.77) and yolk index (0.23), but in negative connection ($p<0.01$) with yolk proportion (-0.54), yolk/albumen ratio (-0.41) and albumen index (-0.25). Determined egg quality indicated good potential of this species in extensive rearing, which could be improved and used in more favorable rearing conditions.

Key words: Guinea fowl, egg quality, phenotypic correlation

Introduction

Guinea fowl (*Numida meleagris*) is one of rarely reared poultry species, usually kept in extensive or semi-intensive conditions. Regardless of its long presence in Bosnia and Herzegovina, Guinea fowl is still kept as an exotic bird, with unrecognized production potential. Egg productivity of gray variety of Guinea fowl in those conditions reaches nearly 100 eggs or less *(Kuzniacka et al., 2004; Nickolova, 2009; Bernacki et al., 2013)* with specific quality characteristics (e.g.

lower egg weight, pyriform egg shape, thicker and stronger shell) different from eggs of other poultry species *(Song et al., 2000).* Differences in egg production and quality among different varieties *(Nowaczewski et al., 2008; Bernacki et al., 2013)* as well variations in egg quality during laying season *(Nickolova, 2009)* in semi-extensive conditions or during egg storage *(Banaszewska et al., 2015)* are observed. However, recent studies regarding egg quality in extensive rearing of this species are rare, especially in conditions of Bosnia and Herzegovina. Therefore, aim of this study was to determine certain egg quality parameters and its phenotypic correlation in gray Guinea fowl reared in extensive conditions.

Materials and methods

Study was performed from May to August 2015, using one flock of grey Guinea fowl reared in extensive condition in mountain region of western part of Republic of Srpska – Bosnia and Herzegovina. Birds were kept in provisional object under natural light, with all day open access to forested field and feed only with cereals given in morning hours. During five examined laying months, a total of 150 eggs were used for quality analysis. In the last week of every month 30 eggs were collected and then individually marked and stored one day at 6°C prior to analysis. Egg weight was measured with electronic scale (0.00 g), and egg height and width with electronic caliper (0.00 mm). Those data were used for calculation of egg shape index, according to formula: Egg shape index (%) = (egg width / egg length) x 100. Eggs were then broken and the following internal quality parameters were measured: yolk and albumen height, with tripod micrometer (0.00 mm), yolk diameter and albumen minimal and maximal diameter, with electronic caliper (0.00 mm) and yolk color, with yolk color fan. Obtained data were used for following calculations: Yolk index (%) = (yolk height / yolk diameter) x 100; Yolk / Albumen ratio = yolk weight / albumen weight; Albumen index (%) = [albumen height / (minimal + maximal albumen diameter / 2)] x 100 and Haugh unit = 100 log x (albumen height + 7.57 – 1.7 x egg weight $^{0.37}$) *(Haugh, 1937).* Electronic scale (0.00 g) was used for measuring of yolk weight after its separation from albumen and shell weight after one day long drying at room temperature. Weight and proportion of egg components was calculated as: Albumen weight (g) = Egg weight – (yolk weight + shell weight); Yolk proportion (%) = (yolk weight / egg weight) x 100; Albumen proportion (%) = (albumen weight / egg weight) x 100 and Shell ratio (%) = (shell weight / egg weight) x 100. Shell thickness was measured at blunt, equatorial and sharp region with electronic caliper (0.00 mm). Shell parameters were calculated as follows: Average shell thickness = (thickness of blunt part + thickness of equatorial part + thickness of sharp part) / 3; Shell weight per unit surface (g/cm^2) = shell weight / shell surface area (*Curtis et al*., 1985). Statistical analysis was performed using methods of descriptive statistics and simple linear correlation in software package SPSS.

Results and Discussion

External quality parameters of examined Guinea fowl eggs are presented in table 1. Average value for egg weight in our study was relatively lower when compared to results got in eggs from extensive rearing in study of *Kuzniacka et al. (2004)*. Higher average weight are reported for eggs originated from semi-intensive, e.g. 39.19 g *(Wilkanowska and Kokoszyński, 2010)*; 40.14 g *(Alkan et al., 2013)* and 40.38 g *(Nickolova, 2009)* and intensive conditions, e.g. 48.9 g *(Ancel and Girard, 1992)*. Mean egg length and width found in this study was comparable with 49.4 and 37.47 mm found by *Wilkanowska and Kokoszyński (2010)*, or 49.47 and 37.89 mm reported by *Alkan et al. (2013)*. Especially higher values of egg length and width (55.4 and 51.7 mm) were reported by *Ancel and Girard (1992)*.

Table 1. Parameters of egg external quality

Parameters	Mean	Min	Max	SE	CV
Egg weight (g)	38.14	30.32	45.99	0.355	10.02
Egg length (mm)	49.24	44.67	52.10	0.136	2.98
Egg width (mm)	37.42	34.58	40.04	0.105	3.03
Egg shape index (%)	76.03	71.27	80.71	0.184	2.61
Shell thickness (mm)	0.49	0.29	0.64	0.006	14.10
Shell weight per surface area (g/cm^2)	0.11	0.06	0.14	0.002	14.82

Egg shape index values indicate normal shape of analyzed eggs, which is comparable with average of 76.60% reported by *Alkan et al. (2013)*. Lower findings are 73.7% in meat or 74.7% in domestic type of Guinea fowl, reported by *Nowaczewski et al. (2008)*, but there are also higher values in the literature, as 77.8% found by *Kuzniacka et al., (2004)* or 79.4% *(Ancel and Girard, 1992)*. Average shell thickness and shell weight per surface area and their coefficients of variation were the highest among all external parameters in this study. Similar values for thickness (0.48 mm) are reported by *Kuzniacka et al. (2004)*. Lower value of egg shell thickness (0.440 mm) was found by *Bernacki et al. (2013)*, but higher (0.55 mm) by *Nickolova (2009)*. Average unit surface shell weight was equivalent to 0.11 found by *Alkan et al. (2013)* or 102 in white and 101 mg/cm^2 in pearl variety *(Bernacki et al., 2013)*.
Weight and proportions of egg parts are presented in Table 2.

Table 2. Weight and proportion of egg parts

Parameters	Mean	Min	Max	SE	CV
Shell weight (g)	5.83	2.84	8.14	0.101	18.62
Albumen weight (g)	20.23	13.97	24.60	0.241	12.19
Yolk weight (g)	12.26	9.27	14.48	0.098	8.21
Shell share (%)	15.23	8.94	19.85	0.198	14.03
Albumen share (%)	52.69	43.60	59.20	0.267	5.19
Yolk share (%)	32.10	28.77	38.05	0.215	6.87

Average egg structure, regarding shell, albumen and yolk weight, and their shares was similar to results found by *Nowaczewski et al. (2008)* with 15.6, 53.0 and 31.4%, respectively, *Banaszewska et al. (2015)* with 15.63, 51.66 and 32.71%, respectively, as well as *Alkan et al. (2013)* and *Bernacki et al. (2012)*. Higher shell (22.0%) and lower albumen proportion (46.1%) were found by *Kuzniacka et al. (2004)*, while *Nickolova (2009)* got higher shell (20.18%) and lower yolk proportion (29.29%).

Quality parameters of yolk and albumen are presented in table 3.

Table 3. Parameters of internal egg quality

Parameters	Mean	Min	Max	SE	CV
Yolk height (mm)	16.54	14.20	18.30	0.086	5.51
Yolk diameter (mm)	39.95	34.66	42.77	0.135	3.63
Yolk index (%)	41.50	35.15	47.21	0.237	6.06
Yolk color	13.76	10.00	15.00	0.103	8.05
Albumen diameter (mm)	59.30	47.59	71.56	0.435	7.87
Albumen height (mm)	5.67	4.50	6.78	0.040	7.66
Albumen index (%)	9.62	6.99	12.08	0.086	9.60
Haugh units	82.58	74.72	89.75	0.265	3.46
Yolk/albumen ratio	0.61	0.49	0.87	0.007	11.57

Average yolk height and diameter were comparable to findings of several authors, as 16.4 and 37.3 mm in study of *Bernacki et al., (2012)*, 15.7 and 38.2 mm found by *Kuzniacka et al. (2004)* or 15.20 and 39.83 mm found by *Banaszewska et al. (2015)*. Average yolk index in our study indicated satisfying quality of analyzed eggs. Lower values were reported by *Wilkanowska and Kokoszyński (2010)* for pearl (37.86%) and white variety of Guinea fowl (39.80%). *Bernacki et al. (2012)* found higher values in white (43.4%) and pearl variety (44.0%). Yolk color was

darker, compared with findings of 9.69 *(Bernacki et al., 2012)* or 10.25 *(Nickolova, 2009)*. *Banaszewska et al. (2015)* also found darker color (14.60), similar to our findings.

Average value of albumen height in our study was similar to report of *Kuzniacka et al. (2004)*, who got average value of albumen height of 5.6 mm, while *Alkan et al. (2013)* found average albumen height of 4.77 mm. Average value of albumen index in our study was higher than 7.05%, found in pearl, or 8.29%, found in white variety *(Wilkanowska and Kokoszyński, 2010)* or 6.79% found by *Alkan et al. (2013)*. Haugh units in this research indicated high egg quality, comparable with values of 82.1 found in white or 82.7 found in pearl variety by *Bernacki et al. (2012)*, and was better than value of 74.97 found by *Alkan et al. (2013)*. *Nickolova (2009)* got values of Haugh units above 95.61 during entire laying season. Calculated mean yolk/albumen ratio in our study was higher than 0.55 observed by *Song et al. (2000)*, or 0.51 in meat and 0.59 in domestic type, found by *Nowaczewski et al. (2008)*. Higher mean yolk/albumen ratio (0.68) was found by *Alkan et al. (2013)*.

Phenotypic correlation among internal and external egg quality parameters are presented in table 4.

Table 4. Coefficients of simple correlation (r) between egg quality parameters

	EL	EW	SI	ST	SW	SP	AW	AP	YW	YP	YA	YI	AI	HU
EM	.76**	.92**	.22**	.60**	.81**	.44**	.92**	.11	.77**	-.54**	-.41**	.23**	-.25**	-.09
EL		.62**	-.39**	.28**	.53**	.20*	.70**	.07	.68**	-.27**	-.21*	.13	-.21*	-.07
EW			.48**	.52**	.75**	.42**	.82**	.03	.75**	-.43**	-.30**	.22**	-.27**	-.13
SI				.30**	.28**	.26**	.18*	-.04	.11	-.20*	-.12	.11	-.08	-.06
ST					.89**	.89**	.36**	-.42**	.46**	-.33**	-.03	.02	-.28**	-.35**
SW						.88**	.59**	-.32**	.62**	-.44**	-.14	.13	-.28**	-.24**
SP							.16	-.59**	.35**	-.22**	.13	.01	-.22**	-.31**
AW								.49**	.53**	-.73**	-.70**	.25**	-.16	.08
AP									-.37**	-.66**	-.88**	.13	.14	.40**
YW										.12	.22**	.14	-.27**	-.26**
YP											.93**	-.17*	.03	-.19*
YA												-.17*	-.04	-.31**
YI													.31**	.22**
AI														.65**

EM – egg weight; EW – egg width; EL – egg length; SI – shape index; ST – shell thickness; SM – shell weight; SP – shell proportion; AW – albumen weight; AP - albumen proportion; YW – yolk weight; YP – yolk proportion; YA – yolk/albumen ratio; YI – yolk index; AI – albumen index; HU – Haugh units. * $p<0.05$; ** $p<0.01$

The lowest coefficients of correlation in our study was found for albumen proportion vs. yolk/albumen ratio and the highest for yolk proportion vs. yolk/albumen ratio. Egg weight was positive correlated with egg length and width, which is in agreement with *Bernacki et al. (2012), Tebesi et al. (2012)* and *Alkan et al. (2013)*. Moreover, positive correlation of egg weight with shape index in this research are analogous with *Kuzniacka et al. (2004), Bernacki et al. (2012)* and *Alkan et al. (2013)*, but contrary to *Nowaczewski et al. (2008)*. Significant correlation of egg weight with shell, albumen, and yolk weight was also found by *Bernacki et al. (2012), Nowaczewski et al. (2008)* and *Alkan et al. (2013)*. Regarding egg part proportions, egg weight was in positive correlation with shell and in negative correlation with yolk, which is also described by *Alkan et al. (2013)*. *Kuzniacka et al. (2004), Bernacki et al. (2012)* and *Nowaczewski et al. (2008)* reported different positive and negative correlation of egg weight with egg part proportions. Positive correlation was also detected for egg weight with shell thickness and yolk index. Positive correlation of egg weight with shell thickness is also reported by *Nowaczewski et al. (2008)* and *Alkan et al. (2013)*. Negative connections of egg weight in this study were found with yolk/albumen ratio and albumen index. Negative correlation of egg weight with yolk/albumen ratio was also noticed by *Nowaczewski et al. (2008)*.

Increase in shell index in this study was followed with increase in egg width and decrease in egg length. This is in accordance with results of *Bernacki et al. (2012), Alkan et al. (2013)* and *Tebesi et al. (2012)*. Also, shell index was in positive correlation with shell thickness, weight and proportion, as well as with albumen weight, but was in negative correlation with yolk proportion. Positive correlation of shape index with shell and yolk weight was found by *Alkan et al. (2013)*. Increase in shell, albumen, and yolk weight as well as shell thickness, according to *Nowaczewski et al. (2008)*, resulted in decrease of egg shape index.

