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DIVERSITY STUDY ANALYSIS OF LEPTIN GENE IN SOME RUMINANT AND NON-RUMINANT SELECTED ANIMAL SPECIES

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Original scientific paper

Abstract. The key element of the system regulating food intake has proven to be the Leptin. It act as hunger centre in the hypothalamus and affects the regulation of appetite. It has also been shown that Leptin gene influence milk performance in sheep, cattle and reproduction performance in beef cattle. Genetic characterization to assess the existing biodiversity and differences among the important livestock breeds is an essential pre-requisite to facilitate the conservation program in an effective and meaningful way. This paper sought to study the diversity analysis of Leptin gene in some ruminant and non-ruminant animal species. A total of twenty three (23) Leptin gene sequences belonging to eight (8) species: Cattle (3), Sheep (3), Goat (3), Swine (3), Horse (2), Camel (3), Mouse (3) and Rabbit (3) were retrieved from Genbank (www.ncbi.nlm.nih.gov). Sequences alignment, translation and comparison were done using ClustalW of the MEGA 6.0. The minimum distance matrix (Dxy) value (0.02) was observed between the sequence of cattle and goat while the maximum Dxy value (2.72) was seen between cattle and sheep in ruminant species. In non-ruminant species the highest Dxy value (17.61) was seen between rabbit and camel while the minimum Dxy value (0.18) was observed between mouse and camel respectively. The smaller the distance matrix value, the closer the sequence of the species and the lesser the genetic distance among or between species whereas the larger the Dxy value, the higher the genetic distance among and between species investigated. This finding could provide basis for selection when considering evolution and differentiation among species.

Keywords: diversity study, leptin, ruminant, non-ruminant, sequences, phylogenetic analysis

Introduction

Leptin is a 16-kDa protein hormone belonging to the class-1 helical cytokine family of proteins *(Trombley et al., 2012).* Leptin was first discovered in the mouse *Mus musculus* and has a central role in the regulation of appetite, energy metabolism, body composition, immune functions and reproduction in mammals *(Trombley et al., 2012).*

Leptin is primarily produced in adipose tissue and is secreted into the blood stream after cleavage of the 21 amino acid signal peptide *(Barb et al., 2001),* secretion occurs in response to changes in body fat levels or energy status *(Barb et al., 2001).* Leptin acts as an anorexigenic signal through a negative feedback loop to the appetite centre in the hypothalamus causing long term and short-term effects on feed uptake and energy homeostasis *(Trombley et al., 2012).*

Expression of gene which encodes a Leptin receptor has been confirmed in pituitary, adipose tissue, granulosa and theca cells of the ovary, interstitial cells in testis, in heart, liver, lung, kidney, adrenal gland, small intestine and lymph nodes *(Hoggard et al., 1997).* In mammals the Leptin is considered as a hormone that regulates the body weight by maintaining the balance between food intake and energy expenditure through signalling to the brain and brings the changes in stored energy level *(Friedman et al., 1998).*

Elevated plasma Leptin levels inhibit continued feeding and regulate body weight in the long term as well as promoting postprandial satiety *(Trombley et al., 2012).* Low Leptin levels are associated with low body fat levels and starvation, signalling energy insufficiency and stimulating appetite in humans, rats *Rattus spp* and pigs *Sus spp.* The Leptin gene is highly conserved across species and is located on chromosome 7q31.3 in humans and on chromosome 4q32 in cattle *(Fatima et al., 2011)*. Leptin gene DNA sequence includes 15,000 base pairs and contains 3 exons, which are separated by 2 introns. Out of 3 exons and 2 introns, only two exons are translated into protein.

In mammals, Leptin informs the hypothalamus *(Barb et al., 2001)* about the amount of fat stored in the body through short and long forms of Leptin receptor. Leptin also plays a major role in control of body growth, adaptability, immune function, angiogenesis, renal function, haematopoiesis, reproduction, and not only acts as an endocrine signal in brain and different peripheral tissues in which Leptin receptors are expressed in fatal tissue, mammary gland, rumen, abomasum, duodenum and pituitary gland. The Leptin expression is also modulated according to different physiological and growth stages of animal *(Wallace et al., 2014).* Therefore, the Leptin could act as marker for animal growth, feed conversion efficiency and health and therefore the present study sought to explain a form of diversity study analysis of Leptin gene in-silico in some selected ruminant and non-ruminant animal species.

Materials and Methods

A total of twenty three (23) Leptin gene sequences of some selected ruminant and non-ruminant animal species as thus: Cattle (3), Sheep (3), Goat (3), Swine (3), Horse (2), Camel (3), Mouse (3) and Rabbit (3) were retrieved from the GenBank (www.ncbi.nlm.nih.gov). The GenBank accession number of these cattle, sheep, goat, swine, horse, camel, mouse and rabbit sequences were: NM_173928.2, Y11369.1, NM_001034741.1 (Cattle), NM_001009763.1, XM_004002049.3, XM_004021753.3 (Sheep), XM_018045213.1, XM_018045217.1,NM_001159756.1 (Goat), AY079082.1, EU189935.1, GBZA01000352.1 (Swine), XM_014738998.1, XM_014736686.1 (Horse), XM_010949533.1, XM_010949543.1, XM_006180441.2 (Camel), NM_026609.2, NM_025961.5, NM_145541.5 (Mouse), XM_008258163.2, XM_002709552.3, XM_002715941.3 (Rabbit).

Sequence alignments, translations and comparisons were done using ClustalW as described by *(Larkin et al., 2007).*

Neighbor-Joining trees were constructed each using P-distance model and pair wise deletion gap/missing data treatment. The construction was on the basis of genetic distances, depicting phylogenetic relationships among the Leptin nucleotide sequences of the investigated species. The reliability of the trees was also calculated by bootstrap confidence values *(Felsenstein, 1985)*, with 1000 bootstrap iterations using MEGA 6.0 software *(Tamura et al., 2013).*

Unweighted pair group method using arithmetic average (UPGMA) trees for the gene was constructed with consensus sequences using same model as that of the tree. All sequences were trimmed to equal length corresponding to same region before generating the tree.

Results

Table 1.**Leptin sequence variation between and among species**

Species	Number of sequences	Sequence length variation (bp)
Cattle	3	2042, 2060, 2930
Sheep	3	2586, 2757, 2836
Goat	3	2205, 2643, 2767
Swine	3	2060, 2123, 2642
Horse	2	2597, 2935
Camel	3	1383, 2556, 2839
Mouse	3	2357, 2474, 2609
Rabbit	3	2433, 2526, 2680

bp= base pair

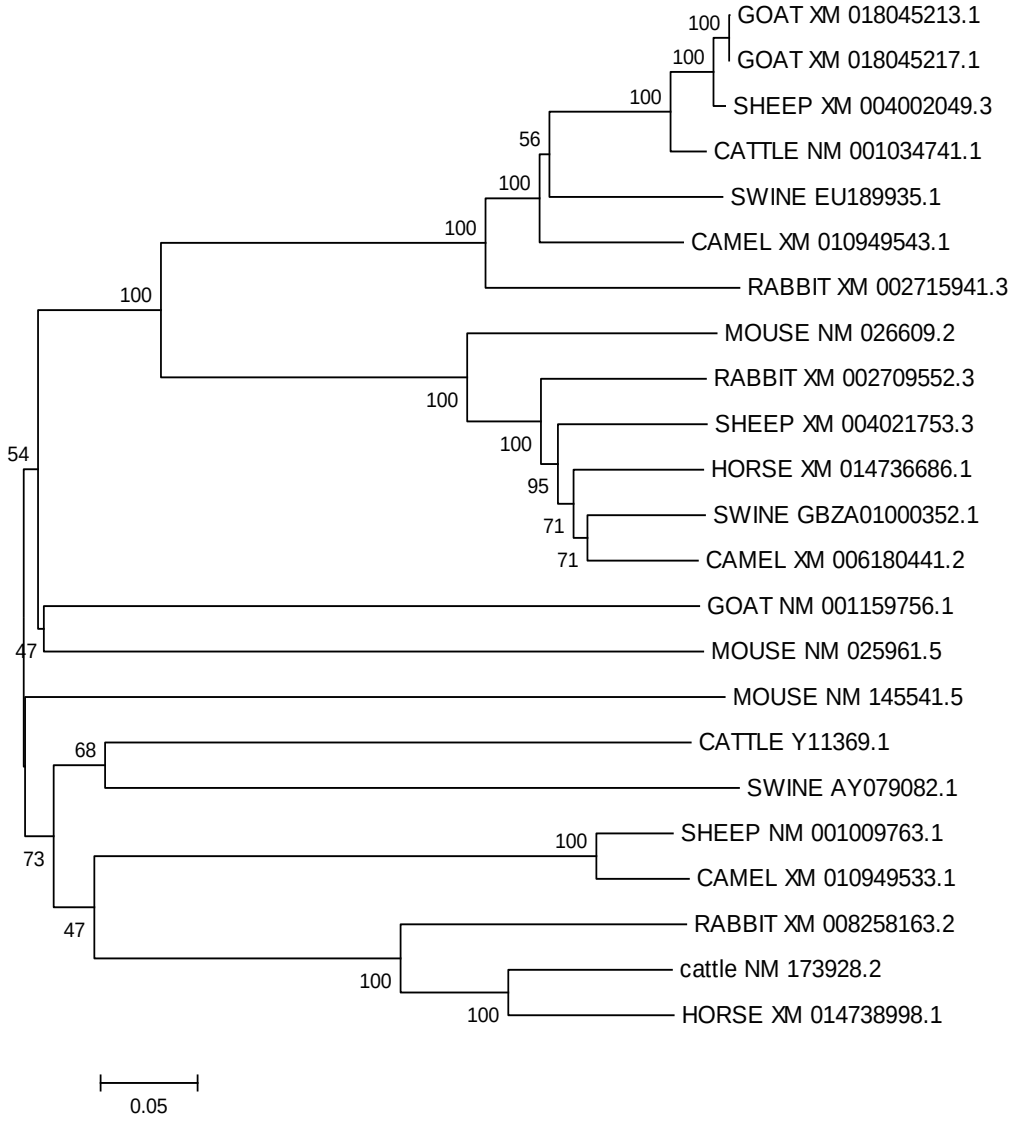

Fig 1. Phylogenetic tree of leptin gene sequences of the species selected.