Our results showed that shell weight was good predictor for egg length, egg width, shell thickness, shell proportion and albumen weight. *Alkan et al. (2013)* found parallel increase in shell weight with egg width and length, shell thickness and proportion, but also inverse direction with albumen proportion. Positively correlated parameters with shell weight were albumen weight, shell thickness and proportion *(Bernacki et al., 2012)*, yolk and albumen weight, shell proportion and thickness *(Nowaczewski et al., 2008)*, egg length, shell proportion and thickness *(Tebesi et al., 2012)*. Negative correlation with shell weight was found for albumen and yolk proportion, as well as albumen index and Haugh units. Negatively correlated parameters with shell weight were yolk proportion in study of *Bernacki et al. (2012)*, yolk proportion and yolk/albumen ratio, according to *Nowaczewski et al. (2008)*, and shape index *(Tebesi et al., 2012)*.

Albumen weight was in positive correlation with egg length and width, albumen proportion, yolk weight, yolk index and shell thickness. Negative correlation of albumen weight was found for yolk proportion and yolk/albumen ratio. *Bernacki et al. (2012)* found positive connection among albumen weight with egg length, shape index and albumen proportion, but also negative with egg width, yolk and shell proportion and Haugh units. *Alkan et al. (2013)* found positive correlation with egg width and albumen index, but negative with yolk/albumen ratio, albumen and yolk proportion. *Nowaczewski et al. (2008)* recorded shell and yolk weight, albumen proportion and shell thickness as positive, and yolk proportion and yolk/albumen ration as negative correlated to albumen weight. *Kuzniacka et al. (2004)* reported that weight and proportion of albumen and yolk was positive correlated, but shell proportion was negative correlated to albumen weight. *Tebesi et al. (2012)* found that egg width and length, and albumen proportion are in positive, whereas yolk weight and proportion, yolk and albumen index, and Haugh units were in negative correlation to albumen weight.

Yolk weight was in positive correlation with egg length and width, shell weight and proportion, albumen weight, shell thickness, and yolk/albumen ratio. Negative relation of yolk weight was with found with albumen proportion, albumen index and Haugh units. *Bernacki et al. (2012)* reported that yolk weight had positive correlation with egg width and length, and yolk proportion, but negative with albumen and shell proportion. *Alkan et al. (2013)* found that yolk weight had positive direction with egg width and length, shell weight and thickness, shape index, shell and yolk proportion, and yolk/albumen ratio, but negative with albumen proportion. Positively correlated parameters to yolk weight were albumen weight, and shell weight and thickness *(Nowaczewski et al., 2008)*; or shape index, albumen weight, and shell and yolk proportion *(Kuzniacka et al., 2004)*. *Tebesi et al. (2012)* found egg length and Haugh units as positive, and albumen weight and proportion as negative correlated to yolk weight.

Conclusion

Evaluation of Guinea fowl egg quality showed satisfactory result obtained by determination of standard parameters such as Haugh units, albumen and yolk index or yolk color. Also, these results indicated good potential of this species in extensive rearing, which could be improved and used in more favorable rearing conditions. Significant phenotypic correlations observed among majority of quality parameters can be used in indirect egg quality evaluations. This research contributes to knowledge on Guinea fowl egg quality and therefore can be useful for breeders in field of reproduction or table egg production in order to improve productivity of this species.

Pokazatelji kvaliteta jaja sive biserke u ekstenzivnom gajenju

Marinko Vekić, Stoja Jotanović, Đorđe Savić

Rezime

U radu su prikazani rezultati određivanja pokazatelja kvaliteta i njihove fenotipske korelacije kod jaja sivog soja biserke gajene u ekstenzivnim uslovima. Trideset jaja je sakupljeno u svakom od pet ispitivanih meseci nošenja, tako da je ukupno 150 jaja korišćeno za određivanje kvaliteta i statističku analizu primenom metoda deskriptivne statistike i proste linearne korelacije. Prosečna masa jaja, dužina jaja, širina jaja, indeks oblika ljuske, debljina ljuske i masa ljuske po jedinici površine iznosili su 38,14 g; 49,24 mm; 37,42 mm; 76,03%; 0,49 mm i 0,11 g/cm^2, redom. Prosečna masa ljuske, belanca i žumanca bila je 5,83; 20,23 i 12,26 g, redom, a njihov udeo 15,23; 52,69 i 32,10%, redom. Prosečna visina, prečnik, indeks i boja žumanca iznosili su 16,54 mm; 39,95 mm; 41,50% and 13,76, redom. Prosečan prečnik, indeks i visina belanca, kao i Haughove jedinice i odnos žumance/belance iznosili su 59,30 mm; 9,62%; 5,67 mm; 82,58 i 0,61, redom. Većina ispitivanih pokazatelja kvaliteta pokazala je značajnu fenotipsku korelaciju sa drugim pokazateljima. Masa jaja bila je u pozitivnoj korelaciji ($p<0,01$) sa dužinom (0,76), širinom (0,92) i indeksom oblika jaja (0,22), zatim debljinom (0,60), masom (0,81) i udelom ljuske (0,44), masom belanca (0,92) i žumanca (0,77), te indeksom žumanca (0,23), ali u negativnoj ($p<0,01$) sa udelom žumanca (-0,54), odnosnom žumance / belance (-0,41) i indeksom belanca (-0,25). Kvalitet jaja biserke određen u ekstenzivnom gajenju upućuje na njen dobar potencijal koji bi se mogao unaprediti i koristiti u povoljnijim uslovima gajenja.

Ključne reči: biserka, kvalitet jaja, fenotipska korelacija

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NUTRITIONAL AND ENERGETIC VALUE OF HARD CHEESE

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Abstract: Insufficient intake of dairy product, especially of hard cheese, in Serbia is a nutritional problem of concern. It is caused not only by income but also with low commercial availability of the product and consumer knowledge and preferences. This study assesses nutritional and microbiological parameters of hard cheese made from pasteurized cow milk. Standard chemical analyzes were performed and cheese were analyzed on the 1st, 30th and 60th days of ripening. The following microbiological indicators were monitored: *Listeria monocytogenes*, *coagulase-positive Staphylococci*, *Escherichia coli* and *Enterobacteriaceae*. Furthermore, ripened cheeses were analyzed on amino and fatty acid profile. All cheese samples presented satisfactory microbiological and nutritional characteristics for most of the assessed parameters. Ripened cheese contained on the average 29.08% milk fat, 25.29% proteins, 0.98% lactose and pH value was 5.23. The fat content on dry matter basis (FDM) and moisture in non fat substance (MNFS) were 49.11% and 55.84 %, respectively. The energy value of cheeses amounted to 366.80 kcal /1523.22 kJ. Mean values of fatty acids content (g/100 g) showed that cheese most contained saturated fatty acids, following with monounsaturated and polyunsaturated fatty acids: 66.92%, 30.13% and 2.95%, respectively. The most common essential amino acids were leucine, lysine and isoleucine. This paper confirms that hard cheese is an important source of valuable nutrients and energy and should possess priority in human diet.

Key words: hard cheese, nutritional value, microbiological quality

Introduction

The nature of dairy products has changed dramatically in recent decades, with an increased orientation towards the production of "value added products", some of which are segmented into the "health and wellness" market (*FAO, 2013*).

At a same time, the popularity of cheese is enhanced by its healthy and positive image (*Fox et al., 2000*). Even that, cheese consumption in Serbia is very small and amounts 2.76 kg/per capita/per year (*FAOSTAT, 2013*). Cheese is a rich source of essential nutrients; in particular, proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins and minerals (*Walther et al., 2008*). The role of milk and dairy products in human nutrition has been increasingly debated in recent years. Numerous studies have indicated that cheese consumption has beneficial effects on bone and dental health and is important in osteoporosis prevention (*Pampaloni et al., 2011*; *Sahni et al., 2017*). However, there is a reasonable amount of literature indicating an association between cheese intake and the incidence of some chronic disease such as cancer and cardiovascular disease (CVD), mainly owning to high content of saturated fatty acids (*Rashidinejad et al., 2017*). The relationship between dairy foods intake and various health-related CVDs is controversal and needs further assessment and confirmation (*Bonthuis et al., 2010*; *Chen et al., 2016*; *Miraghajani et al., 2017*).

The nutritional value of food depends mainly on it having the appropriate content of compounds necessary for the proper functioning of the human body. Although a particular food may provide considerable amounts of a particular nutrient, it does not always follow that the nutrient will be available for absorption and utilization. *Pampaloni et al. (2011)* stated that extra hard Parmigiano Reggiano (P-R) cheese can be considered as a "functional food" because it is easy digested with probiotic and prebiotic effect and is recommended in all feeding age groups. Cheese ripening typically involves the progressive breakdown of casein and this process, which is essential for the development of flavor and texture also increases the digestibility of cheese protein to almost 100% (*Fox et al., 2000*). The determination of free amino acids plays an important role in assessing the nutritional quality of foods (*Casella and Contursi, 2003*). If the essential amino acid index of total milk protein is assigned a value of 100, the corresponding value for cheese protein varies from 91 to 97, depending on the variety (*Fox et al., 2000*). Milk fat is highly complex, consisting of a large number of fatty acids and other lipid molecules that have various effects on human health. One portion (50 g) of full-fat cheese provides about two-thirds of the recommended daily intake of fat (*Walther et al., 2008*). Linoleic acid (omega-6) and linolenic (omega-3) acid are called the essential fatty acids and they are what the body uses to construct a variety of substances that are important to the functioning of the cardiovascular, immune, and nervous systems. These essential fatty acids are not produced in the body and must be obtained solely from the diet (*Finnegan and Gray, 1990*). Ripened cheese do not usually contain lactose, and its content in cheese is generally less than 1 g/100 g, with a few exceptions (*FAO, 2013*).

Microorganisms play essentials role in the manufacture and ripening of

cheese. Cheeses are currently considered to be one of the safest foods consumed, however, pathogenic bacteria that can be transmitted by dairy products, including cheese, are important to the dairy industry (*Little et al., 2008*). Pasteurization will eliminate *L. monocytogenes*, but cheeses made from raw milk could be contaminated by milk-borne *L. monocytogenes*. In research *Little et al*. (2008) raw or thermized milk cheeses were of unsatisfactory quality due to levels of *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogenes*, whereas pasteurized milk cheeses were of unsatisfactory quality due to *S. aureus* and *E. coli*. The control of spoilage yeasts and moulds has been traditionally done by chemical additives, but the application of new antifungal protective cultures is very promising, especially for the cheese industry. It has also been recently shown that naturally established cheese microflora can efficiently prevent the growth of pathogenic or spoilage microorganisms (*Grattepanche et al*., *2008*). Analysis of nutrition survey data in research *Paulin et al. (2015)* indicates that hard and semi-hard types of cheese present a lower risk for exposure to *L. monocytogenes* due to low water activity and low pH.

Quality control of dairy products is particularly important for public health and safety. The quality of cheese is influenced by its composition, especially moisture content, NaCl concentration, pH, moisture in nonfat substances (MNFS), and percentage fat in dry matter (FDM) (*Fox et al., 2000*). Considering these aspects, our study focused on investigation of chemical parameters during cheese ripening, including determination of amino and fatty acid profile of mature cheese. The other objective was to evaluate the microbiological safety of hard type cheese during ripening.

Material and methods

Cheese samples were produced from cow pasteurized milk. Samples (n=3) were analyzed on chemical composition on the 1st, 30th and 60th days of ripening. The composition of cheese was determined by standard methods. Total protein of cheese was determined by measuring total nitrogen in the cheeses using the Kjeldahl method. Dry matter was measured by drying the sample to a constant weight. Fat content was determined according to Van Gulik (IDF, 2008) and pH was measured with a pH meter, WTW, Type pH inoLab 720. To determine fatty acids (FA) in cheese samples, gas chromatograph (GC) with FID detectors was used, while for detection of amino acids (AA) in cheese was used HPLC method with fluorescence detection. Total and soluble nitrogen were determined according to Kjeldahl standard method, while the coefficient of maturity was measured by Kjeldahl / Kjeldahl-Van Slyke standard method. The energy value was determined in accordance with *Serbian Regulations (2013)*. Furthermore, the following microbiological indicators were tested and used methods for discovering and

counting *Listeria monocytogenes* (SRPS EN ISO 11290-1:2009) and *coagulase-positive Staphylococci* (SRPS EN ISO 6888-1:2009). *Escherichia coli* (SRPS ISO 16649-2:2009) and *Enterobacteriaceae* (SRPS ISO 21528- 2: 2009) were investigated as hygienic parameters. Data were statistically processed using Microsoft Excel 10 and have been used methods of descriptive statistic. Results were presented as mean ± standard deviation.

Results and discussion

Chemical composition

The results of the chemical analyses of hard-type cheese during ripening are summarized in Table 1.The obtained values presented as the means of three replicates ± standard deviation. During 60 days of ripening total content of fat in dry matter (FDM) increased from 47.04 % to 49.11 % which was supported by the increased of a dry matter content. In opposite, moisture content continually decreased, from 50.32 % to 39.58 %. According to *Serbian Regulations (2014)* the mature cheese in this study was classified as a high-fat hard cheese, since its FDM and moisture content in non fat substance (MNFS) were 49.11% and 55.84 %, respectively. This was in accordance with the results obtained previously from *Popović-Vranješ et al. (2016),* while some others authors found higher values of fat, protein, FDM and pH (Livanjski hard cheese, industrially produced of cow's milk) (*Matić et al.,2014*).