The tree above showed a kind of proximity and differentiation among the ruminant and non-ruminant animal species selected.

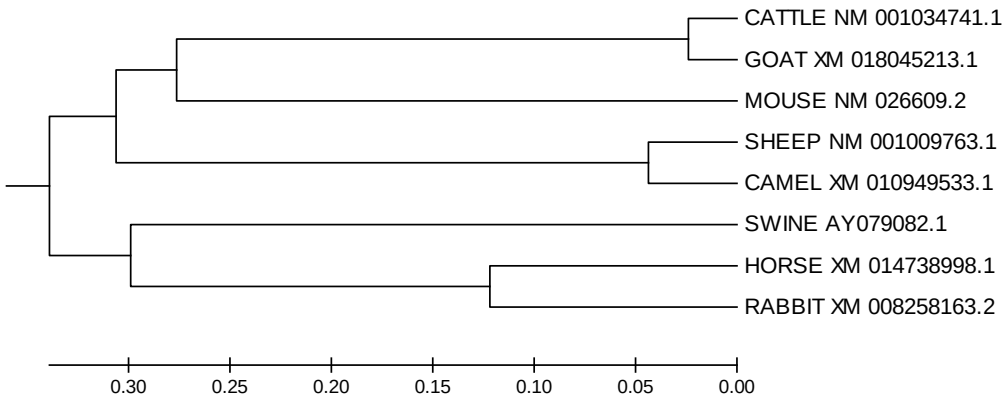

Fig.2. UPGMA tree from the consensus sequence of the phylogenetic tree

This figure showed that the sequence of Leptin gene of cattle clustered more closely with those of goats than mouse. Sequence of sheep from this figure appeared closer to those of camel than those of swine. Whereas Leptin gene sequence of horse and rabbit clustered closely than those of swine. In ruminant species, cattle and goats Leptin sequences clustered closely than those of sheep. While of those of non-ruminant, Leptin sequences of horse and rabbit clustered closely followed by those of swine and then mouse respectively and this could be explained due to species specific residues and such patterns of the sequences may be explained by gene conversion and balancing selection.

Table 2. Test of the Homogeneity of Substitution Patterns between Sequences selected

	Cattle	Sheep	Goat	Swine	Horse	Camel	Mouse	Rabbit
Cattle		0.00	0.22	0.00	0.00	0.03	1.00	0.00
Sheep	2.72		0.00	0.00	0.00	0.02	0.03	0.00
Goat	0.02	2.46		0.00	0.00	0.04	1.00	0.00
Swine	8.58	14.80	9.67		0.04	0.00	0.00	0.02
Horse	4.80	7.38	5.66	1.03		0.00	0.00	0.00
Camel	1.14	0.20	1.04	11.82	5.46		0.27	0.00
Mouse	0.00	1.37	0.00	7.56	3.35	0.18		0.00
Rabbit	14.26	21.19	16.01	1.40	3.37	17.61	13.08	

Standard error estimate is presented at the upper diagonal while average genetic distances between species is presented at the lower diagonal**.**

This distance matrix table explained better the distance between and among the leptin gene sequence of the selected animal species. The standard error above the diagonal ($P<0.05$) are considered significant.

The smaller the distance matrix value, the closer the sequence of the species and the lesser the genetic distance among or between species whereas the larger the Dxy value, the higher, the genetic distance among and between species.

Distance matrix (Dxy) of the sequence of cattle and goat (0.02) was minimum while while the maximum Dxy value (2.72) was seen between cattle and sheep in ruminant species. In non-ruminant species the highest Dxy value (17.61) was seen between rabbit and camel while the minimum Dxy value (0.18) was observed between mouse and camel respectively.

Discussion

The LEP is a cytokine-like hormone that regulates appetite, energy homeostasis, body composition, reproduction, immunity, and metabolic functions *(Ahima and Flier, 2000).* Whereas in wild animals, adaptive evolution has been shown to have occurred in pika (*Ochotona curzoniae*) Leptin in response to environmental stress (extreme cold) *(Yang et al., 2008),* in livestock, polymorphism in the Leptin gene has been found to be associated with variations in traits of economic importance *(Zhou et al., 2009).* In sheep, products of the different allele variants in the Leptin gene have been shown to differ in their biochemical and biological properties *(Reicher et al., 2011).* The presence and maintenance of Leptin genetic polymorphism in the livestock population suggests that different forms of the protein might have differential functional abilities.

The Leptin protein circulates in the serum as a free hormone or as a complex with Leptin soluble receptor (bound form). It was found that the proportion of circulating free Leptin to bound Leptin varies in different physiological conditions. In addition, it has been suggested that this variation might disrupt the binding of Leptin to its receptor *(Buchanan et al., 2002).*

Leptin gene sequence length variation of the selected species ranged from 1383–2930 base pair. The Dxy value inferred closeness and distance of the sequences of the various species.

The length variation of the Leptin gene within and among species might result from evolution and differentiation. Many length variations caused by insertions and deletions resulting in amino acid variation within species have been found by comparison with known sequences *(Faith and Owoeye, 2017).*

The presence of numerous alleles at a particular Leptin locus is evidence of the long-term evolutionary persistence of the locus. This is suggested by the fact that the alleles in one species are often more closely related to the alleles in closely related species than to the other alleles in the same species. The species wise clustering might be due to species specific residues *(Takahashi and Nei, 2000)* and such patterns of the sequences may be explained by gene conversion and balancing selection.

It has also been shown that Leptin gene influence milk performance in sheep, cattle and reproduction performance in beef cattle *(Mahmoud et al., 2014).* Studies on Leptin gene polymorphism and production traits in dairy cattle, sheep and poultry has been reported with promising results and can be considered as one of the best biological markers in animals and human beings *(Nassiry et al., 2008).*

Conclusion

The presence of numerous alleles at a particular Leptin locus is evidence of the long-term evolutionary persistence of the locus. This is suggested by the fact that the alleles in one species are often more closely related to the alleles in closely related species than to the other alleles in the same species. The species wise clustering of Leptin gene might be due to species specific residues and such patterns of the sequences may be explained by gene conversion and balancing selection.

Ispitivanje raznovrsnosti leptin gena u odabranim vrstama preživara i nepreživara

Faith Elijah Akumbugu, Abubakar Ibrahim Zanwa

Rezime

Leptin se pokazao kao ključni element sistema koji reguliše unošenje hrane. Deluje kao centar gladi u hipotalamusu i utiče na regulaciju apetita. Takođe je utvrđeno da leptin gen utiče na prinos mleka kod ovaca, goveda, kao i na reprodukciju u govedarstvu. Genetska karakterizacija za procenu postojećeg biodiverziteta i razlika među važnim stočarskim rasama je suštinski preduslov za olakšanje programa konzervacije na efikasan i značajan način. Ovaj rad je pokušao da prouči analizu raznolikosti leptin gena u određenoj vrsti preživara i monogastričnih životinja. Ukupno dvadeset tri (23) sekvence leptin gena koje pripadaju osam (8) vrsta: goveda (3), ovce (3), koze (3), svinje (3), konj (2), kamila (3) i zečevi (3) su preuzeti iz Genbank-e (www.ncbi.nlm.nih.gov). Usaglašavanje, prevođenje i upoređivanje sekvenci obavljeno je pomoću ClustalW - MEGA 6.0. Utvrđena je vrednost minimalne matrica rastojanja (Dxy) (0,02) između sekvence goveda i koza, dok je maksimalna vrednost Dxy (2,72) utvrđena između goveda i ovaca, kod preživara. U monogastričnim vrstama, najveća Dxy vrednost (17,61) je utvrđena između zeca i kamile, dok je minimalna Dxy vrednost (0,18) primećena između miša i kamile, respektivno. Što je manja matrica udaljenosti, to je bliža sekvenca vrste i manja je genetička razdaljina unutar ili između vrsta, dok veća vrednost Dxy, ukazuje na veću genetička razdaljina unutar i između ispitanih vrsta. Ovaj rezultat bi mogao da bude osnova za selekciju kada se razmatra evolucija i diferencijacija među vrstama.

Ključne reči: studija raznolikosti, leptin, preživari, nepreživari, sekvence, filogenetska analiza

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EFFECTS OF HYDROXYCINNAMIC ACIDS (FERULIC AND P-COUMARIC ACIDS) IN BARLEY CULTIVARS ON CELL WALL COMPONENTS DEGRADABILITY IN RUMEN

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Original scientific paper

Abstract. Barley grain contains hydroxycinnamic acids especially Ferulic (FA) and p-Coumaric acid (pCA) become cross-linked to cell wall polysaccharids as lignification commences that are the major inhibiting factors of biodegradability of plant cell walls in the rumen. Chemical characteristics, FA and pCA content of 11 Iranian barley cultivars determined. Using 3 fistulated ewes, the effects of FA and pCA content on ruminal degradation of dry matter (DM), neutral and acid detergent fiber (NDF and ADF) and lignin were studied. In barley cultivars, DM varied from 82.52 to 90.90 %; NDF varied from 9.64 to 27.34 % DM; ADF varied from 2.03 to 8.13 % DM and lignin varied from 0.87 to 3.03 % DM. The FA content ranged from 151.2 to 354.2 μg/g; and pCA content ranged from 114.5 to 444.4 μg/g of DM. Ruminal degradation parameters for DM, NDF, ADF and lignin were different between barley cultivars. The soluble fraction, slowly degradable, potential degradable, and undegradable fraction of DM were 2.92 to 56.33%; 42.64 to 91.45%; 65.68 to 98.97%, and 1.02 to 34.31%, respectively. The rate of ruminal degradation for DM varied among barley cultivars from 3.64 to 27.81% h^{-1}. The FA was related to rumen indigestible DM, NDF, ADF and lignin, while pCA correlated with ADF. Using multi-regression, FA and pCA were inhibiting factors of ruminal degradability for DM and cell wall components; and FA was the most effective factor to predict DM degradability, while both FA and pCA affected NDF and ADF ruminal degradability.

Key words: hydroxycinnamic acid, Ferulic acid, p-coumaric acid, barley, rumen, degradability

Introduction

Recently, increasing corn prices resulted in using more barely grain as main starch sources in dairy cattle rations. In barley *(Hordeum vulgare L.)*, the starch- and protein-laden endosperm is surrounded by a pericarp encased in a fibrous hull both of which are extremely resistant to damage by chewing and microbial degradation *(Beauchemin et al. 1994)*. Barley grain contains predominant phenolic compounds or low molecular weight hydroxycinnamic acids including FA and pCA *(Hernanz et al. 2001)*. The rate and extent of ruminal degradation of plant cell wall is negatively impacted by complex components such as lignin, cellulose, lignin-carbohydrate, and phenolic-carbohydrate, as well as FA and pCA is believed to be the major inhibiting factors to the ruminal biodegradability of plant cell walls *(Yu et al. 2005)*. However, livestock performance can be improved by increasing the digestibility of feeds.

The FA rapidly deposits in the cell walls at the early stage of lignification, subsequently pCA residue deposits continuously throughout the lignification *(Brett et al. 1999)*. The acylation of polysaccharides was done via feruloyl-CoA, coumaroyl-CoA, and the secretion of phenolic precursors, such as hydroxycinnamates amides and esters into the cell wall of dicotyledons, which were oxidatively linked to the cell wall polymers. The cell walls polysaccharids become cross-linked to monolignols via Hydroxycinnamic acids as lignification commences *(Santiago et al. 2006).* As bifunctional molecules with carboxylic and phenolic bonding sites, these Hydroxycinnamic acids can be involved in both ester and ether linkages. The presence of esterified phenolic compounds may protect the plant against pathogen infestation and generate a chemical barrier that improves disease resistance *(Santiago et al. 2006).* Furthermore, increases in dimeric and monomeric compound content following exposure to light were reported. These compounds influence the mechanical properties of the cell walls, such as rigidity during plant growth *(Miyamoto et al. 1994)*.

Barley grain contains 8% lignin *(NRC, 2001)*. There is no apparent lignin-degrading microorganisms or enzymes in the rumen therefore, its digestibility is relatively low and variable *(Van Soest, 1994)*. Lignin plays a negative role in ruminant nutrition, feed digestion and utilization through three ways *(Moore and Jung, 2001)* :1) lignin inhibits ruminal digestion as a physical barrier to restrict rumen microbes and enzymes acting; 2) lignin reduces plant energy availability by limiting animal fiber utilization, and 3) lignification restricts animal DM intake because it slows down plant DM digestibility and increases the rumen fill effect. The action of lignin seems to depend not only on their amount but also on other factors like cross linking and because of the chemical nature of this heterogeneous compound, it is nearly impossible to extract lignin in any pure form–especially once it polymerizes into ADL *(Raffrenato and Van Amburgh, 2010).* The relative abundance of lignin and the frequently of phenolic compounds cross-links with

polysaccharids appear to be the most important factors limiting energy utilization in barley grain and hull by rumen microorganism *(Casler, 2001)*. Variation of the content of hull, FA, pCA, NDF, ADF, ADL and characteristics of particle size reduction in various barley varieties may cause differences in the digestibility of barley grain. Therefore, greater knowledge about the relationship between the digestibility in the rumen and the specific chemical and physical profiles of barley grain will provide useful information for barley breeders and cattle producers. The objectives of this research were to identify interrelationships among FA and pCA and cell wall component of 11 barley cultivars and to determine their influence on DM, NDF, ADF and ADL ruminal degradation.

Material and Methods

Barley cultivars

Eleven barley cultivars were used as substrates in this experiment. These cultivars (Table 1) were grown at Karaj Research Station, Iran, in one field under the similar soil and environmental conditions.

Table 1. Variety and growing condition of eleven barley samples utilized in this study

	Variety [a]	Seed coat	Climate	Winter/spring variety
1	Bahman	Hulled	Cold mountains	Winter
2	Fajr30	Hulled	Mediterranean	Moderate
3	Kavir	Hulled	Mediterranean	Spring- autumn
4	Makooei	Hulled	Cold mountains	Winter
5	Nimrooz	Hulled	Hot coastal dry	Spring
6	Nosrat	Hulled	Mediterranean with spring rains	Moderate
7	Reyhan03	Hulled	Mediterranean	Spring- autumn
8	Sahra	Hulled	Caspian mild and wet	Spring-autumn
9	UH-12	Hulless	Mediterranean	Spring
10	Usef	Hulled	Mediterranean	Spring
11	Valfajr	Hulled	Mediterranean	Spring- autumn

[a] Eleven varieties of barley were grown at Karaj Research Station Farm (Karaj, Iran) using standard agronomic production practices for barley production.

Animals and diet

Three fistulated ewes (approximately 2 years old, Body weight = 35 ± 2 kg) those were equally fed a total mixed ration at maintenance level that included alfalfa hay

and barely grain with 75:25 ratios were used. Diets also contained vitamin-mineral premix, limestone, and salt. Water and mineral block were available over the experiment. The diets were offered in two equal meals at 0700 h and 1900 h. The animals were adapted to the basal rations for two weeks prior to ruminal incubation of the bags. All procedures used in this study were approved by the Animal Care and Use Committee of Proposing a National Ethical Framework for Animal Research in Iran *(Mobasher et al. 2008).*

Chemical Analyses

Feed samples were analyzed for dry matter (DM) by drying at 105°C. The neutral (NDF) and acid (ADF) detergent fibers were determined according to the procedure described by *Van Soest et al. (1991)*, and acid detergent lignin (ADL) was determined *(Feldsine et al., 2002)*. Two hydroxycinnamic acids (FA and PCA) in barley cultivars were determined using High Performance Liquid Chromatography (HPLC) and barley pretreatment for HPLC analysis was done using the method of *Hernanz et al. (2001)* with some modifications. For extraction, whole barley grain was cleaned, ground through a 1-mm mesh screen, hydrolyzed by adding 2 *M* NaOH solution (100 mL) per 1gr followed by incubation at ambient temperature for 16 h while samples wrapped with Aluminum foil. Then samples acidified with 6 *M* HCl to pH 2.6, and then extracted five times with equal volumes of ethyl acetate. The solutions were combined and evaporated to dryness with rotary evaporator at 45ºC. The residue was dissolved in 1 mL methanol HPLC grade and filtered through a 0.45 μm syringe filter (Millipore) and 20 μL samples were analyzed by HPLC using standard FA (46278) and pCA (C9008) that were purchased from Sigma. A Knaure smartline 1100 HPLC system and UV detector was employed. Separation was performed by isocratic elution with a mobile phase of water-acetic acid (98:2; v/v) (A) and methanol-butanol (92:8; v/v) (B), in a column C18 (250×4.6 mm, 5 mm). The gradient conditions were as follows 0 -10 min, 85% A and 15% B; 10 - 20 min, 50 % A and 50% B; 20 - 30 min, 85% A and 15 % B. Flow rate was 1 mL/min; and injection volume was 20 μL. The content of FA and pCA were calculated from chromatograms that were recorded at 245 nm.

Rumen incubation

Using the nylon bag technique, the barley samples were ground to pass a 2 mm screen. Then approximately 3 g of dry samples were weighed into 7×14 cm^2 and 40 ± 5 μm pore size nylon bags. Bags were incubated in the rumens of three ewes and were removed after 0, 1, 3, 6, 9, 12, 24, 36 and 48 h of incubation. Immediately after removing from the rumen, the bags were washed with cold tap water until clear and then were dried at 55°C for 48 h. The bags were weighed and residues were removed and then analyzed for DM, NDF, ADF and ADL. The disappearance of DM, NDF, ADF and ADL at each incubation time was calculated from the

proportion remaining in the bag after incubation in the rumen. The disappearance rate was fitted to the following equation given *(Orskov and McDonald, 1979)*:
Disappearance (%) = $a + b\times(1 - e^{-ct})$
where, a = soluble fraction (% of total), b = degradation fraction (% of total), t= time of rumen incubation (h), and c = rate of degradation (% h^{-1}). The effective degradability of DM, NDF, ADF and ADL was calculated by the equation of *Orskov and McDonald (1979):*
Effective degradability = $a + [(b \times c)/(c + k)]$
Where, k is the estimated rate of outflow from the rumen. Effective degradability of DM, NDF, ADF and ADL was estimated at ruminal outflow rates of 6% h^{-1}.