Table 1. Chemical and physical composition of the hard cheese during ripening (*Mean* ± SD)

Samples	Dry matter (%)	Moisture (%)	Protein (%)	Fat (%)	Lactose (%)	FDM (%)	MNFS (%)	pH
Cheese 1st day	49.68 ± 1.73	50.32 ± 1.73	19.29 ± 0.66	23.37 ± 0.95	2.03 ± 0.08	47.04 ± 0.62	65.55 ± 1.52	5.37 ± 0.14
Cheese 30th days	56.31 ± 1.84	43.69 ± 1.84	23.69 ± 1.14	27.11 ± 0.56	1.04 ± 0.16	48.14 ± 2.50	59.94 ± 2.96	5.17 ± 0.06
Cheese 60th days	60.42 ± 0.98	39.58 ± 0.53	25.29 ± 0.56	29.08 ± 1.44	0.98 ± 0.06	49.11 ± 2.45	55.84 ± 1.80	5.23 ± 0.04

FDM - fat in dry matter; MNFS - moisture in non fat solids; SD- standard deviation

In the experiment, pH value changes little during ripening and in the second month of ripening slightly increased up to 5.23. The amino acids released by proteolysis reaction cause a slight increase in pH during cheese ripening (*Waagner-Nielsen., 1993; Fox et al., 2000*). The pH of cheese is influenced by two major factors: how much acid is formed and how much calcium phosphate, or other

buffers, remain accessible in the cheese. The less calcium phosphate remains in the curd leading to less buffering in the cheese and to lower pH. The pH and acid content also play a major role in flavor perception. For example, the pH of 1- day Cheddar cheese may range from 5.3 to 4.9 (*Fox et al., 2004)*. Hard cheese contain small amount of lactose and therefore suitable for the nutrition of lactose-intolerant individuals. In the current study, estimated mean value of lactosa decreased from 2.03% to 0.98%. Contrary, *Pampaloni et al*. (2011) reported total absence of lactose in extra-hard P-R cheese.

Table 2 shows the effects of ripening on total and soluble N, coefficient of ripening and energy value. In monitored samples, coefficient of ripening increased during the cheese ripening from 8.95 to 13.73. Total N increased during first month of ripening and after that slightly decreased (3.80, 4.13 and 4.08%, respectively).The content of soluble N compounds reflect the "width" of ripening (*Fox et al., 2004*). Cheeses are different depending on the production technology and ripening conditions and they also differ from each other due to the extent of proteolysis and other changes that occur during the ripening period. Different content of soluble N in cheese (a widely-used proteolysis index) occurs because of the difference in moisture content, pH value, the ripening duration and curd drying temperature *(Popović-Vranješ et al., 2017*). Regarding to *Fosnerič,* (*1967*) the amount of soluble nitrogen compounds in hard cheese (Cheddar, Emmentaler, etc.) is up to 20-25%.

Table 2. Total and soluble N, coefficient of ripening and energy value of hard cheese during ripening (*Mean* ± SD)

Samples	Total N	Soluble N	Coefficient of ripening	Energy value kcal/KJ
Cheese 1st day	3.80 ± 0.11	0.34 ± 0.04	8.95 ± 1.12	295.61± 10.45/ 1227.13 ± 43.25
Cheese 30th days	4.13 ± 0.05	0.51 ± 0.04	12.35 ± 0.86	342.91 ± 6.26/ 1423.48 ± 31.60
Cheese 60th days	4.08 ± 0.07	0.56 ± 0.05	13.73 ± 1.06	366.80 ± 12.02/ 1523.22 ± 49.90

Hard cheese is a particularly good source of energy. The cheese 60 days old possessed an energy value of 366.80 kcal/ 1523.22 KJ. *Pampaloni et al.* (2011) reported that reduced water content of the P-R cheese (30% approximately) and the presence of as many as 70% nutrients, first of all protein and fat, caused the high energy value, equal to 388 kcal per 100 grams of product. Comparing with others cheese types, approximate energy value for Cheddar is 412 kcal/100g, Gruyere 409

kcal/100g and Cottage cheese only 98/100g kcal *(Fox et al., 2000).*

Fatty acid profile

One of the main factors affecting cheese quality is the fatty acid profile. Based on the results, main determined FA were palmitic ($C_{16:0}$) and oleic acid ($C_{18:1}$) with 27.99 and 25.51 %, respectively, followed by, stearic ($C_{18:0}$) and myristic ($C_{14:0}$) acids, 11.76 and 9.13% respectively. Saturated fatty acid amounted 66.92%, monounsaturated 30.13 % and polyunsaturated 2.95% (Table 3.).

These results support findings from several authors. *Walther et al.* (*2008*) reported that saturated fatty acids accounted of the total fatty acid content in analyzed cheese and the most common saturated FA is palmitic acid (16:0), in second place myristic acid (14:0) and in third place stearic acid (18:0). All other saturated FA are less present. FA composition of cheese fat is roughly proportional to that of the milk used in its production so the FA composition reflects the composition of milk fat, with a ratio of saturated fatty acids and unsaturated 3:1 (*Pampaloni et al., 2011*). Cheese fat (except in mold cheese, fat does not change during ripening), has an average content of 600 g · kg−1 fat of saturated fatty acids (SFA), 235 g · kg−1 fat of monounsaturated fatty acids (MUFA) and 46 g · kg−1 fat of polyunsaturated fatty acids (PUFA) (*Walther et al., 2008*). In addition, *Fox et al.* (*2000*) recommended that 50 g of Cheddar cheese provides 17 g fat, in which approximately 66% of the fatty acids are saturated, 30% are monounsaturated, and 4% are polyunsaturated.

Table 3. Fatty acid profile of hard cheese (60 days old, g/100g)

Parameter	Butyric C4:0	Capric C8:0	Caprinic C10:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Stearic C18:0	Oleic C18:1	Linolec C18:2	Linolenic C18:3	Arachidic C20:0
Mean ± SD	0.94 ± 0.10	1.16 ±0.19	2.96 ± 0.63	2.48 ± 0.18	9.13 ± 0.33	27.99 ±0.35	11.76 ±0.07	25.51 ±0.38	1.80 ± 0.39	0.70 ± 0.39	0.25 ± 0.11
Fatty acid content expressed as % in weight of total fatty acids: SSCFA 2.48 % * SFA 66.92% ; MUFA 30.13 %; PUFA 2.95 % SMCFA 17.20 % SLCFA 47.24 %											

SSCFA- saturated short-chain fatty acids ($C_{4:0}$, $C_{8:0}$)
SMCFA-saturated medium-chain fatty acids ($C_{10:0}$, $C_{12:0}$, $C_{14:0}$)
SLCFA-saturated long-chain fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{20:0}$)
*SFA-(sum) saturated fatty acids
MUFA-monounsaturated fatty acids ($C_{18:1}$)
PUFA-polyunsaturated fatty acids ($C_{18:2}$, $C_{18:3}$)

Amino acids profile

Free amino acid composition of cheese was evaluated in order to determine the quantity and the ratios of particular amino acids which significantly influence

the texture and organoleptic properties of cheese as well as its digestibility and easy assimilation. Among essential amino acids, leucine, lysine, isoleucine and valine, were more concentrated in cheese samples and among non-essential, aspartic and glutamic acid were dominant. Regarding to the ratio of essential in relation to non-essential amino acids, it amounted 44.45 / 55.55 % (Table 4.).

Table 4. Amino acids profile of hard cheese (60days old, g/100g)

Amino acids			
Essential	*Mean* ± SD	Non-essential	*Mean* ± SD
THR	0.885 ± 0.24	ASP	3.593 ± 0.96
VAL	1.897 ± 0.16	GLU	8.323 ± 0.63
MET	1.220 ± 0.28	SER	1.589 ± 0.06
PHE	1.074 ± 0.19	GLY	0.927 ± 0.16
ISO	2.815 ± 0.75	ALA	0.841 ± 0.02
LEU	3.384 ± 0.02	TYR	2.589 ± 1.06
LYS	3.018 ± 0.68		
Ratio of essential / non-essential AA (%)		44.45/55.55	

In hard cheese large part of free amino acids are essential (leucine, valine, isoleucine, lysine) and also the level of non-essential is very high which effectively reduces the metabolic energy expended on biosynthetic reactions. For example, except for metionine+cystine, 50 g of Grana Padano and P-R cheese are enough to meet the daily requirements of the other essential amino acids (*Fox et al., 2004)*. Long-ripened cheese may be differentiated from young cheese by the content of glutamic acid, glycine, serine and threonine, while cheese produced from raw or pasteurized milk can be differentiated by the concentration of asparagine and glutamine (*Frau et al., 1997*). Branched amino acids (leucine, isoleucine, valine) are necessary in the muscle cells to promote protein synthesis and they are metabolized to generate energy in muscles rather than in liver (*Poltronieri et al., 2012*). The amino acid composition is ideal for the absorption due to profound changes that protein fraction undergoes during the long maturation period, which contributes to the separation of the milk casein into compounds of molecular weight smaller and smaller and finally into free amino acids (about 25% of total nitrogen) (*Pampalon*i *et al., 2011*).

Microbiological quality

The results of the microbiological analyses of cheeses during ripening showed that foodborne pathogens *Escherichia coli*, *Listeria monocytogenes, Enterobacteriaceae* and *coagulase-positive Staphylococci*, were not detected in any of the tested samples. This result indicates the good microbiological quality of raw milk, proper milk-handling and manufacturing practices.

In many studies, the microbiological safety of dairy products was considered. *Fox et al.* (*2000*) suggested that in modern factories, where enclosed vats and other equipment is used, the level of contamination from the environment is very low. Most of foodborne outbreaks of *E coli* O157:H7 have been associated with the consumption of foods contaminated with cattle feces and enterohemorrhagic *Escherichia coli* 0157:H7 is relatively an acid tolerant microorganism (*ICMSF, 2006*). *Enterobacteriaceae* and coliforms, microorganisms in many cases showed postpasteurization contamination of the cheese from the environment. The impact of factors on the fate of pathogens during cheese manufacture varies significantly between cheesemaking processes and cheese types. Compliance with microbiological criteria at the end of cheese production (final product) doesn't guarantee that microbiological hazards are excluded. *Listeria monocytogenes* is a ubiquitous bacteria and secondary contamination of products is possible under poor hygienic conditions. Despite the fact that the growth of the pathogen is limited in semi-hard and hard cheeses by low water activity, the secondary (surface) contamination could result in hazardous products (*Vrdoljak et al., 2016*).

Conclusion

Results of this study showed that investigated hard cheese belonged to the group of hard, full fat cheese and could provide a wide range of essential nutrients to the diet. Particularly, it is a good source of high-quality protein, amino acids, fat, fatty acids and energy essential for growth and maintenance of various body functions. In addition, cheese is very suitable source of protein for people who are not eat meat and considered to be one of the main food groups important in a healthy balanced diet. The unique nutrients and important bioactive compounds of hard cheese make it a product of added value. The greatest impact on the quality of the final product had variables connected to microbiological quality. In our investigation, microbiological safety is achieved by focusing on the prevention, adhering to the good hygiene practice and due to good control of ripening conditions.

Nutritivna i energetska vrednost tvrdog sira

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Rezime

Nedovoljna konzumacija mlečnih proizvoda, posebno tvrdog sira, u Srbiji predstavlja nutritivni razlog za zabrinutost. Osim slabe kupovne moći, ovo je prouzrokovano i lošom komercijalnom dostupnošću proizvoda ali i znanjem i afinitetom potrošača. Osnovni cilj ovog istraživanja je bio da se determinišu nutritivni i mikrobiološki parametri tvrdog sira proizvedenog od pasterizovanog mleka. Standardnim hemijskim analizima sir je ispitivan prvog, 30-og i 60-og dana zrenja. Mikrobiološki parametri koji su utvrdjivani su: *Listeria monocytogenes,* koagulaza pozitivnih *Staphylococci, Escherichia coli* i *Enterobacteriaceae*. Osim toga, utvrdjivan je profil amino i masnih kiselina kod uzoraka sira nakon 60 dana zrenja. Rezultati su pokazali da su svi ispitivani uzorci pokazali zadovoljavajuće mikrobiološke i nutritivne karakteristike za većinu ocenjivanih parametara. Zreo sir je raspolagao sa prosečno 29.08% mlečne masti, 25.29% proteina, 0.98% laktoze i pH vrednošću od 5,23. Udeo mlečne masti u suvoj materiji sira i sadržaj vode u bezmasnoj materiji sira su posedovali prosečne vrednosti od 49.11% i 55.84%, pojedinačno. Energetska vrednost je iznosila 366.80 kcal / 1523 KJ. Srednje vrednosti sadržaja masnih kiselina su pokazale da je tvrdi sir raspolagao najviše sa zasićenim masnim kiselinama, zatim mononezasićenim i najmanje polinezasićenim sa pojedinačnim udelima od: 66.92%, 30.13% i 2.95%. U pogledu esencijalnih aminokiselina sir je najviše raspolagao sa leucinom, lizinom i izoleucinom. Ovim istraživanjem potvrdjena je činjenica da tvrdi sir predstavlja bogat izvor hranljivh sastojaka i energije i treba da poseduje prioritet u ljudskoj ishrani.

Ključne reči: tvrdi sir, nutritivna vrednost, mikrobiološki kvalitet

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APPLICATION OF PLANT PROTEOLYTIC ENZYMES FOR TENDERIZATION OF RABBIT MEAT

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Abstract: The purpose of this study is to assess the tenderizing effect of plant proteolytic enzymes upon raw rabbit meat. Tests are performed on rabbit meat samples treated with papain and two vegetal sources of natural proteases (extracts of kiwifruit and ginger root). Two variants of marinade solutions are prepared from each vegetable raw materials– 50% (w/w) and 100 % (w/w), with a duration of processing 2h, 24h, and 48h. Changes in the following physico-chemical characteristics of meat have been observed: pH, water-holding capacity, cooking losses and quantity of free amino acids. Differences in values of these characteristics have been observed, both between control and test samples, as well as depending of treatment duration. For meat samples marinated with papain and ginger extracts, the water-holding capacity reached to 6.74 ± 0.04 % (papain), 5.58 ± 0.09 % (variant 1) and 6.80 ± 0.11 % (variant 2) after 48 hours treatment. In rabbit meat marinated with kiwifruit extracts, a significant increase in WHC was observed at 48 hours, 3.37 ± 0.07 (variant 3) and 6.84 ± 0.11 (variant 4).
The test samples also have reduced cooking losses compared to control samples. In control samples, cooking loss is increased from 13.79% (2 h) to 20.78 % (48 h). SDS-PAGE of meat samples after 48 h of treatment shows a reduction in the intensity of actin and myosin bands in all variants with papain and vegetal extracts. Electrophoretic pattern of test samples depicts proteolysis and degradation of muscle proteins.