Statistical Analysis

Using a completely randomized design with eleven treatments with three replicates, the data were analyzed with the PROC GLM of SAS® (20). Duncan Multiple Range test were used for means comparison when significance was declared at $P<0.05$. In addition, PROC CORR procedure of SAS® (2002) was used to examine the correlations among the barley components such as FA, PCA, NDF, ADF and ADL and ruminal degradation parameters of DM, NDF, ADF and ADL. A multi-regression analysis was carried out using the PROC REG of SAS ® (2002) in order to develop prediction equations to show the effects of FA, pCA on ruminal degradation parameters of DM, NDF, ADF and ADL.

Results

Chemical compositions

The DM, FA, PCA, NDF, ADF and ADL content of the eleven barley cultivars were significantly different ($P<0.0001$; Table 2). Dry matter content varied from 82.52 to 90.90 % with a mean value of 88.35 %. Kavir and UH-12 had the highest and the lowest DM content. NDF varied from 9.64 to 27.34 % DM with mean of 22.55 % DM. ADF was much lower than NDF, ranging from 2.03 to 8.13 % DM, with an average of 6.26 % DM. In addition, ADL varied from 0.87 To 3.03 % DM with a mean of 2.18% DM. Bahman, Valfajr and Nimrooz had higher NDF, ADF and ADL. UH-12 had the lowest NDF. The FA content ranged from 151.2 to 354.2 µg/g with the highest in Bahman and lowest in UH-12, and PCA content ranged from 114.5 to 444.4 µg/g of DM with the highest in Valfajr and lowest in UH-12. In addition, the starch, CP, EE, ash and NFC content of were significantly different among the varieties (Table 2).

Table 2. Chemical compositions of different cultivars of barley grains cell wall [1, 2]

Cultivar	Unit	Bahman	Fajr30	Kavir	Makooei	Nosrat	Nimrooz	Reyhan03	Sahra	Valfajr	UH-12	Usef	SEM	P-value
DM	%	8910^{e}	90.39^{d}	90.90^{c}	88.08^{i}	88.08^{gh}	88.41^{h}	97.35^{a}	88.51^{g}	88.96^{f}	82.52^{j}	96.68^{b}	0.001	<0.0001
NDF	% DM	27.34^{a}	24.40^{c}	24.69^{b}	23.86^{f}	23.54^{g}	23.51^{gh}	24.26^{d}	23.41^{i}	23.46^{hi}	9.64^{j}	24.12^{e}	0.001	<0.0001
ADF	% DM	6.57^{e}	7.52^{b}	6.75^{d}	6.26^{h}	6.52^{f}	6.34^{g}	7.24^{c}	6.04^{i}	8.13^{a}	2.03^{k}	5.17^{j}	0.0004	<0.0001
ADL	% DM	2.02^{c}	2.01^{c}	2.00^{c}	3.01^{a}	2.50^{b}	3.03^{a}	3.00^{a}	1.5^{d}	2.03^{c}	0.87e	2.00c	0.004	<0.0001
FA	µg/g DM	354.2^{a}	222.5^{i}	277.1^{f}	277.6^{e}	329.4^{c}	265.4^{g}	277.5^{e}	257.3^{h}	333.4^{b}	151.2j	308.6^{d}	0.04	<0.0001
PCA	µg/g DM	192.2^{d}	237.7^{b}	178.4^{e}	219.4^{c}	162.3^{f}	118.2^{i}	222.5^{c}	131.5^{h}	444.4^{a}	114.5i	147.3^{g}	11.30	<0.0001
Starch	% DM	56.79^{l}	57.64^{j}	57.15^{K}	58.07^{h}	59.43^{c}	59.26^{d}	58.44^{f}	59.24^{de}	59.95^{b}	62.52^{a}	58.48^{g}	0.0001	<0.0001
CP	% DM	10.34^{f}	10.03^{f}	10.50^{e}	11.50^{a}	9.50^{h}	11.16^{c}	10.53^{e}	10.68^{d}	10.46^{e}	11.32^{b}	9.87^{g}	0.003	<0.0001
EE	% DM	2.86^{i}	4.17^{a}	3.58^{c}	2.99^{g}	3.51^{d}	3.08^{f}	2.62^{j}	2.91^{h}	3.65^{b}	2.75^{e}	3.11^{e}	0.0001	<0.0001
Ash	% DM	2.08^{gh}	2.23^{e}	2.38^{d}	2.49^{b}	2.11^{g}	2.44^{c}	2.82^{a}	2.01^{h}	2.10^{h}	1.97^{j}	2.16^{f}	0.0004	<0.0001
NFC	% DM	57.38^{j}	59.17^{i}	58.85^{f}	59.16^{i}	61.34^{b}	59.81^{g}	59.77^{h}	60.99^{c}	60.33^{d}	74.32^{a}	60.74^{c}	0.001	<0.0001

[a-k] means within a raw with differing superscripts are significantly different (P<0.05).

[1] DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; FA, Ferulic acid; PCA, p-coumaric acid, CP, crud protein, EE, ether extract, NFC, non-fiber carbohydrate.

Rumen degradation kinetics

Ruminal degradation parameters were significantly different between barley cultivars for DM, NDF, ADF and ADL (Table 3). The soluble fraction ranged between 2.92 to 56.33 % of DM. Nimrooz had the highest (91.45%) slowly degradable fraction than others, and UH-12 had the lowest (42.64%) slowly degradable fraction (Table 2; $P<0.0001$). However, Bahman had higher (34.31%) and UH-12 had lower (1.02%) undegradable fraction for DM ($P<0.0001$). The potential degradable of DM in barley cultivars ranged between 65.68 to 98.97% ($P<0.0001$), that was highest for UH-12 and lowest for Bahman. The rate of degradation varied among barley cultivars and was lowest for Fajr30 cultivar (3.64% h^{-1}) and highest for UH-12 cultivar (27.81% h^{-1}; $P<0.0001$).

The soluble fraction for NDF differed ($P<0.0001$) from 0.16 in Valfajr to 10.61% in Nimrooz. This fraction for ADF ranged from 0.11 in Kavir to 3.0 % in Bahman, and in ADL differed ($P<0.0001$) from 0.03 in Fajr30 to 5.86 % in Bahman (Table 3). Also, the *b* fraction of NDF, ADF and ADL differed ($P<0.0001$) from 16.87 % in Usef to 42.80 % Bahman; 19.33 % in Valfajr to 10.07% in UH-12 and 4.36 % in Bahman to 23.85% in UH-12, respectively. The undegradable fraction for NDF, ADF and ADL ranged 54.39 % in Fajr30 to 82.06 % in Bahman; 79.78 % in UH-12 to 89.67 % in Valfajr, and 74.67 % in UH-12 to 89.77 % in Bahman, respectively. In contrary, the degradable fraction of NDF, ADF and ADL in barley cultivars were 17.93 % in Fajr30 to 45.60 % Bahman; 10.32 % in Valfajr to 20.22 % in UH-12, and 10.22 in Bahman to 25.33 % in UH-12, respectively. In addition, the lowest and greatest degradation rate of NDF were observed in Sahra (3.76 $\%h^{-1}$) and Fajr30 (35.83$\%h^{-1}$), respectively. Also, K_d for ADF ranged from 3.38 to 14.08$\%h^{-1}$ for UH-12 and Nimrooz, respectively; and for ADL ranged from 3.09 to 16.71$\%h^{-1}$ in Makooei and Bahman, respectively. In addition, the barley cultivars differed in effective degradability of DM, NDF, ADF and ADL (Table 3).

Table 3. Dry matter, neutral detergent fiber, acid detergent fiber and acid detergent lignin ruminal degradability parameters of barley cultivars

Item	Fajr-30	Nimrooz	Bahman	Sahar	Rey	Usef	Makooei	Kavir	Nosrat	Valfajr	UH-12	SEM	P-Value
Dry matter													
a	7.08^{e}	2.92^{g}	4.82^{f}	10.35^{d}	7.13^{e}	10.13^{d}	17.70^{b}	19.62^{b}	12.80^{c}	3.85^{f}	56.33^{a}	0.002	<0.0001
b	87.25^{ab}	91.45^{a}	60.86^{d}	87.6^{ab}	85.12^{ab}	60.72^{d}	72.20^{c}	73.38^{c}	81.85^{b}	89.01^{a}	42.64^{e}	0.006	<0.0001
a +b	94.34^{ab}	92.37^{ab}	65.68^{c}	97.95^{a}	92.25^{ab}	70.85^{c}	89.91^{b}	93.00^{ab}	94.65^{ab}	92.86^{ab}	98.97^{a}	0.006	<0.0001
c	5.66^{bc}	7.62^{bc}	34.31^{a}	2.04^{c}	7.74^{bc}	29.15^{a}	10.08^{b}	6.99^{bc}	5.34b^{c}	7.13^{bc}	1.02^{c}	0.006	<0.0001
K_d	3.64^{f}	5.13^{f}	9.74^{de}	11.46^{d}	21.39^{b}	8.12^{e}	12.13^{d}	15.15^{c}	11.14^{d}	12.06^{d}	27.81^{a}	0.002	<0.0001
EDDM[1]	39.63^{ef}	43.18^{ef}	42.37^{ef}	67.80^{c}	73.60^{b}	45.03^{e}	65.97^{cd}	72.17^{b}	65.99^{cd}	63.27^{d}	84.99^{a}	0.004	<0.0001
Neutral detergent fiber													
a	5.90^{b}	0.16^{d}	1.05^{d}	0.67^{d}	0.90^{d}	0.34^{d}	5.71^{b}	0.32^{d}	1.49^{d}	10.61^{a}	2.89^{c}	0.001	<0.0001
b	39.70^{ab}	32.02^{cd}	16.87^{g}	25.70^{e}	34.05^{c}	42.80^{a}	39.70^{ab}	41.33^{ab}	30.29^{d}	21.37^{f}	38.13^{b}	0.003	<0.0001
a +b	45.60^{a}	32.18^{c}	17.93^{e}	26.37^{d}	34.95^{c}	43.14^{ab}	45.41^{a}	41.65^{b}	31.78^{c}	31.98^{c}	41.03^{b}	0.003	<0.0001
c	54.39^{e}	67.81^{c}	82.06^{a}	73.62^{b}	65.04^{c}	56.85^{de}	54.58^{e}	58.34^{d}	68.21^{c}	68.01^{c}	58.97^{d}	0.003	<0.0001
K_d	12.29^{a}	7.12^{d}	9.29^{bc}	3.76^{e}	7.97^{cd}	4.53^{e}	10.49^{b}	4.52^{e}	8.82^{c}	8.05^{cd}	5.12^{e}	0.001	<0.0001
EDNDF	32.55^{a}	17.55^{g}	11.30^{h}	10.39^{h}	20.32^{d}	18.73^{ef}	30.92^{b}	18.06f^{g}	19.44^{de}	22.66^{c}	20.39^{d}	0.001	<0.0001
Acid detergent fiber													
a	1.25^{cd}	2.39^{b}	1.48^{c}	1.48^{c}	0.21^{e}	0.78^{d}	3.00^{a}	0.11^{e}	0.20^{e}	0.25^{e}	0.88^{d}	0.0005	<0.0001
b	13.17^{bcd}	11.94^{bcd}	11.27^{bcd}	14.50^{bcd}	15.17^{abc}	15.73^{ab}	14.87^{abcd}	10.68^{cd}	15.51^{abc}	10.07^{d}	19.33^{a}	0.004	<0.0001
a +b	14.43^{bcde}	14.33^{bcde}	12.76^{cde}	15.98^{abc}	15.38^{bcd}	16.51^{abc}	17.88^{ab}	10.79^{de}	15.71^{abc}	10.32^{e}	20.22^{a}	0.004	0.003
c	85.57^{abcd}	85.67^{abcd}	87.24^{abc}	84.01^{cde}	84.61^{bcd}	83.48^{cde}	82.12^{de}	89.21^{ab}	84.28^{cde}	89.67^{a}	79.78^{e}	0.004	0.003
K_d	4.65^{cd}	14.08^{a}	9.25^{b}	4.95^{cd}	4.25^{cd}	3.87^{cd}	4.52^{cd}	7.49^{bc}	7.65^{bc}	9.19^{b}	3.38^{d}	0.004	<0.0001
EDADF	6.62^{ef}	10.69^{a}	8.22^{cd}	7.95^{d}	6.23^{fg}	6.93^{ef}	9.21^{b}	5.59^{g}	8.88^{bc}	6.32^{f}	7.19^{e}	0.0009	<0.0001
Acid detergent lignin													
a	0.03^{e}	1.55^{c}	5.86^{a}	4.76^{b}	0.50^{de}	4.75^{b}	0.40^{de}	0.87^{cde}	0.97^{cd}	0.68^{cde}	0.47^{c}	0.0008	<0.0001
b	12.34^{bc}	13.16^{b}	4.36^{e}	6.58^{de}	10.64^{c}	6.83^{d}	13.71^{b}	10.04^{c}	10.19^{c}	13.36^{b}	10.96^{a}	0.002	<0.0001
a +b	12.38^{cd}	14.71^{b}	10.22^{d}	11.34^{d}	11.14^{d}	11.59^{d}	14.11^{bc}	10.91^{d}	11.16^{d}	14.04^{bc}	25.33^{a}	0.002	0.0002
c	87.61^{ab}	85.29^{c}	89.77^{a}	88.65^{a}	88.85^{a}	88.40^{a}	85.88^{bc}	89.08^{a}	88.83^{a}	85.95^{bc}	74.67^{d}	0.002	0.0002
K_d	4.47^{cd}	4.09^{cd}	16.71a	12.49^{b}	7.36^{c}	12.74^{b}	3.09^{d}	5.23^{cd}	5.04^{cd}	4.52^{cd}	6.78^{c}	0.003	<0.0001
EDADL	5.24^{ef}	6.84^{c}	9.05^{b}	9.11^{b}	6.33^{cde}	9.22^{b}	5.03^{f}	5.54^{def}	5.62^{def}	6.39^{cd}	14.12^{a}	0.001	<0.0001

[a-h] means within a raw with differing superscripts are significantly different (P<0.05).

[1] DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; FA, Ferulic acid; PCA, p-coumaric acid.

[2] *a*, Soluble fraction (%); *b*, slowly degraded fraction (%); *c*, undegradable fraction, *a +b*, degradation fraction (%); K_d, rate of degradation (% h^{-1}); EDDM, EDNDF, EDADF and EDADL, effective degradability of dry matter, neutral detergent fiber, acid detergent fiber, and acid detergent lignin, respectively (0.06 h^{-1}).

Discussion

Chemical compositions

The DM level of the barley grain cultivars used in the present study was lower than those reported by *Ghorbani and Hadj-Hussaini (2002)* who showed that the DM level of 10 barley grain cultivars ranged from 92 to 94 %. *Abdi et al. (2011)* reported that the DM values for 16 cultivars of barley grains and indicated it ranged from 92.5 to 93.5%. The NDF, ADF and ADL concentrations of the barley grain cultivars used in the present study had more variance than those of reported by *Du et al. (2009)*, that examined six Canadian barley varieties and reported NDF, ADF and ADL values varied from 17.6 to 21.9, 5.5 to 7.0 and 1.7 to 2.1 %DM. Also, the FA and pCA content ranged from 555 to 663 and 283 to 345 μg/g of DM, respectively *(Du et al. 2009)*. *Holtekjolen et al. (2006)* studied five varieties of hulled two-row barley grown in Norway in 2002 and observed that FA content varied from 512 to 723 μg/g of DM, and pCA content varied from 114 to 244 μg/g of DM. The pCA content in the present study was similar, but FA content was lower. This variation might be due to the difference between cultivars and growing conditions. The cultivars used this study were grown in the same field under the same soil and environmental conditions. Thus, variation between them is likely a result of the different cultivars type. *Hernanz et al. (2001)* indicated that the concentrations of FA and pCA in barley were influenced by the genotype. *Du et al. (2009)* showed that barley variety had a significant effect on the content of FA, pCA, NDF, ADF, ADL and hull contents in various barley cultivars, and concluded barley variety plays an important role in determining the quality of barley as a feed.

Rumen degradation kinetics

Ruminal degradation parameters were significantly different between barley cultivars for DM, NDF, ADF and ADL (Table 2) that were comparable to the results outlined by *Du et al. (2009)*. In contrary, the potential degradable fraction provides the major source of slowly fermenting starch for rumen microbes *(Ghorbani and Hadj-Hussaini, 2002)*. However, the quantitative importance of lignin in the cell wall, their variable structure, and a variety of cross-linkages between cell-wall components all have variable depressive effects on cell-wall carbohydrate degradation by microorganisms. *Bunzel et al. (2003)* suggested that FA, pCA, and other hydroxycinnamic acids, like Sinapic acid, may also play a similar role to FA in plant cell walls forming crosslinkages. The FA may also conjugate to cell wall nitrogenous compounds or proteins, and in this way FA regulates cell wall rigidity and decreases cell wall digestibility *(Van Soest 1994)*.

Also in present study, the disappearance kinetics of DM, NDF, ADF and ADL in the rumen differed among barley cultivars. Large differences in degradability among barley varieties can be attributed to broad vary in composition such as cell