Key words: tenderization, rabbit meat, papain, kiwifruit, ginger root

Introduction

Quality of meat is determined as a combination of sensory and technological characteristics like tenderness, color, water-holding capacity and texture (*Istrati et al., 2014).*

Tenderness has been identified as the most important factor affecting consumer satisfaction and perception of taste *(Naveena et al., 2004).*

Toughness of meat depends on the amount of intramuscular connective tissue, the length of sarcomere, and also the activity of endogenous proteolytic enzymes. There are two different components to meat toughness: actomyosin toughness and background toughness. Actomyosin toughness is attributed to myofibrillar proteins, whereas background toughness is due to connective tissue presence *(Chen et al., 2006)*. Reduction of meat toughness during maturing "post mortem" or by additional treatment is a process characterized by changes in the actomyosin complex and connective tissue and is defined as tenderization (*Bekhit et al., 2012; Rawdkuen and Benjakul, 2012; Kemp and Parr, 2012).*

Proteolytic enzymes like papain, bromelain, ficain, zingibain, actinidain, etc., derived from plants are widely used in most parts of the world as tenderizers for meat. The most frequently used vegetal sources of proteolytic enzymes are papaya, ginger, pineapple, kiwi *(Ahlawat et al., 2008; Naveena and Mendiratta, 2001; Liu and Hongjun, 2001).*

These natural proteases have the potential to reduce toughness of meat so it may acquire the desired organoleptic characteristics (*Naveena and Anjaneyulu, 2004).*

Natural products containing proteolytic enzymes are defined as natural tenderizers. It has been proven that such natural products may be used for processing meat.

Juices or extracts of numerous fruits, vegetables or vegetal materials containing proteolytic enzymes may be successfully applied as marinade for various types of meat and meat products. In this case marination is a process of soaking in or injecting in meat a proteases-containing solution for achieving improved quality of the meat *(Maiti et al., 2008).*

The purpose of this study is to investigate, evaluate and compare the tenderizing effect of natural vegetal proteases upon raw rabbit meat.

Materials and Methods

Materials

Meat – rabbit (*Oryctolagus cuniculus*) *biceps femoris* muscle, from the local market.

Enzyme preparations – papain (Merck)

Vegetal material: Kiwifruit (*Actinidia deliciosa)*, ginger root (*Zingiber officinale*) from the local market.

Methods

Enzyme solutions marinade type

The two vegetal sources of natural proteases (kiwifruit and ginger roots) are pilled, cut and homogenized for 1-2 minutes. This homogenate is filtered through four layers of lint. The following solutions are prepared: *variant 1* (50% w/w) and

variant 2 (100 %w/w) from ginger and *variant 3* (50%w/w) and *variant 4* (100 % w/w) from kiwifruit. Additionally, a papain solution with 50 U/ml caseinolytic activity is prepared. Distilled water is used for the control.

Enzyme activity

Caseinolytic activity of proteolytic enzymes is determined using casein as substrate in 50 mM Tris / HCl buffer at pH 7.0 with 1 mM $CaCl_2$, as per the Chen et al. (2003) method. One unit of enzyme activity is defined as the quantity of enzymes needed to release 1μg tyrosine from casein for 1 min. (*Chen et al., 2003).*

Marination of rabbit meat samples

Pieces of rabbit meat, about 3x3x3 cm in dimension, are weighted and then soaked in already prepared marinade solutions with different proteolytic activity. After stirring, the samples are placed in plastic containers and kept refrigerated at 4°C for 2, 24 and 48 hours. Besides the test variants, controls are prepared for the three different durations of processing.

pH measurement

10 g meat samples are being weighted from every variant and then homogenized with 50 ml of cold distilled water. pH values are measured using pH – meter (Jenway 3310).

Determining water-holding capacity (WHC)

Meat samples with weight of 3-5 g are wiped from surface water using filter paper and weighted. This value is marked initial weight. Samples are treated with the corresponding marinade solutions for 2, 24 and 48 hours and then surface water is removed using filter paper. Besides the test variants, controls are prepared for the three different durations of processing. Processed meat is weighted and this value shows the weight after enzyme treatment (final weight). Percentage of water holding capacity is calculated with the following formula:

WHC% = 100 x (final weight – initial weight) / final weight

Cooking loss

Samples weight is measured before and after thermal processing in order to determine cooking losses defined as weight of every sample subtracted from initial weight, then divided by initial weight and multiplied by 100. The parameter "cooking loss" is calculated in percent.

Concentration of free amino acids in soluble fractions post enzyme hydrolysis is determined by ninhydrin test *(Murariu et al., 2003).*

Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is performed using the Laemmli method (1970).

Polyacrylamide gel - 6% stacking gel and 10 % separating gel. Electrolyte buffer: Tris – glycine, pH 8.5 with 0.1 % SDS. Electrophoresis is performed at 25 mA current. Gel is colored using 0.1% Coomassie blue (30-40 min), and zones in gel with no protein bands are discolored for 24 h. (*Laemmli, 1970).* Bovine serum albumin (BSA) and LMW protein marker was purchased from SERVA.

Statistical analysis - All experiments were conducted in five replications. The data are presented as means ±SD (standard deviation). Statistical analysis was performed using two-sample t-test. The results are considered to be significant when $P<0.05$. All statistical analyses were performed using Microsoft Excel 2013.

Results and Discussion

Rabbit meat samples are treated with papain and vegetal extracts from ginger and kiwi. A change has been observed in the following physical and chemical characteristics of meat during tenderization: pH, water-holding capacity, loss in cooking and quantity of free amino acids. Tables 1, 2 and 3 list the physical and chemical characteristics of treated rabbit meat.

Table 1. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 2 hours

Properties	Control	Papain	Variant 1 ginger 50%	Variant 2 ginger 100%	Variant 3 kiwi 50%	Variant 4 kiwi 100%
pH	6.22±0.02	6.21±0.02	6.23±0.02	6.19±0.05	5.83±0.01 ***	5.50±0.01 ***
WHC %	3.11±0.03	3.91±0.04 ***	3.07±0.02 *	3.42±0.09 ***	0.99±0.04 ***	1.45±0.06 ***
Cooking loss %	13.79±0.21	35.18±0.56 ***	25.65±0.65 ***	27.52±0.88 ***	29.25±0.37 ***	28.40±1.29 ***
Free amino acids mg/ml	0.566±0.033	0.906±0.01 ***	0.752±0.053 ***	0.791±0.005 ***	0.822±0.018 ***	0.804±0.015 ***

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

Table 2. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 24 hours

Properties	Control	Papain	Variant 1 ginger 50%	Variant 2 ginger 100%	Variant 3 kiwi 50%	Variant 4 kiwi 100%
pH	6.22±0.05	6.17±0.01	6.18±0.02	6.13±0.03	5.64±0.03 ***	5.42±0.02 ***
WHC %	3.30±0.10	6.38±0.04 ***	4.95±0.04 ***	5.53±0.06 ***	1.37±0.03 ***	2.31±0.01 ***
Cooking loss %	14.27±0.25	23.89±0.67 ***	22.28±0.68 ***	25.25±0.50 ***	20.68±0.86 ***	22.30±0.89 ***
Free amino acids mg/ml	0.641±0.003	1.315±0.070 ***	0.988±0.074 **	1.119±0.033 ***	1.043±0.019 **	1.070±0.083 ***

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

Table 3. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 48 hours.

Properties	Control	Papain	Variant 1 ginger 50%	Variant 2 ginger 100%	Variant 3 kiwi 50%	Variant 4 kiwi 100%
pH	6.22±0.02	6.17±0.01 **	6.13±0.07	6.11±0.04 ***	5.63±0.02 ***	5.37±0.02 ***
WHC %	5.91±0.08	6.74±0.04 ***	5.58±0.09 ***	6.80±0.11 ***	3.37±0.07 ***	6.84±0.11 ***
Cooking loss %	20.78±0.98	22.28±0.74 *	18.70±0.58 ***	23.76±0.50 *	20.23±0.30 ***	20.16±0.52 *
Free amino acids mg/ml	0.631±0.008	1.672±0.007 ***	1.297±0.015 ***	1.344±0.023 ***	1.182±0.029 ***	1.283±0.096 ***

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

One of the main properties determining the quality of meat is water-holding capacity – its ability to retain inherent water. Other factors like juiciness, aroma and color are directly related with water-holding capacity (*Gokuglu et al., 2017*).

The percentage of water-holding capacity increases in rabbit meat samples treated with papain and vegetal extracts, when duration of treatment is increased. In the samples marinated with papain and ginger, this effect was observed at 2 h of treatment and the tendency was maintained within 48 hours reaching to 6.74 ± 0.04 (papain), 5.58 ± 0.09 (variant 1) and 6.80 ± 0.11 (variant 2). In rabbit meat marinated with kiwifruit, a significant increase in WHC % was observed at 48 hours - 3.37 ± 0.07 (variant 3) and 6.84 ± 0.11 (variant 4).

Data about effect of enzyme tenderization upon meat WHC is contradictory. Some authors establish a significant increase of this indicator after 48-hour treatment of buffalo meat using ginger homogenate (*Naveena et al 2004). Gokuglu et al. (2017*) report about insignificant increase in water-holding capacity in tenderizing squid (*Loligo vulgaris*) muscle using bromelain and papain solutions. Other authors report significant reduction of this indicator when tenderizing various types of meat using bromelain extracts (*Ketnawa and Rawdkuen, 2011).*

Higher WHC percentage in test samples treated for 48 hours might be due to increase of protein-reactive groups that are available for bonding with water after partial enzymatic hydrolysis. These samples (after 48 h processing) also have reduced cooking losses compared to control. In control samples cooking loss is increased from 13.79% (2 h) to 20.78 % (48 h). On the other hand, some researchers have found higher cooking loss in meat samples treated with higher protease dosage. *Zhang et al. (2017)* investigates the tendering effects of actinidin and the commercial papain preparation using rabbit muscle. They consider that higher cooking loss is due to more extensive rupture of the muscle tissue at higher dosage.

In variants 3 and 4 a lower WHC percentage is observed compared with control and samples treated with papain and variants 1 and 2. This might be due to lower pH, which results in reduction of protein-reactive groups available for bonding with water.

A test for determining free amino acids in reaction solutions was performed in order to identify whether and to what extent enzyme solutions in selected concentration provoke protein hydrolysis to amino acids. Obtained results are listed in tables 1, 2 and 3.

Statistically significant differences in concentration of free amino acids are established between control and test variants. Similar results were noted in our previous study of tenderization of buffalo meat using papain and bromelain (*Doneva et al., 2016)*.

In test variants similar content of free amino acids in reaction solution after enzyme hydrolysis is observed. Highest content of free amino acids is observed in samples treated with papain. Also, this indicator increases when duration of meat samples treatment is increased. More intense hydrolysis of meat proteins leads to worsening both the appearance and taste qualities of meat.

The color of meat is of great importance as this is the first qualitative freshness indicator for customers. In images presented on figure 1 and 2 it is visible that meat samples treated with ginger extracts preserve their color and fresh appearance, and being thermally processed they have preserved their juiciness compared to control samples. In samples treated with papain there is deformation, meat color has faded and its surface becomes slimy.

The plant proteolytic enzyme papain is probably the most effective tenderizing agent with the highest rate of tenderization. However, papain has a tendency to over-tenderize the meat surface since it has broad specificity and indiscriminately decompose connective tissue and myofibrillar proteins, worsens the quality of the meat (*Ashie et al., 2002)*. The vegetal extracts from ginger and kiwifruit can be used as alternatives to papain for tenderization of rabbit meat.

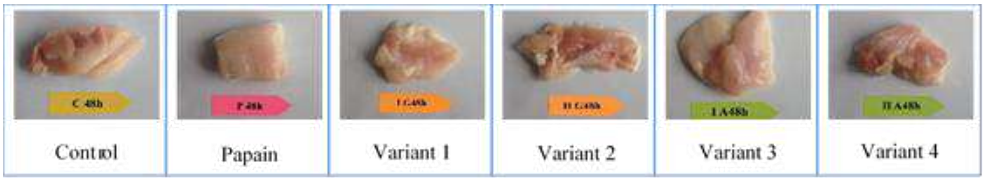

Figure 1. Control and test variants treated with vegetal juices marinade type

Figure 2. Control and test variants treated with vegetal juices marinade type after thermal processing

Figure 3 shows the result from electrophoresis of meat samples after treatment with solutions of papain and vegetal extracts for 2 and 48 hours. SDS-PAGE analysis is used for assessment of the effect of enzyme treatment upon electrophoretic profile of rabbit meat. SDS-PAGE specifies two main bands with molecular mass corresponding to contractile protein – actin and myosin. Actin and myosin are the two predominant proteins in muscles. Proteolysis of these meat proteins plays a significant part in tenderization of meat (*Kanat, et al., 2015).*

The improved meat tenderization with vegetable cysteine proteases is due to the higher breakdown of myofibril proteins and the disruption of the muscular fibril structure in the experimental samples compared to the control ones *(Jorgova et al.,1989).*

Myosin heavy chain has a molecular weight of about 200 kDa. Actin is a globular protein with molecular weight of about 44 kDa. In all variants of samples treated for 48 hours a reduction in the intensity of actin and myosin bands is observed. Intensity reduction of these two bands is more expressed when using papain solutions and ginger extracts. Also, in these samples there is an increase in meat proteins fragmentation.