wall components in barley or its hull. A good feed barley variety should have these traits: high in nutrients, good nutrient availability, slow rate of rumen starch fermentation and maintaining large particle size after mechanical processing *(Du and Yu, 2011)*. The DM soluble fraction had more variance than those of reported by *Khorasani et al. (2000)* that reported solubility of DM ranged from 35.2 to 59.4% in sixty Canadian barley cultivars. Also, *Lehmann et al. (1995)* reported solubility values of 25 to 40.7%. The difference in the proportion of the soluble fraction is related to a number of factors including bag pore size, particle size of the grain, and the ratio of the sample weight: bag surface area and the washing technique *(Ghorbani and Hadj-Hussaini, 2002).* Since the bag pore size was standardized across the trial, it can be assumed that the differences in the results may be attributed to variations in washing technique and an element of variation in grain particle sizes, resulting in different amounts of small particles being washed out rather than being truly soluble. *Ghorbani and Hadj-Hussaini (2002)* reported that DM slowly degradation fraction for 10 Iranian barley grain cultivars ranged from 42.2 to 49.0%, whereas, *Cleary et al. (2011)* reported the b values of DM varied from 46.6 to 63.1%, however in the present study had more variance than them (42.64 - 91.45%). Also, *Cleary et al. (2011)* reported DM undegradable fraction ranged from 5.3 to 27.6%, whereas, *Ghorbani and Hadj-Hussaini (2002)* showed that DM c fraction ranged from 13.5 to 36.0%. The degradable fraction is the portion of the grain which is slowly digested within the rumen when allowed sufficient time. It is an important source of slowly fermenting starch providing energy for the rumen microbes *(Cleary et al., 2011). Khorasani et al. (2000)* reported degradable values of 25 to 40.7%, whereas, *Du and Yu (2011)* reported *a* + b fraction ranged from 79.3 to 82.8%. In present study, UH-12 provided more nutrients for ruminants than others cultivars, because of its higher (98.97 %DM) potential digestible fraction and lower (1.02 %DM) undegradable fraction of DM. Also, UH-12 had lowest content of NDF, ADF, ADL, FA and pCA than the others (Table 2). UH-12 is a hull-less barley cultivar; and had the lowest fiber and phenolic components. The hull fraction of barley seed is usually high in fiber that is made up of cell wall polysaccharides such as cellulose and hemicellulose that are usually more resistant to degradation. Hull-less barley does have surrounding hull during its life cycle, but it is very loosely attached to the kernel and sheds readily, and therefore the kernel becomes naked during threshing. Also, it had highest rate of degradation in rumen and effective degradability of DM in comparison with other cultivars (27.81%). The rate of DM degradation within the rumen is influenced by a number of interactions between the rumen microorganisms and barley kernel tissue. The rate at which digestion occurs influences the rate of passage, site of digestion, form of substrates and the efficiency of feed utilization. The rate and extent of ruminal digestion is important as a high rate of degradation within the rumen causes the higher production of VFA for absorption, drop in pH which can result in ruminal acidosis, a reduction in microbial protein synthesis,

fiber digestion and feed intake *(Van Soest, 1994)*. Therefore, when hull-less cultivars such as UH-12, it is important to consider balancing the extent and rate of fermentation in the rumen. Fajr30 had lowest rate of DM degradation, therefore using Fajr30 in ration could decline occurrence of acidosis. *Cleary et al. (2011)* studied tow malting barley varieties and reported the K_d from 12.7 to 16.5 $\%h^{-1}$. Also, *Khorasani et al. (2000)* found that the K_d ranged from 20 to 62$\%h^{-1}$, whereas, *Ghorbani and Hadj-Hussaini (2002)* reported that the K_d varied from 25.6 to 31.5$\%h^{-1}$. UH-12 showed higher EDDM (84.99 %0.06h^{-1}), which indicated that UH-12 tended to be more extensively degraded in the rumen. *Ghorbani and Hadj-Hussaini (2002)* found the EDDM ranged from 75.4 to 79.5%0.08h^{-1}, and *Khorasani et al. (2000)* reported that it ranged from 73.8 to 89.0%0.09h^{-1}. In our study, EDDM had ranged from 39.63 to 84.99 % when we considered the passage rate 0.06%/h; Table 3).

There was a large variation between chemical compositions and DM, and NDF rumen degradability in Iranian barley cultivars. Chemical compositions were useful in some cases in making inferences about diet digestibility, but could not be used as the sole means of predicting nutritional quality. Digestibility of NDF is a major factors contributing to differences among barley cultivars that has higher fiber and lower starch content than most other grains. A range of variation for NDF digestibility exists. The NDF represents the total structural cell wall components (cellulose and hemicellulose as well as lignin except pectin), so rumen indigestion of NDF residue was lower than ADF and ADL, and averaged 64.35% (from 63 to 68% total tract undigested NDF for whole barley grain (*Feng et al., 1995)*). *Beauchemin et al. (2001)* found it was 53% for the whole barley grain. *Du and Yu (2011)* observed different effects of variety on the rumen undigested residues of barley NDF and ADF, except for ADL residues. Among the eleven Iranian cultivars, Bahman showed considerably higher NDF residue than others (82.6% of DM) that probably related to the highest NDF content in the Bahman (27.34% of DM, Table 2). In contrast, Fajr30 had the lowest NDF residue and the highest NDF potential degradable among cultivars, which might imply that most NDF in Fajr30 was degraded in rumen.

The ADF contains principally cellulose and lignin, which is less digestible than NDF. *Du and Yu (2011)* found that rumen undigested ADF for stream-rolled barley was 80% compared to 50 to 65% of undigested NDF. In this study, ADF residue averaged 85.05 and its potential degradable averaged 14.93%. Among the eleven cultivars, Valfajr had the highest ADF residue than others, and UH-12 had the lowest. Less ADF is always preferred in feed barley selection, whereas Valfajr had the highest original ADF.

Although ADL is thought of as low in digestibility, in the present study, roughly 2% of ADL was soluble in the rumen. Although lignin content in most plants and barley is relatively low, it is the most recalcitrant fiber component. *Du et al. (2009)* reported 10% of ADL was soluble in rumen. The ADL content of barley was quite

low (about 0.87 to 3.03% of DM). In practice, ADL digestibility of barley grain is seldom analyzed. Nevertheless, results showed that Bahman had highest ADL residue than others, and UH-12 had the lowest. Lignin is the typical complex phenolic polymer which impedes animal digestion of plant cell walls. In the animal alimentary tract, proanthocyanidins can inhibit protein digestion and utilization by forming an insoluble complex *(Slafer et al. 2001)*. There are also small quantities of simple phenolic acid residues such as FA and ρCA *(Slafer et al. 2001).*

The presence of excessive hydroxycinnamic acids (especially FA, pCA) in plant cell walls may reduce animal digestibility and productivity. Although phenolic acids (mainly FA and pCA) are present in comparatively low levels, they impose effective and important effects on the physical and chemical properties of barley. Free phenolic acids have oxidative properties and antibacterial functions which help to defend the kernel from micro-organism attack. When these phenolic acids form intricate cross-linkages with lignin and cell wall polysaccharides, they become the inhibitory factors for plant cell wall rumen degradation. Since most esterified pCA on lignin are not covalently attached to other cell wall polymers, they should not directly influence cell wall rumen degradability. Some cell wall models show how they can interfere with ferulate-lignin cross linking and in some cases reduce the proportion of lignin bound to cell wall. Ether linkage between FA and lignin has been used a measure of cross-linking between lignin and arabinoxylans and defined as the most important factor limiting energy utilization *(Casler, 2001)*. Ester-linked FA had generally a negative relationship except in Brown Mid Rib (BMR) corn hybrids for 24h and positive for 96h NDF digestibility *(Raffrenato and Van Amburgh, 2010).* The ferulate primarily form as esters of arabinoxylans and later they cross-link through ether linkages with lignin. So esters of FA should not necessarily limit NDF degradation. This has probably more to do with the degree of arabinoxylans substitution. *Raffrenato and Van Amburgh (2010)* found that forage groups demonstrated different relationships for digestibility from positive to negative in NDF residues, but the ADF residues were instead characterized by a consistent negative relationship among all forage groups and similar results were obtained for 96 h NDF digestibility. However, in this study, we obtained consistent negative relationship with potential degradable of DM, NDF, ADF, and ADL (Table 4). *Raffrenato and Van Amburgh (2010)* found that negative effect of etherified FA on NDF digestibility has been found in elongating internodes in maize but not in internodes that had stopped the elongation process and confirms the hypothesis that secondary cell wall development may mask the negative impact of etherified FA on NDF digestibility. Also, BMR corn shows higher content of etherified FA compared to conventional corn in NDF residues, demonstrating that etherified FA is not always a good indicator of cross-linking between lignin and arabinoxylans. However, this relationship changes when ADF residues were analyzed for ether linked FA, showing how the solubilization or branching of the lignin structure has in this case more importance than linkages.

Acid detergent solution in this case might dissolve those FAs that only etherified (instead of having and ester-ether linkage).

Table 4. Correlation between DM, NDF, ADF, ADL, FA and pCA of eleven varieties and ruminal degradability parameters

Item	NDF (g/kg)	ADF (g/kg)	ADL (g/kg)	FA	PCA
Chemical characteristics					
NDF(g/kg)					
ADF(g/kg)	0.830***				
ADL (g/kg)	0.704***	0.578***			
FA	0.679***	0.635***	0.441*		
pCA	0.292	0.629***	0.132	0.392*	
Degradation parameters of DM					
a	-0.843***	-0.860***	-0.613***	-0.715***	-0.350*
b	0.621***	0.792***	0.531**	0.288	0.362*
a +b	-0.276	-0.060	-0.090	-0.568***	-0.031
c	0.276	0.060	0.090	0.568***	0.031
K_d	-0.672***	-0.563***	-0.345*	-0.445**	-0.098
Degradation parameters of NDF					
a	0.037	0.314	-0.051	0.026	0.842***
b	-0.071	-0.320	0.065	-0.520**	-0.343
a +b	-0.056	-0.196	0.043	-0.505**	-0.015
c	0.056	0.196	-0.043	0.505**	0.015
K_d	0.343	0.464**	0.437*	0.163	0.391*
Degradation parameters of ADF					
a	0.188	-0.102	0.282	-0.140	-0.254
b	-0.475**	-0.615***	-0.227	-0.460**	-0.419*
a +b	-0.402*	-0.615***	-0.140	-0.477**	-0.470**
c	0.4022*	0.615***	0.140	0.477**	0.470**
K_d	0.200	0.324	0.322	0.367*	0.094
Degradation parameters of ADL					
a	-0.003	-0.220	-0.337	0.315	-0.347*
b	-0.710***	-0.539**	-0.256	-0.728***	0.004
a +b	-0.858***	-0.757***	-0.472**	-0.726***	-0.163
c	0.858***	0.757***	0.472**	0.726***	0.163
K_d	-0.029	-0.164	-0.339	0.288	-0.260

*P< 0.05, **P< 0.01, ***P< 0.001;

[1]*a*, Soluble fraction (%); *b*, slowly degraded fraction (%); *c*, undegradable fraction, *a +b*, degradation fraction (%); K_d, rate of degradation (% h^{-1}).