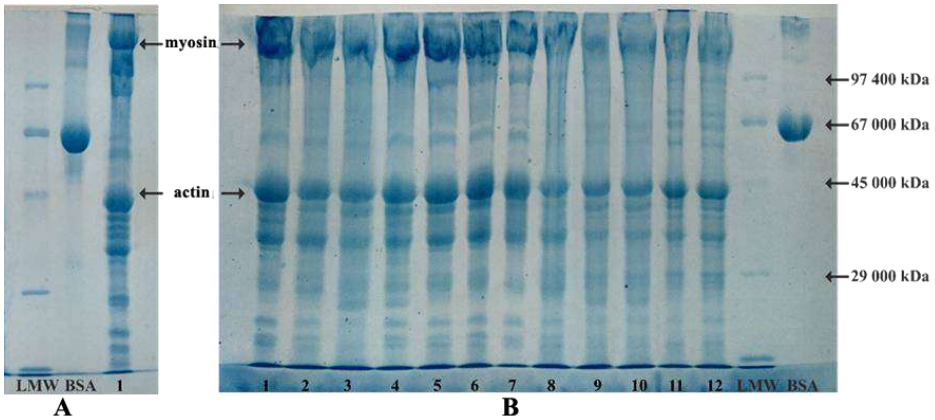

Figure 3. A - LMW - a protein marker; BSA - standard - bovine serum albumin, 1-control rabbit meat
B – (1 to 6 - samples treated for 2 hours): 1-control, 2-papain, 3-variant 1, 4-variant 2, 5-variant 3, 6- variant 4; (7 to 12 - samples treated for 48 hours): 7 - control, 8-papain, 9- variant 1, 10 - variant 2, 11- variant 3, 12- variant 4

Conclusion

The effect of tenderizing enzymes upon the appearance of meat is a primary factor for choosing enzymes and methods of treatment. Preservation of juicy and fresh appearance is an important indicator for customers. Treatment of

rabbit meat with papain and kiwi fruit and ginger root extracts has a significant effect upon the physico-chemical characteristics of meat: pH, water-holding capacity, cooking loss and quantity of free amino acids. Differences in values of these characteristics have been established both between control and test variants and between treatment duration. In raw rabbit meat samples treated with papain and ginger and kiwi homogenates, the percentage of water-holding capacity increases when duration of treatment is increased. Statistically significant differences in concentration of free amino acids between control and test samples have been observed, as the highest values are in samples tenderized with papain.

Electrophoretic profile of control and tenderized meat samples shows reduction in intensity of actin and myosin bands in all variants treated with papain and vegetal extracts. Tests results show that ginger and kiwi extracts may be successfully used for enzyme tenderization of rabbit meat.

Primena biljnih proteolitičkih enzima u tenderizaciji mesa zeca

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Rezime

Cilj ove studije je bio procena efekta biljnih proteolitičkih enzima na sirovo meso zeca. Ispitivanje je urađeno na uzorcima mesa zeca tretiranim papainom i biljnim prirodnim proteazama (ekstrakti kivija i korena đumbira). Dve varijante rastvora za mariniranje su bile pripremljene od svake biljne sirovine - 50% (w/w) i 100% w/w), sa trajanjem tretmana od 2h, 24h i 48h. Uočene su promene sledećih fizičko-hemijskih karakteristika mesa: pH, kapacitet zadržavanja vode, kalo kuvanja i količina slobodnih aminokiselina. Razlike u vrednostima ovih karakteristika su zabeležene, kako između kontrolnih i ispitnih uzoraka, tako i zavisno od trajanja terapije. Uzorci mesa marinirani ekstraktima papaine i đumbira, kapacitet zadržavanja vode dostigao je 6.74 ± 0.04% (papain), 5.58 ± 0.09% (varijanta 1) i 6.80 ± 0.11% (varijanta 2) nakon 48 sati tretmana. Kod mesa zeca mariniranog ekstraktima kivija, značajno povećanje kapaciteta zadržavanje vode je primećeno nakon 48 sati, 3.37 ± 0.07 (varijanta 3) i 6.84 ± 0,11 (varijanta 4).
Uzorci korišćeni za testiranje su takođe pokazali manji kalo kuvanja u poređenju sa kontrolnim uzorcima. U kontrolnim uzorcima, kalo kuvanja se povećao sa 13.79% (2 sata) na 20.78% (48 sati). SDS-PAGE uzoraka mesa posle 48 h tretmana pokazuje smanjenje intenziteta aktina i miozinskih opsega u svim varijantama sa

papain i biljnim ekstraktima. Elektroforetski obrazac uzoraka prikazuje proteolizu i degradaciju mišićnih proteina.

Ključne reči: tenderizacija, meso zeca, papain, kivi, koren đumbira

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TOXIGENIC FUNGAL AND MYCOTOXIN CONTAMINATION OF MAIZE SAMPLES FROM DIFFERENT DISTRICTS IN SERBIA

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Original scientific paper

Abstract: This study was carried out in order to investigate the natural occurrence of toxigenic fungi and levels of zearalenone (ZEA), deoxynivalenol (DON) and aflatoxin B_1 (AFB_1) in the maize stored immediately after harvesting in 2016 and used for animal feed in Serbia. A total of 22 maize samples were collected from four different districts across the country: City of Belgrade (nine samples), Šumadija (eight samples), Podunavlje (four samples) and Kolubara (one sample). Toxigenic fungi were identified according to the morphological characteristics whereas the mycotoxins contamination were detected using biochemistry enzyme-linked immuno-sorbent (ELISA) assay. The tested samples were mostly infected with *Aspergillus, Fusarium* and *Penicillium* spp., except that one sample originated from Kolubara was not contaminated with *Aspergillus* species. *Fusarium graminearum* was the most common species in the maize sample from Kolubara district (60%), *F. verticillioides* in the maize samples from Podunavlje (43.75%) and City of Belgrade (22.4%) districts, and *Penicillium* spp. in the maize samples from Šumadija district (26.38%). In the analysed maize samples the presence of *Aspergillus* species was low (0-1.78%). Mycotoxicological analysis revealed the presence of zearalenone (ZEA), deoxynivalenol (DON) and aflatoxin B_1 (AFB_1) in all the investigated samples, except that DON and AFB_1 were not recorded in the samples from Podunavlje and Kolubara districts, respectively. The investigated samples were highly contaminated with ZEA, with incidence of 100% for the samples from Šumadija, Podunavlje and Kolubara districts and 88.89% for the samples from City of Belgrade district. In addition, the samples contamination with DON was 100% and 22.2% for the samples from Šumadija, Kolubara and City of Belgrade, districts, respectively. The highest number of AFB_1 positive samples was found in Šumadija district (87.5%), while in the City of Belgrade and Podunavlje districts, 55.56% and 50% AFB_1 positive

samples were established, respectively. Generally, remarkable infection of all the tested samples with toxigenic fungal species from *Aspergillus*, *Fusarium* and *Penicillium* genera were recorded. In addition, high contamination with mycotoxins ZEA, DON and AFB_1 were also recorded; nevertheless, only in one sample the level of DON exceeded the allowed legal limit (1750 μg kg^{-1}) according to Regulation for unprocessed maize. Therefore, permanent mycological and mycotoxicological analyses of maize grain are necessary for risk assessment of fungal and mycotoxin contamination throughout the food chain.

Key words: maize, toxigenic fungi, mycotoxins

Introduction

Maize as the most important crop in diet for humans and animals is grown on an area of about 1.03 million hectares in Serbia (*Statistical Yearbook of Serbia, 2017*). Maize grain as a rich source of nutrients represents a very good substrate for the development of toxic fungi (moulds) from the genera *Aspergillus, Fusarium* and *Penicillium*. These moulds are producers of secondary metabolites (mycotoxins). The most commonly detected mycotoxins in maize grain are fumonisins, deoxynivalenol (DON), zearalenone (ZEA), and aflatoxins (*Covarelli et al., 2011*). Among aflatoxins, aflatoxin B_1 (AFB_1) is a potent hepatotoxin and carcinogen that is a common contaminant of cereals and feeds. Aflatoxin M_1 (AFM_1) is a 4-hydroxylated metabolite of AFB_1, which is excreted into milk through diet for dairy cows and represents a potential human carcinogen (*Britzi et al., 2013*). Zearalenone (ZEA) and deoxynivalenol (DON) are produced mainly by *F. graminearum*, and aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus* and *A. parasiticus (Nuryono et al., 2005; Zain, 2011*). The harmful effects of aflatoxins, ZEA and trichothecenes on human and animal health are globally known (*Khatoon et al., 2012*).

Temperature, humidity and light are the key factors for *Fusarium* infection (*Doohan et al., 2003*), while the main factor for *Aspergillus* infection is the presence of primary inoculum at the time of maize ripening (*Tédihou et al., 2012*). In addition to field grain infestation, the development of these toxic species can be continued even during the storage period. Frequent adverse abiotic (high humidity and temperature) and biotic factors, including microorganisms, insects, mites, rodents and birds, can greatly contribute to increase contamination of maize grain with moulds and mycotoxins during storage conditions (*Santin et al., 2005).*

The occurrence of moulds and their mycotoxins in food is unpredictable and therefore can sometimes lead to adverse effects (mycotoxicoses) when consuming mouldy food (*Nugmanov et al., 2018*). Mycotoxicoses can be with acute and chronic symptoms. Farm animals, such as cattle, sheep, pigs and poultry

are very sensitive to increased mycotoxin concentrations in food. Intoxication with ZEA, DON and AFB_1 leads to disorders of reproductive functions and functions in the gastrointestinal tract in animals (*Biagi, 2009; Liew and Mohd-Redzwan, 2018*).

Due to the inevitable fungal and mycotoxin contamination of maize grain, it is necessary to propose preventive measures in the field in order to increase food safety. The application of maize hybrids less sensitive to fungal infection is one of the ways to reduce mycotoxin level in grain (*Iglesias et al., 2010*).

The aim of this research was to establish the presence of toxigenic fungal species and the level of some mycotoxins (ZEA, DON and AFB_1) in maize grain samples which were used for animal feed and to assess the risk of possible harmful effects of these contaminants in four districts of Serbia.

Materials and Methods

A total of 22 maize samples were collected from the maize stored immediately after harvesting, during November and December in 2016, from four different districts of Serbia, City of Belgrade (nine samples), Šumadija (eight samples), Podunavlje (four samples) and Kolubara (one sample). Most maize samples (eight samples from the City of Belgrade, four samples from Šumadija and Podunavlje districts, each, and one maize sample from Kolubara district) were collected from ventilated maize cribs, while fewer samples (one sample from City of Belgrade and four samples from Šumadija district) were collected from closed concrete warehouses in which the temperature and relative humidity conditions are not controlled. In both types of maize warehouses, maize was dried naturally. Maize grains were harvested manually from cob samples which were collected from maize cribs. The samples of maize grains of about 1 kg were stored in the paper bags in a refrigerator at 4°C prior to fungal and mycotoxin analyses.

Mycological analyses of maize grain samples were conducted according to the previously described methods by *Krnjaja et al. (2015*). Based on morphological properties (colony and spore appearance), toxigenic species have been identified according to fungal keys of *Burgess et al. (1994)* and *Singh et al. (1991).* The incidence of toxigenic species was calculated per sample according to *Lević et al. (2012).*

In order to determine the moisture content and the level of mycotoxins, the tested maize samples were first ground in an analytical mill (IKA A11, Germany). The moisture content was determined in laboratory conditions using a moisture analyser (OHAUS MB35, USA). Prior to the mycotoxicological analysis, maize samples were dried at 60°C for 72h and then the mycotoxin level was determined by a competitive ELISA method. The ELISA assay was done according to the manufacturer's instructions Celer Tecna® ELISA kits, at a wavelength of 450 nm.

The limit of detection for ZEA, DON and AFB_1 were 10 μg kg^{-1}, 40 μg kg^{-1} and 1 μg kg^{-1}, respectively.

The Pearson correlation coefficients between investigated variables (moisture content, incidence of toxigenic fungal species and the level of mycotoxins) were done in Excel 2010.

Results

The average moisture content of tested maize samples from City of Belgrade, Šumadija, Podunavlje and Kolubara districts were 13.57%, 15.45%, 14.35% and 15.38%, respectively.

Mycological analyses confirmed the presence of *Aspergillus*, *Fusarium* and *Penicillium* spp. in the maize grain samples from all investigated districts, except in the maize grain sample from Kolubara district, in which *Aspergillus* species were not identified. Considering the average values, the most frequent species were *F. graminearum* in the maize grain sample from Kolubara district (60%) and *F. verticillioides* (43.75%) in the samples of maize grain from the Podunavlje district. *Penicillium* species were most prevalent in maize grain samples from Šumadija district (26.38%). *Aspergillus* species were present from 0 to 1.78% in the tested maize grain samples. Among the identified *Aspergillus* species, the species *A. flavus* was the most prevalent in maize samples from the City of Belgrade district (1.78%), while *A. niger* was most prevalent in samples of maize grain from the Podunavlje district (1%) and *A. parasiticus* was equally represented in maize samples from all districts (0.22-0.25%) except for Kolubara district (0%) (Table 1).

Table 1. Average incidence (%) of potentially toxigenic fungal species in tested maize samples from four Serbian districts

	Districts			
Fungal species	City of Belgrade	Šumadija	Podunavlje	Kolubara
A. flavus	1.78	1.50	0.5	0
A. niger	0.44	0	1	0
A. parasiticus	0.22	0.25	0.25	0
F. graminearum	12.33	10.13	8.50	60
F. proliferatum	0.33	0.38	0	0
F. verticillioides	22.4	12.38	43.75	5
F. subglutinans	1.11	1.75	0	2
Penicillium spp.	9	26.38	1.50	1

In mycotoxicological analyses, a high percentage of ZEA positive samples of maize grain originating from all districts was established (88.89-100%). DON was detected in 100% of the samples of maize grain from Šumadija and Kolubara

districts and in 22.2% of the samples of maize grain from the City of Belgrade district. AFB_1 was detected in 50, 55.6, and 87.5% of maize grain samples from Podunavlje, City of Belgrade and Šumadija districts, respectively (Table 2).