Correlation between chemical components and ruminal degradation parameters

Correlation between NDF with ADF, ADL, and FA and between ADF with ADL, FA and pCA was significantly high (Table 4). Also correlation between ADL with FA was significant, but between ADL with pCA was not statistically significant. However, correlation between FA and pCA was significant. The FA correlated to the content of NDF, ADF and ADL, but pCA only were significantly correlated to ADF. The correlation between FA and cell wall components such as NDF, ADF and ADL was relatively stronger than pCA**.** The high correlation could be explained by the different bonding models between FA and pCA in plant cell walls. The pCA is heavily esterified to lignin, and seldom linked to cell wall polysaccharides, while FA is esterified to polysaccharides, etherified to lignin, and forms cross-linkages between polysaccharides and lignin, and among polysaccharides *(Van Soest, 1994).* There is some evidence which suggests that phenolic acids may limit the digestibility of the plant cell wall in the ruminants. The FA and pCA are covalently linked to plant cell wall polysaccharides by ester bonds and to lignin by both ester and ether bonds *(Hernanz et al., 2001; Lam et al., 1992)* and directly or indirectly involved in affecting the digestibility of cell wall polysaccharides *(Grabber et al., 2004).* These phenolic acids are esterified to arabinoxylans within the plant cell wall, and digestibility of plant cell walls has been related to amounts of phenolic acids released by alkali treatment. Formation of ester bonds between phenolic acids and plant wall polysaccharides through *in vitro* syntheses, while not entirely representative of naturally occurring esters, reduced biodegradation of carbohydrates, further supports the contention that phenolic esters limit carbohydrate degradation by ruminal microorganisms.

Also, FA had positive correlation with rumen indigestible DM, NDF, ADF and ADL while pCA had just positive correlation with rumen indigestible ADF, and both had similar but negative effect on potential degradable fraction. The FA and pCA had effect on rapidly degradable fraction of DM, which for FA is relatively stronger than that pCA. FA and pCA are both esterified and etherified to plant cell wall components *(Du and Yu, 2011).* Also, FA negatively corrected with slowly degradable fractions of NDF, ADF and ADL, but pCA alone had significantly effect on slowly degradable of DM and ADF. The FA corrected with rate of degradability (K_d) fraction of DM and ADF, and pCA only corrected with rate of degradation fraction of NDF. Generally, results can be meaning that FA and pCA in barley grain reduce the degradability of barley grain in the rumen. The negative effects of barley fiber have been studied extensively. The NDF, ADF and ADL contents were significantly correlated to in situ rumen degradation kinetics of DM, except fraction of a+b and c were not significantly affected by NDF, ADF and ADL. These relations were negative with *a*, *a*+b and K_d fraction and positive with *b* and c fraction of DM. Cell wall fiber contents were a little correlated to *in situ*

rumen degradation kinetics of NDF, and showed no correlation effect with the ruminal degradability kinetics, except K_d. Ruminal degradability kinetics of ADF includes *b*, *a*+b and c significantly corrected with NDF and ADF, but had no correlation with ADL. The b, and *a+b* fractions of ADF negatively corrected to NDF and ADF; and the ADF c fraction positively corrected to NDF and ADF. Ruminal degradability kinetics of ADL includes b, a+b and c significantly corrected with NDF ADF and ADL.

The FA and pCA of barley grain reduce the ruminal degradability parameters of barley grain NDF, ADF and ADL. The rumen degradability of plant cell walls are improved by releasing FA and pCA from plant cell walls and by reducing FA cross-linking in the plant *(Jung and Phillips, 2008). Khorasani et al. (2002)* observed that FA content in barley grain positive effects on *in situ* residue of DM, NDF and ADF, but pCA positive effects only on residue of DM and NDF, which means that FA and pCA in barley grain had negative correlation on ruminal degradability of barley grain. *Jung and Phillips (2008)* also observed the negative correlation between the content of FA and the degradation of Lucerne cell walls. Our results showed that FA had more inhibitory effects than pCA. This probably results from the differences in bonding models. *Grabber et al. (2004)* reported that FA is extensively and covalently linked to cell wall components, forms ester/ether bridges between polysaccharides and lignin, and forms ester/ester bridges among polysaccharides, while pCA is esterified to lignin. Therefore, FA inhibits the degradability of plant cell wall polysaccharides while pCA is deemed to be an indicator of lignification and exerts a negative influence directly or indirectly through lignin. In addition, *Grabber et al. (2004)* suggested that lignin composition does not directly affect the degradability of cell walls by fungal enzymes or by rumen microorganisms. According to current information, barley cultivars with less FA and pCA content would be a good candidate for feed barley and the correlation analysis results implied that reduction of barley FA and pCA content could increase the degradability of barley grain in ruminants.

Prediction of ruminal degradability kinetics using FA and pCA

The multi-regression analysis to find the most important variable to predict of ruminal degradability kinetics using FA, pCA with a tested multi-regression model as follows:

Y (degradation kinetics) = $FA + pCA + FA^2 + pCA^2 + FA \times pCA + FA^2 \times pCA^2$

The best models deduced from the stepwise multi-regression analysis are presented in Table 5.

Table 5. The best models deduced from the stepwise multi-regression analysis

Predicted variable (y)	Prediction equations best model	R^2	Partial $R^2_{f,p}$	Partial $R^2_{p,f}$	p-value
DM (a)	y=67.09 – 0.19×FA	0.51	-	-	<0.0001
NDF (a)	y=2.66 – 0.00003×FA^2+0.00006×pCA^2	0.79	0.71	0.08	<0.0001
ADF (a+b)	y=2.66 – 0.02×FA– 0.01×pCA	0.36	0.22	0.09	0.002
ADL (b)	y=29.80 – 0.08×FA– 0.01×pCA	0.62	0.52	0.09	<0.0001

The linear and quadratic regression analysis further shows that the content of FA in barley grain accounted for 51% variation in DM rumen degradability. Variation in NDF rumen degradability was explained when a quadratic regression coefficient of FA^2 and pCA^2 were used in model (R^2 = 0.79; P<0.0001). Although the R^2 of partial regression for FA^2 was 0.79 alone and pCA^2 just explained 8% of variation in DM rumen degradability. In addition, the total variation in the rumen undegradable fraction FA and pCA against ADF degradability 0.36 % explained by model and content of FA and pCA in barley grain accounted for 22% (P=0.002) and 9% of the variation in the rumen degradation, respectively. Multi-regression linear and quadratic analysis also shows that both FA and pCA accounted for 62% (P<0.0001) variation in the rumen degradability of ADL.

Efekti hidroksikinamičnih kiselina (ferulnih i p-kumarnih kiselina) u sortama ječma na razgradivost komponenti ćelijskog zida u rumenu

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Rezime

Zrno ječma sadrži hidroksikinamične kiseline, posebno ferulnu (FA) i p-kumarnu (pCA), koje postaju ukrštene sa polisaharidima ćelijskog zida, kako počne lignifikacija, i koji su glavni inhibitorni faktori biorazgradljivosti zidova biljnih ćelija u rumenu. Određene su hemijske karakteristike, FA i pCA sadržaj 11 iranskih sorti ječma. Korišćenjem 3 fistulisane ovce, ispitivani su efekti sadržaja FA i pCA na vlaknastu degradaciju suve materije (SM), vlakna neutralnog i kiselog deterdženta (NDF i ADF) i lignina. U sortama ječma, DM je varirao od 82,52 do 90,90%; NDF je varirao od 9,64 do 27,34% SM; ADF je varirao od 2,03 do 8,13% SM, a od 0,87 do 3,03% SM. Sadržaj FA se kretao od 151,2 do 354,2 μg/g; i

sadržaj pcA JE varirao od 114,5 do 444,4 μg/g SM. Parametri degradacije u rumenu za SM, NDF, ADF i lignin su bili različiti zavisno od sorti ječma. Rastvorljiva frakcija, polako razgradiva, potencijalno razgradiva i nerazgradiva frakcija SM su bile 2,92 do 56,33%; 42,64 do 91,45%; 65,68 do 98,97% i 1,02 na 34,31%, respektivno. Stopa ruminalne degradacije za SM varirala je između sorti ječma od 3,64 do 27,81% h^{-1}. FA je bio povezan sa nerazgradivim u rumenu SM, NDF, ADF i ligninom, dok je pCA u korelaciji sa ADF-om. Koristeći multi-regresiju, FA i pCA su bili inhibirajući faktori razgradljivosti ruminalnih komponenti SM i komponenti ćelijskog zida; a FA je bio najefektivniji faktor za predviđanje razgradljivosti SM, dok su FA i pCA uticali na razgradivost NDF i ADF u rumenu.

Ključne reči: hidroksikinamična kiselina, ferulinska kiselina, p-kumarinska kiselina, ječam, rumen

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MORPHOMETRIC SIMILARITIES AND DIFFERENCES BETWEEN TREE GENOTYPE OF PRAMENKA SHEEP FROM CENTRAL BOSNIA

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Abstract. Morphometric characterization of three strains: Dub, Privor and Kupres was done in order to obtain the genetic characterization of autochthonous sheep strains in Central Bosnia. Total of 205 ewes and rams was measured in order to determine similarities and differences between them. The eight, most important, morphometric trait were determined: wither height, rump height, body length, shoulder width, chest depth, hip width, chest perimeter and shin perimeter. Ewes of Dub Pramenka in relation to Privor and Kupres strains had pronounced morphometric measures, and established differences were statistically significant and highly significant. Statistically significant differences in all measures was observed between rams, expect for hip width. The obtained results show a significant difference in morphometric measures of three autochthonous Pramenka strains from Central Bosnia.