In the tested samples of maize grain, the mean level of ZEA was from 16.82 (Podunavlje district) to 26.97 μg kg^{-1} (Šumadija district), DON from 445 (City of Belgrade district) to 1977 μg kg^{-1} (Kolubara district) and AFB_1 of 1.3 (Podunavlje district) to 1.39 μg kg^{-1} (City of Belgrade district). DON and AFB_1 were not detected in maize grain samples from Podunavlje and Kolubara districts. In all tested samples, the levels of ZEA, DON, and AFB_1 were not above the allowed limits of 350, 1750, and 5 μg kg^{-1}, respectively, adopted by the European Commission (EC, 2007, 2010), except for DON level in maize grain sample from Kolubara district (1977 μg kg^{-1}) (Table 3).

Table 2. Incidence (%) of mycotoxin positive maize samples from four Serbian districts

	Districts			
Mycotoxin	City of Belgrade	Šumadija	Podunavlje	Kolubara
ZEA	88.89	100	100	100
DON	22.2	100	0	100
AFB_1	55.56	87.5	50	0

Table 3. Mean mycotoxin levels (μg kg^{-1}) in positive maize samples from four Serbian districts

	Districts			
Mycotoxin	City of Belgrade	Šumadija	Podunavlje	Kolubara
ZEA	20.46	26.97	16.82	17.56
DON	445	998.38	ND*	1977
AFB_1	1.39	1.36	1.3	ND*

*ND – non detectable

Examination of the correlation ratios considered for a total of 22 tested maize samples showed positive correlations between moisture content and incidence of *F. graminearum* (r=0.28), *F. proliferatum* (r=0.12) and *Penicillium* spp. (r=0.58) and levels of ZEA (r=0.56), DON (r=0.49) and AFB_1 (r=0.16). Also, a positive correlation was established between the incidence of *F. graminearum* and DON (r=0.13) and between the incidence of *A. parasiticus* and AFB_1 (r=0.52). Likewise, positive correlations were established between the levels of ZEA and DON (r=0.52) and the level of DON and AFB_1 (r=0.42). Negative correlations were found between moisture content and incidence of *A. flavus* (r=-0.01), *A. parasiticus* (r=-0.16), *A. niger* (r=-0.01), *F. verticillioides* (r=-0.11) and *F subglutinans* (r=-0.09) and between the incidence of *A. flavus* and AFB_1 (r=-0.20) and incidence of *F. graminearum* and ZEA (r=-0.09).

Discussion

In the present study, the presence of toxigenic fungal species from *Aspergillus, Fusarium* and *Penicillium* genera and mycotoxins such as ZEA, DON and AFB_1 was confirmed in most of the maize samples originated from four Serbian districts. In all investigated districts, among *Fusarium* species, *F. verticillioides* was the most prevalent, except in maize sample from Kolubara district where the most dominant species was *F. graminearum* (Table 1). Similarly, in Argentina, in mycological studies including 52 maize samples, *Pacin et al. (2001)* have found that in all examined departments *F. verticillioides* was the prevalent toxigenic species, whereas the incidence of *F. graminearum* was low. Furthermore, in the biennial (2006-2007) mycological studies of maize grain samples originating from different locations in central Italy *Covarelli et al. (2011)* have established the dominance of the species *F. verticillioides* in relation to other *Fusarium* species. Likewise, among *Fusarium* spp., *Czembor et al. (2015)* isolated *F. verticillioides* as commonly presented, with a mean incidence of 16.19% in 30 maize grains samples from three locations in 2011 and in seven locations in 2012, in Poland, while *F. graminearum* was isolated in maize grain samples in only one location (3.57%).

In the present study, *F. graminearum* highly infected the maize sample from Kolubara district with incidence of 60%, while relatively high incidence were recorded (8.5-12.33%) in the samples from other investigated districts. These results may be explained with high average values of grain moisture content (13.57-15.45%). Moisture content is consider as one of the most important physiological factors for successful and safe storage of maize. The recommended moisture content for the safe maize storage is around 13% and below (*Alptekin et al., 2009*). The higher values of moisture content promote the favourable condition for development and proliferation of fungi and the appearance of insects leading to the storage problems (*Weinberg et al., 2007*). Also, *Logrieco et al. (2002)* reported that in some geographic regions the incidence of *F. graminearum* varies considerably from the investigated years and locality, which is highly connected with abiotic and biotic conditions.

Among toxigenic fungal genera, *Covarelli et al. (2011)* found out the dominance of *Fusarium* species in the tested maize samples, followed by *Aspergillus* species of *Flavi* and *Nigri* sections and *Penicillium* spp., which is similar to our findings. In contrast, *Alptekin et al. (2009)* demonstrated a significantly higher incidence of *Penicillium* spp. in the maize samples collected from various counties in Turkey during the 2005-2006 growing season, relative to the species from *Fusarium* and *Aspergillus* genera.

Incidence of positive maize samples for all investigated mycotoxins was relatively high (50-100%) in all examined districts, except for DON in maize

samples from City of Belgrade district (22.2%) and Podunavlje district (0%) and for AFB_1 in maize samples from Kolubara district (0%). Mean levels of ZEA, DON and AFB_1 were not above the allowed limits of 350, 1750 and 5 μg kg^{-1}, respectively, prescribed by the European Commission (EC, 2007, 2010) for unprocessed maize, except for DON level in maize sample from Kolubara district (1977 μg kg^{-1}). Similar results were reported by *Covarelli et al. (2011)* in Italy, with DON and AFB_1 levels in some samples of maize grain being very high, 14,000 μg kg^{-1} and 820 μg kg^{-1}, respectively. *Czembor et al. (2015)* detected the incidence of DON and ZEA of 66.67% and 43.33%, respectively in the 30 tested maize samples originated from Poland, with the mean levels for positive samples of 50.77 μg kg^{-1} and 18.39 μg kg^{-1}, for DON and ZEA, respectively. In Turkey, *Alptekin et al. (2009)* detected AFB_1 in 72.4% of maize samples, with a concentration in the range of 0.63-108.86 μg kg^{-1}, with 43% of maize samples having an AFB_1 concentration above the permitted limit (5 μg kg^{-1}). In Argentina, *Pacin et al. (2001)* have not detected ZEA and DON, while AFB_1 was detected in only one maize sample at a concentration of 16.8 μg kg^{-1}. In North Korea, *Kim et al. (1993)* have established 30% DON and 8% ZEA positive maize samples with an average concentration of 310 and 151 μg kg^{-1}, respectively. In Indonesia, *Nuryono et al. (2005)* have established a low percentage (3%) of ZEA positive maize samples for feed with an average concentration of 25.5 μg kg^{-1}.

Considering the correlation values for the total number of 22 samples examined, medium positive correlations between moisture content and incidence of *Penicillium* spp. were found (r=0.58) and levels of ZEA (r=0.56) and DON (r=0.49), also between the incidence of *A. parasiticus* and AFB_1 (r=0.52), ZEA and DON levels (r=0.52). Slight positive correlations were found between moisture content and incidence of *F. graminearum* (r=0.28) and *F. proliferatum* (r=0.12) and levels of AFB_1 (r=0.16), and between incidence of *F. graminearum* and DON levels (r=0.13). Slight negative correlations were found between moisture content and incidence of *A. flavus* (r=-0.01), *A. parasiticus* (r=-0.16), *A. niger* (r=-0.01), *F. verticillioides* (-0.11) and *F. subglutinans* (-0.09) and between incidence of *F. graminearum* and ZEA (r = -0.09) and incidence of *A. flavus* and AFB_1 levels (r = -0.20). In similar studies, *Alptekin et al. (2009)* have established slight positive correlations between relative humidity (RH) and fungal count (r=0.378) and AFB_1 level (r=0.258) and slight negative correlations between fungal count and AFB_1 level (r=-0.249). In contrast, *Gourama and Bullerman (1995)* have established a positive correlation between fungal growth and AFB_1 production.

Conclusion

Based on the obtained results, it can be concluded that the considerable presence of certain potentially toxic fungi species, especially from the *Fusarium*

genus, was determined in the maize samples, depending on the district tested. *F. graminearum* was the most common species (60%) in the maize sample from Kolubara district, followed by *F. verticillioides* in the maize samples from Podunavlje (43.75%) and City of Belgrade districts (22.4%) and *Penicillium* spp. in the maize samples from Šumadija district (26.38%). In regard to the tested mycotoxins, ZEA, DON and AFB_1, only DON exceeded the allowed limit (1750 μg kg^{-1}; EC, 2007) in the maize sample from Kolubara district. This was expected due to the high incidence of *F. graminearum* in the maize sample from this district and a positive correlation between incidence of *F. graminearum* and DON level.

These studies have confirmed the potential danger and risk from toxigenic species, primarily *Fusarium* species and their mycotoxins in the production of maize in agro-ecological conditions in Serbia. The obtained results justify the need for constant fungal and mycotoxin analyses of maize grain and other types of feeds, in order to find preventive measures for reducing these contaminants in the food chain. Future research should focus on the examination of the incidence and analysis the greater number of the samples from a number of localities, as well as a more detailed examination of the dependence of fungal and mycotoxin contamination from climatic factors in order to more accurately assess the effect of the locality (district) on the natural occurrence of these contaminants in the production of maize.

Kontaminacija toksigenim vrstama gljiva i njihovim mikotoksinima uzoraka kukuruza iz različitih regiona u Srbiji

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Rezime

Ispitivanja u ovom radu izvedena su s ciljem da se odredi prirodna pojava potencijalno toksigenih gljiva iz rodova *Aspergillus*, *Fusarium* i *Penicillium* i sadržaj mikotoksina zearalenona (ZEA), deoksinivalenola (DON) i aflatoksina B_1 (AFB_1) u kukuruzu uskladištenom neposredno posle berbe u 2016. godini i korišćenom za ishranu životinja. Ukupno 22 uzoraka zrna kukuruza sakupljeni su iz četiri regiona u Srbiji: Beogradski (devet uzoraka), Šumadijski (osam uzoraka), Podunavski (četiri uzorka) i Kolubarski (jedan uzorak). Toksigene vrste gljiva su identifikovane na osnovu morfoloških osobina, a sadržaj mikotoksina određen je pomoću biohemijske, imunoadsorpcione enzimske metode (ELISA).

Ispitivani uzorci kukuruza većinom su bili inficirani sa *Aspergillus*, *Fusarium* i *Penicillium* spp., izuzev što u uzorku iz Kolubarskog regiona nisu bile identifikovane *Aspergillus* vrste. *Fusarium graminearum* bila je najučestalija vrsta

u uzorku kukuruza iz Kolubarskog regiona (60%), *F. verticillioides* u uzorcima iz Podunavskog (43,75%) i Beogradskog regiona (22,4%) i *Penicillium* spp. u uzorcima iz Šumadijskog regiona (26,38%). U ispitivanim uzorcima kukuruza zastupljenost *Aspergillus* vrsta bila je niska (0-1,78%).
Mikotoksikološkim analizama ustanovljeno je prisustvo zearalenona (ZEA), deoksinivalenola (DON) i aflatoksina B_1 (AFB_1) u svim ispitivanim uzorcima kukuruza, izuzev što DON nije detektovan u uzorcima iz Podunavskog a AFB_1 u uzorku iz Kolubarskog regiona. Ispitivani uzorci su visoko kontaminirani sa ZEA, 100% uzoraka iz Šumadijskog, Podunavskog i Kolubarskog regiona i 88,89% uzoraka iz Beogradskog regiona. Isto tako, sa DON bilo je kontaminirano 100% uzoraka iz Šumadijskog i Kolubarskog regiona i 22,2% iz Beogradskog regiona. Najveći broj AFB_1 pozitivnih uzoraka ustanovljen je u Šumadijskom regionu (87,5%), dok je u Beogradskom i Podunavskom regionu ustanovljeno 55,56% i 50% AFB_1 pozitivnih uzoraka, respektivno.
Uopšteno razmatrajući, u ovim analizama ustanovljena je visoka zastupljenost toksigenih vrsta u svim ispitivanim uzorcima kukuruza. Isto tako, ustanovljena je visoka kontaminiranost uzoraka sa mikotoksinima ZEA, DON i AFB_1, iako je samo u jednom uzorku sadržaj DON premašio dozvoljeni limit (1750 μg kg^{-1}) prema zakonskoj regulativi za neprerađeni kukuruz. Zbog toga, stalne mikološke i mikotoksikološke analize zrna kukuruza neophodne su radi ocene rizika od gljivične i mikotoksin kontaminacije u lancu ishrane.

Ključne reči: kukuruz, toksigene gljive, mikotoksini

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EFFECT OF BACTERIAL INOCULANTS APPLICATION AND SEEDING RATE ON COMMON VETCH-OAT SILAGE QUALITY

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Abstract: The experiment was carried out to evaluate the possibility of ensiling common vetch – oat mixtures sown at five different seeding rates. Two effects were studied: seeding rate of common vetch and oat in the mixtures and application of bacterial inoculant. The pH, DM (Dry Matter) content, ammonia nitrogen, soluble nitrogen, lactic, acetic and butyric acids were determined on silages. DLG method was utilized for classification the silage quality. Common vetch and oat were grown in binary mixtures at the experimental field of the Institute for forage crops, Kruševac – Serbia, and were tested at five different mixture rates: pure vetch, 25% vetch + 75% oat, 50% vetch + 50% oat, 75% vetch + 25% oat and pure oat. Application of bacterial inoculant affected higher content of ammonia nitrogen and acetic acid ($P< 0.05$), but lower content of soluble nitrogen ($P< 0.05$). Depending on the seeding rates of common vetch and oat, 75 : 25 common vetch – oat silage had the highest content of lactic acid and the lowest content of butyric acid. Contents of DM, pH and ammonia nitrogen were similar in all silages ranged from 307.2 to 318.5 g kg^{-1}, from 4.27 to 4.54 and from 16.1 to 19.1% ΣN, respectively. According to the DLG method for silage quality evaluation, similar quality grades were founded.

Key words: common vetch – oat silages, quality of fermentation

Introduction

Good quality silage and hay are important for the nutrition of ruminants, as well as for the quality and safety of dairy products. The conservation process involves many steps that should be managed carefully to ensure good quality. This starts in the crop composition, continues with harvest, ensiling, and feed out

management and is influenced by additives. Silage quality depends on many factors. In terms of the nutritive value of the forage, the crop composition at harvest has a major impact on the ensiling process and quality of silage (*Buxton and O´Kiely, 2003*). *Đorđević et al. (2011)* reported that bacterial-enzyme additives reduce fiber and increase the concentration of sugar and lactic acid and digestibility of silage. *Bijelić et al. (2015)* concluded that bacterial inoculants reduced crude protein content, ammonia nitrogen, acetic acid and pH value and increased the proportion of lactic acid relative to the acetic acid.

Experience with cultivation of legume –cereal mixtures for silage is limited, and there have been no enough studies on different mixtures for this purpose. The aim of the present study was to evaluate the fermentative characteristics of common vetch-oat silages depending on different seeding rate in the mixtures and application of inoculant.

Material and methods

Common vetch and oat were grown in binary mixtures at the experimental field of the Institute for forage crops, Kruševac-Serbia (21º 19′ 35″ E, 43º 34′ 58″ N). The experiment was designed with three replication according to a randomized complete block. The common vetch:oat mixtures were ensiled in the experimental containers holding 130 dm^3, with three replications. After compaction, silomass was covered with plastic wrap, and covered with a layer of sand thickness of about 10 cm as the main load. Bacterial inoculant *BioStabil Plus* which contained homo-fermentative lactic acid bacteria (*Enterococus faecium* and *Bacillus plantarum*) and hetero-fermentative lactic acid bacteria (*Bacillus brevis*) with a concentration of 5×10^{10} CFU per gram was added, and ensiled in containers for 45 days (a_1 – treatment with bacterial inoculant; a_2 – treatment without bacterial inoculant). The common vetch and oat were tested at five different mixture rates: b_1) 100% common vetch + 0% oat; b_2) 25% common vetch + 75% oat; b_3) 50% common vetch + 50% oat; b_4) 75% common vetch + 25% oat and b_5) 0% common vetch + 100% oat. Plant samples were taken at forming the first pods on 2/3 plants of common vetch.

The DM content was determined in the silage, the degree of acidity (pH), ammonia and soluble nitrogen, content of acetic, butyric and lactic acids. In order to provide more realistic estimates, DLG method for evaluating the quality of silage was used (*Đorđević et al., 2003*).

The experimental data were analyzed by a two-way analysis of variance for silage samples using a model that accounted for the main effects of addition of inoculant and common vetch : oat mixtures. Effects were considered significant at $P<0.05$ level. The significance of differences between arithmetic means was tested by LSD test.

Results and discussion

The results of fermentation characteristics in common vetch-oat silages are presented in Table 1. The higher numerical content of DM was found for the inoculant treatment (313.3 g kg^{-1}) than in treatment without bacterial inoculant (311.9 g kg^{-1}). However, differences were not significant. Depending on the seeding rate of common vetch and oat in the mixtures, DM content increased from 307.2 g kg^{-1} in pure common vetch silage to 318.5 g kg^{-1} in pure oat silage.

Table 1. Conservation characteristics of common vetch – oat silages

Factors		DM	pH	NH_3-N	H_2O-N	AA	BA	LA
A	B	g kg^{-1}		%ΣN	%ΣN	g kg^{1}DM	g kg^{1}DM	g kg^{1}DM
a_1	b_1	301.3^{d}	4.48^{c}	18.1^{b}	58.5^{d}	53.3^{b}	5.3^{b}	139.2^{b}
	b_2	315.7^{b}	4.61^{b}	19.0^{b}	58.3^{d}	36.9^{f}	5.4^{b}	126.5^{c}
	b_3	312.6^{b}	4.25^{e}	19.8^{a}	48.0^{f}	60.9^{a}	5.4^{b}	118.9^{c}
	b_4	317.0^{b}	4.32^{d}	19.7^{a}	61.6^{c}	55.3^{b}	1.4^{d}	97.2^{d}
	b_5	319.7^{a}	4.17^{f}	17.1^{c}	65.7^{a}	47.7^{c}	3.9^{b}	72.5^{e}
Average A_1		**313.3^{NS}**	**4.36^{B}**	**18.7^{A}**	**58.4^{B}**	**50.8^{A}**	**4.3^{NS}**	**110.9^{NS}**
a_2	b_1	313.0^{b}	4.60^{b}	16.0^{d}	59.7^{d}	51.0^{c}	5.1^{b}	133.0^{b}
	b_2	302.7^{c}	4.13^{f}	19.3^{a}	63.7^{b}	49.7^{c}	3.1^{c}	89.2^{d}
	b_3	312.0^{b}	4.85^{a}	17.6^{c}	59.6^{d}	43.1^{e}	2.2^{c}	79.6^{e}
	b_4	314.7^{b}	4.22^{e}	17.4^{c}	55.2^{e}	45.2^{d}	6.2^{a}	148.3^{a}
	b_5	317.3^{b}	4.89^{a}	15.0^{d}	64.2^{b}	47.6^{c}	4.1^{b}	95.4^{d}
Average A_2		**311.9^{NS}**	**4.54^{A}**	**17.1^{B}**	**60.5^{A}**	**47.3^{B}**	**4.2^{NS}**	**109.1^{NS}**
Average B_1		**307.2^{C}**	**4.54^{A}**	**17.0^{B}**	**59.1^{B}**	**52.1^{A}**	**5.2^{A}**	**136.1^{A}**
Average B_2		**309.2^{C}**	**4.36^{B}**	**19.1^{A}**	**61.0^{B}**	**43.3^{D}**	**4.3^{B}**	**107.9^{C}**
Average B_3		**312.3^{B}**	**4.55^{A}**	**18.7^{A}**	**53.8^{D}**	**52.0^{A}**	**3.8^{C}**	**99.3^{D}**
Average B_4		**315.8^{A}**	**4.27^{B}**	**18.5^{A}**	**58.4^{C}**	**50.2^{B}**	**3.8^{C}**	**122.7^{B}**
Average B_5		**318.5^{A}**	**4.53^{A}**	**16.1^{C}**	**64.9^{A}**	**47.6^{C}**	**4.0^{B}**	**83.9^{E}**

a_1- treatment with bacterial inoculant; a_2- treatment without bacterial inoculant; b_1- silage made from pure common vetch; b_2- silage made from 25 : 75 common vetch – oat mixture; b_3- silage made from 50 : 50 common vetch – oat mixture; b_4 - silage made from 75 : 25 common vetch – oat mixture; b_5 - silage made from pure oat; AA – acetic acid; BA – butyric acid; LA – lactic acid; Different letters denote significantly different means ($P< 0.05$)

The pH of silage with the inoculant was lower (4.36) than the pH of silage without inoculant (4.54). The highest pH values were determined in the silages of

pure common vetch, pure oat and 50:50 common vetch oat mixture. According to *Weissbach (1996)* pH values below 4.2 with 200 g DM kg^{-1} and below 4.45 with 300 g DM kg^{-1} are needed to obtain well-fermented and stable silage. Results of the present study comply with these requirements.

The ammonia nitrogen ratios in common vetch-oat silages were very high (18.7% ΣN in silage with bacterial inoculant and 17.1% ΣN in silage without inoculant). Depending on the seeding rate in the common vetch-oat mixture, the ammonia nitrogen ranged from 16.1% ΣN in pure oat silage to 19.1% ΣN in the 25:75 common vetch-oat silage. It implies on significant activity of proteolytic bacteria. Legume crops, such as vetch species have high protein and low carbohydrate content effect difficulties for fermentation of silage and these protein is rapidly degraded resulted in high ammonia nitrogen *(Balabanli et al., 2010).* So, protein can be inhibit acid to neutralize and prevent pH fallings. The presence of ammonia nitrogen in silages without or with very low level of butyric acid can be explained with the activity of plant enzymes *(McDonald, 1981).* Higher content of the soluble notrogen was recorded in silage without inoculant (60.5% ΣN) and differed significantly from the silage with inoculant (58.4% ΣN). In pure oat silage the ratio of soluble nitrogen of 64.9% ΣN is above the permitted value wich is 60% ΣN *(Ensilage, 1978),* whereas in all other silages ratio of soluble nitrogen was below or equal to the permitted value.

Table 2. Evaluation of silages quality

Factors		DLG method of evaluation		
A	**B**	**Score**	**Class**	**Quality**
a_1	**b_1**	46	I	Very good
	b_2	45	I	Very good
	b_3	46	I	Very good
	b_4	46	I	Very good
	b_5	43	II	Good
a_2	**b_1**	46	I	Very good
	b_2	47	I	Very good
	b_3	43	II	Good
	b_4	47	I	Very good
	b_5	43	II	Good

a_1- treatment with bacterial inoculant; a_2- treatment without bacterial inoculant; b_1- silage made from pure common vetch; b_2- silage made from 25 : 75 common vetch – oat mixture; b_3- silage made from 50 : 50 common vetch – oat mixture; b_4 - silage made from 75 : 25 common vetch – oat mixture; b_5 - silage made from pure oat

Bacterial inoculation caused high content of the lactic acid and the acetic acid, and low content of butyric acid content (Table 1). The lactic acid bacteria

may be classified as homofermentative or heterofermentative based on their by-products of sugar fermentation. Homofermentation gives only lactic acid as the end product of glucose metabolism. In heterofermentation equimolar amounts of lactic acid, carbon dioxide and ethanol or acetic acid are formed from glucose via the phosphoketolase pathway *(Tyrolová and Vyborná, 2011)*. In the present study, the added heterofermentative bacteria might have utilised water soluble carbohydrates more effectively than the homofermentative bacteria. The highest lactic acid conten was determined in pure common vetch silage (136.1 g kg^{-1} DM), followed by silage from 75:25 common vetch-oat mixture (122.7 g kg^{-1} DM). The lactic acid contents decreased as the DM content of the silage increased, with the exception of 75:25 common vetch-oat silage (Table 1). This is consistent with observations reported by *Muck et al. (2003)*. These authors suggest that a high DM content depresses the total amount of fermentation in silages, resulting in a higher final pH and lower concetration of fermentation acids, particularly lactic acid. In this study, quality classification of the silages by DLG scores resulted that silages prepared from common vetch-oat mixtures have very good or good quality (Table 2).

Conclusion

According to the results in performed investigations, it could be concluded that bacterial inoculant application did not significantly have an effect on fermentative process during ensilaging of common vetch-oat mixtures. Only the amount of soluble nitrogen in the treatment with inoculant was below permitted value, while the amounts of ammonia nitrogen were much higher than permitted value, and it implies on significant activity of proteolitic bacteria. However, obtained results show that silage from 75:25 common vetch-oat mixture had the lowest pH, ammonia nitrogen and buturyc acid content and the highest content of lactic acid. And according to the DLG method of evaluation almost all silages achieved very good and good quality. Common vetch-oat mixtures could be successfully ensiled with and without bacterial inoculant.

Uticaj primene bakterijskih inokulanata i strukture smeše na kvalitet silaže grahorice i ovsa

Jordan Marković, Milomir Blagojević, Ivica Kostić, Tanja Vasić, Snežana Anđelković, Mirjana Petrović, Ratibor Štrbanović

Rezime

Istraživanje je sprovedeno da bi se procenila mogućnost siliranja smeša grahorice i ovsa posejanih u pet različitih odnosa. Ispitivana su dva faktora: udeo semena grahorice i ovsa u smeši i primena inokulanta pri siliranju. Sadržaj suve materiije, pH, sadržaj amonijačnog i rastvorljivog azota, kao i sadržaj sirćetne, buterne i mlečne kiseline je utvrđen u silaži. Za ocenu kvaliteta silaže je korišćena DLG i metoda po Weissbach-u. Ogled je postavljen na eksperimentalnom polju Instituta za krmno bilje u Kruševcu, Srbija, i ispitavanja su obuhvatila pet različitih smeša: čist usev grahorice, 25% grahorice + 75% ovsa, 50% grahorice + 50% ovsa, 75% grahorice + 25% ovsa i čist usev ovsa. Rezultati suobrađeni kao dvofaktorijalni ogled, analizom varijanse korišćenjem modela koji objašnjava uticaj structure smeše i primene inokulanta na kvalitet silaže. Primena bakterijskog inokulanta je uzrokovala veći sadržaj amonijačnog azota i sirćetne kiseline ($P< 0.05$), ali niži sadržaj rastvorljivog azota. Smeša u kojoj je odnos grahorice i ovsa bio 75:25 sadržala je najveći udeo mlečne kiseline i najmanji udeo buterne kiseline. Sadržaj suve materije, pH i amonijačnog azota je bio sličan u svim silažama i kretao se od 307,2 do 318,5 g kg^{-1}, od 4,27 do 4,54 i od 16,1 do 19,1% ΣN, respektivno. Na osnovu ocene kvaliteta silaže prema DLG i Weissbach metodi utvrđen je približan kvalitet ispitivanih silaža.

Ključne reči: silaža grahorice i ovsa, kvalitet fermentacije

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THE EFFICIENCY OF THE PRODUCTION OF RABBIT MEAT WITH THE HELP OF MODERN TECHNOLOGY IN THE PERSONAL SUBSIDARY FARM

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Communication

Abstract: It is proposed to grow rabbits in the modern production technology with the use of the developed technology of using recycled materials in the construction of cages for keeping rabbits. It is also proposed to use interbreed crossing of New Zealand White breed and Flanders, and also breeds Californian and Flanders breed.

Key words: rabbit meat, recycle, live weight, average daily gain, slaughter yield.

Introduction

At this time there is a revival of rabbit livestock industry in the world (*Croft et al., 2002; Vere et al., 2004; Senchenko, 2016*). Important for farmers financial support of the regional authorities, which the help of loans, grants, subsidies on the production of meat products, especially in the field of children's and dietary food. The Russian market of rabbit meat is not filled practically. According to statistics, the need of dietary rabbit meat in Russia on average is satisfied less than 0.5%. Unsatisfactory demand for dietary rabbit meat in Russia is great and according to the assessment is more than 300 thousand tons per year, which causes the relevance of our work (*Senchenko, 2016*).

Material and methods

Given the above the aim of our study was to study the dynamics of live weight, average daily growth, slaughter yield, organoleptic and chemical indicators of meat of rabbits of different breeds with the use of modern technology. To achieve this goal we solved the following tasks: -to improve the rabbit meat production technology based on the use of crossbreeding and the use of recycled materials in the construction of cages for keeping rabbits; - to study the dynamics of live weight of purebred and crossbred rabbits; - calculate the average daily growth of rabbits of the experimental groups; - to carry out the slaughter rabbits and to calculate the slaughter yield; - to conduct organoleptic evaluation of bouillon from rabbit meat; - to determine the chemical composition of different parts of rabbit carcass.
The studies are conducted on the private mini-farm of the village of Pruzhinino of the Yaroslavl region of Gavrilov-Yam district (Photo 1).

Photo 1. Cages for keeping rabbits on the mini-farm of the village of Pruzhinino

Object of study: purebred and crossbred rabbits of the first generation, obtained in accordance with the scheme (Table 1).

Table 1. Objects of the study

№ of the group	The breed of DOE-rabbit	The breed of male rabbit	The number of rabbits
1	Flanders	Flanders	8
2	Californian	Flanders	10
3	New Zealand	Flanders	12
4	White Giant	Flanders	9
5	Soviet chinchilla	Flanders	8

To receive rabbits it was taken 25 females of different breeds, average weight of which was – 5617 g of White Giant breed, 4722 g – of California breed, 4834 g of New Zealand White, 7139 g of Flanders and 5154 g of Soviet

chinchilla breed. We used electronic scales of CAS SWII-30 mark to define the live weight of rabbits Weighting was conducted on the day of birth of rabbits, and then we weighed them after every 30 days until the age of 5 months. Average daily growth was determined with the help of formula 1.

$$C = \frac{W_t - W_0}{t}, \text{g} \quad (1)$$

,

where C –is the average daily gain of live weight, g;
W_t – animal live weight at the end of the period, kg;
W_o – is the live weight of animal at beginning of the period, kg;
t – is the time between two weighings, days.

The slaughter of the rabbit was carried out at the age of 5 months. Before slaughter the animals were kept without feed and water systems within 12 to 18 h to release the contents of the gastrointestinal tract and bladder. Slaughter yield was calculated with formula 2.

$$S = \frac{W_2}{W_1} * 100, \% \quad (2)$$

where S – slaughter yield, %;
W_2– weight after slaughter, kg;
W_1 –weight before slaughter, kg.

We cut slices of muscle of 25 g mass of thigh, shoulder, back, chuck with scalpel from each carcass and double-milled them in a meat grinder when we conduct organoleptic evaluation of the bouillon from rabbit meat. To prepare the meat bouillon we weighed 20 g of minced meat on a laboratory scale, placed in a conical flask with 100 cm^3 capacity and filled with 60 cm^3 of distilled water. The contents of the flask were thoroughly mixed. The flask was closed with a watch glass and placed in a boiling water bath for 10 min. The smell of meat bouillon was determined in the heating process up to 80^0C-85^0C at the time of the appearance of vapor coming from an open flask by feeling their perfume. The transparency of the bouillon was determined by visual inspection of 20 cm^3 of bouillon, poured in a measuring cylinder with 25 cm^3 capacity and diameter of 20 mm.
The study of the chemical composition of rabbit meat according to the following indicators was carried out in the research laboratory of monitoring and quality control of FSBI Yaroslavl state agricultural Academy: protein, fat, ash, and moisture content. The crossbred rabbit was the investigated object (New Zealand White*Flanders) at the age of 150 days.

When we conduct sampling we took the rabbit carcass and divided it along the spine into two equal parts, then each part of the sample was taken from the neck, back, abdomen, front and hind legs and the liver, weighing about 10 g. The Evaluation was conducted in two replications.
The determination of the chemical composition of the parts of rabbit carcasses was carried out according to the following indexes:
- moisture content according to GOST 33319-2015;
- ash content GOST 31727-2012 (ISO 936:1998);
- the protein content according to GOST 25011-81;
- fat content, GOST 23042-2015;
- the content of extractive nitrogen-free substances.

To calculate the maintenance BEV in meat from 100% subtracted water content, protein, fat and ash.
Biometric processing of the results of the studies were conducted according to the method *Merkur'eva (1970)*

Results and discussion

To reduce the cost of the production of rabbit meat we have developed a technology that involves the processing of recycled material (plastic bottles, metal profiles) and their further use for the equipment of cells, as drinkers and feeders, *Merkurjeva (1970).* After the introduction of this technology animals were kept in outdoor cages of self modifications with the size 80×50×60 cm from used old pallets. The feature of the feeding was in the diet of rabbits including feeding of mother's milk during the longer period. It was a 2-3 months instead of one, depending on the milk yield of females. In addition, we fed of rabbits after their birth often. Constant access to water and food in the cells was always maintained. Feeding was carried out by juicy feed and pellets for rabbits or pigs. The dynamic of live weight of animals in the period from birth to 5 months was learned in the experiment to study the growth of purebred and crossbred rabbits of different genotypes in accordance with the methodology of the research. The results of the determination of the dynamics of live weight are presented in Table 2.

Table 2. The dynamics of rabbits live weight

№ group	Age, days					
	1	30	60	90	120	150
	Live weight, kg					
1. Flanders*Flanders	0.06±0.004	0.6±0.005	1.5±0.006	2.5±0.011	3.9±0.011	4.9±0.013
2.Flanders*Californian	0.06±0.003	0.6±0.004	1.5±0.008	2.9±0.010	4.5±0.011	5.6±0.013
3. New Zealand*Flanders	0.06±0.003	0.7±0.004	1.7±0.008	3.0±0.010	4.7±0.008	5.7±0.015
4. White Giant *Flanders	0.07±0.004	0.6±0.005	1.6±0.007	3.0±0.009	4.4±0.009	5.8±0.012
5. Soviet chinchilla* Flanders	0.06±0.002	0.6±0.003	1.4±0.005	2.3±0.007	3.5±0.008	4.8±0.010

From Table 2 it can be seen that the highest live weight had the rabbits of the 3rd group (New Zealand White*Flanders) which were kept under proposed technology in the period from 30 day to 120-day age, but the highest live weight of 150 days of age rabbits was observed in the 4th group, obtained by interbreed crossing of the breeds of White Giant and Flanders – 5.8 kg. Also, high live weight at this age had the rabbits of the 3rd group (New Zealand White*Flanders) – 5.7 kg and 2nd group (California*Flanders) – 5.6 kg.

The value of the average daily growth of the experimental groups of rabbits is presented in Table 3. Analyzing the indexes of the table 4 it can be seen that the best average daily growth from 30-day to 120-day age was in group №3 (New Zealand White*Flanders) and in group №2, that is, rabbits, obtained by interbreed crossing of the Californian and Flanders. But in the period of 120-day to 150-day age best average daily growth was among crossbred rabbits of White Giant*Flanders.

Table 3. Rabbits daily growth

№ group	Period, days				
	1-30	30-60	60-90	90-120	120-150
	Average daily growth, g				
1. Flanders*. Flanders	20±3	25±2	27±4	32±4	32±5
2. Flanders * Californian	20±2	25±4	32±4	37±5	37±6
3. New Zealand*. Flanders	23±3	28±4	33±5	39±5	38±6
4. White Giant *. Flanders	20±3	26±3	33±4	36±4	39±5
5. Soviet chinchilla*. Flanders	20±2	23±2	25±3	29±3	30±4

To study the meat quality of rabbits we conducted slaughter of 5 rabbits of each group. The results of the slaughter are presented in the table 4.

Table 4. Results of slaughter of rabbits

Index	№ group				
	1	2	3	4	5
Rabbits live weight before slaughter, kg	4.9±0.03	5.5±0.03	5.6±0.02	5.7±0.02	4.7±0.02
Rabbits live weight after slaughter, kg	2.6±0.03	3.0±0.02	3.1±0.03	2.8±0.02	2.5±0.03
Slaughter yield, %	53	55	56	49	53

According to the table 5 it can be concluded that carcass yield of young rabbits was almost identical and ranged from 53-56% except rabbits obtained by crossing breeds of White Giant*Flanders. The breed of White Giant can give meat and pelt and its carcass yield is 5-10 % less than New Zealand white and Californian breeds.

The evaluation of organoleptic characteristics of meat of rabbits of two groups, number 3 (New Zealand White*Flanders) and number 1 (Flanders*Flanders) was carried out in accordance with the requirements of GOST 20235.0-74 «Rabbit meat. Sampling methods. Organoleptic methods of freshness determination» (*GOST, 1974*).

The results of the evaluation of organoleptic characteristics of rabbit meat are shown in the Table 5.

Table 5. Organoleptic characteristics of rabbit meat

Index	New Zealand*Flanders	Flanders* Flanders
The surface of the carcass	Crust of drying pale pink Wet, shiny	Crust of drying pale pink Wet, shiny
Serous membrane of the abdominal cavity	Slightly moist, pale pink color	Slightly moist, pale pink color
Muscle on the cut Consistency	Muscle is elastic, the hole aligns quickly	Muscle is elastic, the hole aligns quickly
Smell	Specific, peculiar to fresh meat	Specific, peculiar to fresh meat
Transparency and flavor of the broth	Transparent, fragrant	Transparent, fragrant

From Table 5 we can see that the organoleptic characteristics of rabbit meat of group №3 and group №1 do not differ from each other. Visual inspection of rabbit meat in both groups reported good carcasses vascularization. All samples of meat were well-expressed drying crust. After 24 hours of storage of rabbit meat the speed of alignment of the pits on the surface after finger pressure was the same. Thus, the rabbit meat was good, bruises were not found, without foreign smell, without blood clots. Smell – typical of this type of meat, the color pink and white. The bouillon is transparent, without characteristic smell

so the meat was fresh. We assessed the broth in terms of taste, aroma and transparency and identified the following features: the broth from rabbit meat of the group 3 was more fatty, rich, as meat rabbits (New Zealand White breed) have a greater number of minuscule fat, unlike meat of rabbit from group №1
The chemical composition of carcass parts of the rabbit (New Zealand White* Flanders) is presented in table 6.

Table 6. Chemical composition of parts of the carcass of a rabbit (New Zealand White*Flanders)

Part of the carcass	Total moisture, %	Dry matter, %	Fat, %	Protein, %	Ash, %	NFE,%
Neck	75.59±0.70	24.41±0.70	1.73±1.63	21.00±0.62	0.71±0.29	0.98±0.01
Abdomen	75.37±0.44	24.63±0.44	1.76±0.23	21.19±0.53	0.80±0.61	0.88±0.59
Blade	75.33±0.69	24.67±0.69	2.00±1.33	20.81±1.77	0.62±0.21	1.24±1.34
Thigh	78.61±0.40	21.39±0.40	0.74±0.28	14.13±0.30	0.93±0.34	5.60±0.52
Back	77.14±0.17	22.86±0.17	1.75±0.71	19.41±0.57	0.85±0.14	0.86±0.82
Liver	74.65±0.02	25.36±0.02	1.64±0.72	18.06±0.88	1.19±0.19	4.47±1.39

Based on the results of the studies we concluded that the lowest moisture content is in the liver (74.65 %), and the highest is in the thigh (78.61%). More dry matter is in the liver (25.36%), and less is in the thigh (21.39%). Fat is less in the hip (0.74%), but more is in the blade (2.00%). The highest protein content is in the abdomen (21.19%) and less is in the thigh (14.13%). The highest content of ash is in liver (1.19%) and the smallest is in the blade (0.62%). The highest content of nitrogen-free extractives is located in the thigh (5.60%) and the smallest is in the back (0.86%).
Metzger et al. (2003); Szkucik and Libelt (2006); Maj et al. (2008); informed that the level of protein, fat and ash in rabbit meat were in the ranges: 21.36-23.91%, 0.65-1.74% and 1.16-1.30%, ab we can conclude that our results are similar.

Conclusion

Based on our researches we came to the conclusion: the using of our developed technology of application of recyclable materials and interbreed cross breeds of New Zealand White*Californian and Flanders*Flanders increases the efficiency of rabbit meat production in the conditions of private farming.

Efikasnost proizvodnje mesa zeca uz pomoć savremene tehnologije na privatnoj farmi

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Rezime

Predlaže se uzgoj zečeva u savremenoj proizvodnoj tehnologiji uz korišćenje razvijene tehnologije upotrebe recikliranih materijala u izgradnji kaveza za držanje zečeva. Takođe se predlaže korišćenje meleza novozelandske bele rase i flandrijske rase zečeva, kao i meleza kalifornijske i flandrijske rasa.

Ključne reči: meso zeca, recikliranje, živa masa, prosečni dnevni prirast, klanični randman

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EFFECTS OF REARING SYSTEM AND BODY WEIGHT OF REDBRO BROILERS ON THE FREQUENCY AND SEVERITY OF FOOTPAD DERMATITIS

Zdenka Škrbić, Zlatica Pavlovski, Miloš Lukić, Veselin Petričević

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