Key words: sheep, autochthonous Pramenka strains, Central Bosnia, morphometric characterization, differences

Introduction

The highest percentage of sheep breeding from Bosnia and Herzegovina is based on autochthonous Pramenka sheep. The most important Pramenka strain is Dub, Kupres and Privor. They inhabit the area of Central Bosnia. The sheep are traditionally bred in extensive husbandry, on large pastures without supplemented feed in highland areas. Pramenka strains are mainly bred for lamb meat and milk, which is processed to traditionally cheese.
The places that are inhabited by Dub Pramenka sheep are municipalities that are linked to Vlašić Mountain, as follows: Teslić, Kotor Varoš, Kneževo, Travnik and Zenica. During the summer Dub Pramenka are on the large pastures of Vlašić Mountain. The largest percentage of sheep bred for fresh milk, which is processed

to famous Vlašić (Travnik) cheese. Type of sheep productions has been nomadic and it remains in the narrow area of Dub Pramenka breeding. In the former Yugoslavia, the sheep were moved from Vlašić Mountain in the lowland areas (Vojvodina, Posavina and Slavonia) at end of autumn. This type of sheep breeding and crossbreeding with Tsigai, the autochthonous sheep from Vojvodina, influenced on the morphometry of Dub Pramenka.

Privor Pramenka inhabits the municipality of Gornji Vakuf and parts of Bugojno and Prozor. The common name of this area is Privor, and because that she named Privor Pramenka. During the summer Privor Pramenka moving to pasture of Vranica Mountain. They graze on large pastures, milked and from milk are made cheese and cream. At the end of autumn sheep were returned to the lowlands in the countryside and kept in barns. Privor Pramenka in contrast to Dub Pramenka, do not been nomadic, but they have barn and facilities for preparing and storing food for the coming winter.

Kupres Pramenka inhabits Kupres plateau, which is a located at an altitude of 1,100 to 1,200 m above sea level. Kupres fields and the surrounding mountains abound with large number of pastures where sheep graze. A small number of farmers from Kupres milked sheep and preparing milk products, they mainly selling lambs which quality of meat is well known, particularly in Western Herzegovina and Dalmatia. The system of sheep production in Kupres is differs from the system in Vlašić and Privor. In the summer sheep are kept outside of the barns, on the pasture near the farm, and farmers preparing food from meadows and fields, that is used for feeding over the winter.

Variability and differentiation of various Pramenka strains from Balkan has been the subject of numerous studies which have used different methods, from morphometry, polymorphism of hemoglobin to methods of molecular genetics. *Vazić et al. (2015)* investigated the polymorphism of hemoglobin in three Pramenka strains (Dub, Kupres, Privor). The results showed that all three stains have similar frequencies of genotypes polymorphism, or there is not statistically significant difference in polymorphism of hemoglobin. In addition, according the research of genetic variability using microsatellites, *Ćinkulov et al. (2008)* report that Dub Pramenka compared to the other Pramenka strains of Balkan Peninsula such as Svrljig, Bardoka, Piva and Racka showed no significant genetic distance. *Ćurković et al. (2016)* was researched genetic diversity and structure of 18 sheep breeds from Balkan Peninsula and Central and North-western Europe, including seven Pramenka strains from Croatia and Bosnia and Herzegovina. The results also showed low genetic differentiation of Pramenka strains. Morphometric characterization of Pramenka also was a subject of many authors. For example, *Antunović et al. (2013)* and *Vazic et al. (2017b)* measured Dub, *Šmalcelj (1937)* and *Vazic et al. (2016)* Privor, *Ivanković et al. (2009)* and *Vazic et al. (2017a)* Kupres Pramenka. However, in the current literature there is not a paper that describes morphometry of all three Pramenka strains from Central Bosnia.

Therefore, the aim of this study was, on the basic of morphometric measures, compare the ewes and rams of all three Pramenka strains and according that to identify the similarities and differences between them.

Material and methods

Total of 205 ewes and rams was measured, of which there were: 80 sheep of Dub (68 ewes and 12 rams), 63 sheep of Privor (53 ewes and 10 rams) and 62 sheep of Kupres Pramenka (56 ewes and 6 rams). All the animals have completed their growth and development (over 4 years old). The eight, most important, morphometric trait were determined: wither height, rump height, body length, shoulder width, chest depth, hip width, chest perimeter and shin perimeter. Measuring of the height, length and width were taken by Ludtin's stick, and the scope was taken by ribbons. All sheep have completed their growth and development. Sheep were taken randomly from the flock. Obtained morphometric measurement between strains was compared using analysis of variance with unequal number of repetitions where is calculated F- test, and differences between measurements were tested with t-test.

Results and discussion

The most cammon three Pramenka strains from Central Bosnia are grown almost under the same agro-ecological conditions. They are characterized by exellent adaptation to harsh climatic conditions and their resistences to disease. The difference between these strains is in different type of productions. Dub pramenka has been nomadic, but Privor i Kupres Pramenka all year spent on the Privor, respectively Kupres Montain. Table 1 shows morphometric similarity and differences between ewes of three Pramenka strain of Central Bosnia.

Table 1. Morphometric similarity and differences between ewes of three Pramenka strains of Central Bosnia

Measurements	Strain	$\bar{x}$	$F_{calc.}$	x_i-x	x_i-x	$t_{calc.}$	
Wither height	Dub	73,37	10,65**	3,66**	3,09**	4,21**	3,55**
	Privor	70,28		0,57		0,61	
	Kupres	69,71					
Rump height	Dub	73,72	6,67**	3,15**	2,38**	3,50**	2,61**
	Privor	71,34		0,77		0,80	
	Kupres	70,57					
Body length	Dub	74,66	4,84**	1,82*	1,62	2,49*	2,16*
	Privor	73,04		0,20		0,26	
	Kupres	72,84					
Shoulder width	Dub	22,72	23,32**	1,60**	1,89**	5,33**	6,30**
	Privor	20,83		-0,29		0,91	
	Kupres	21,12					
Chest depth	Dub	34,50	45,31**	2,52**	2,01**	9,00**	6,67**
	Privor	32,49		0,51		1,70	
	Kupres	31,98					
Hip width	Dub	21,95	41,25**	1,67**	1,29**	8,35**	6,45**
	Privor	20,66		0,38		1,90	
	Kupres	20,28					
Chest perimeter	Dub	98,72	76,66**	7,97**	9,83	9,49**	11,43**
	Privor	88,89		-1,86*		2,07*	
	Kupres	90,75					
Shin perimeter	Dub	9,31	96,72**	1,40**	0,86**	14,00**	8,60**
	Privor	8,45		0,54**		4,91**	
	Kupres	7,91					

*level of significant 0,05, **level of significant 0,01

The results showed statistically significant difference between ewes of Pramenka strains. Dub Pramenka sheep had larger measurements than the other two strains. T-test showed that the differences between Dub Pramenka ewes on one side and Privor and Kupres on the other hand, statistically significant higher. The values of t-test indicate a certain uniformity of morphometric measurements between Privor and Kupres Pramenka. Statistically highly significant differences was found only for the shin perimeter, and statistically significant diferences tor the chest perimeter. Dub, Privor and Kupres Pramenka compared to autocthtonous sheep from Croatia are much more developed than the following: Lika Pramenka, Dubrovnik Ruda, Krč sheep, Raška sheep, Cres sheep and Dalmatian Pramenka *(Mioč et al., 1998; Mioč et al., 2003; Mioč et al., 2004; Pavić et al., 2005; Pavić et al., 2006; Širić et al., 2009).* Pramenka strains from Central Bosnia had lower wither height only from Istria sheep (73.51 cm) *(Mikulec et al., 2007),* which is caused by crossing autochthonous Istria Pramenka with a different imported races, primarily Italian.

The rams of this strain are strong animals whit robust skeleton. The carcass of rams characterized with emphasized depths and very modest widths. Table 2 shows morphometric similarity and differences between rams of three Pramenka strain of Central Bosnia.

Table 2. Morphometric similarity and differences between rams of three Pramenka strain of Central Bosnia

Measurements	Strain	$\bar{x}$	$F_{calc.}$	x_i-x	x_i-x	$t_{calc.}$	
Wither height	Dub	79,92	13,08**	4,59**	6,12**	3,19**	4,94**
	Privor	73,80		-1,53		1,06	
	Kupres	75,33					
Rump height	Dub	80,16	8,86**	3,83**	5,36**	2,51**	4,09**
	Privor	74,80		-1,53		0,96	
	Kupres	76,33					
Body length	Dub	80,42	4,01*	2,59	4,62*	1,21	2,51*
	Privor	75,80		-2,03		0,92	
	Kupres	77,83					
Shoulder width	Dub	23,75	4,47*	-0,58	2,45*	0,51	2,52*
	Privor	21,30		-3,03*		2,58*	
	Kupres	24,33					
Chest depth	Dub	36,17	5,55*	1,67	2,67**	1,77	3,34**
	Privor	33,50		-1,00		1,03	
	Kupres	34,50					
Hip width	Dub	22,91	3,16	0,91	2,11*	0,93	2,51*
	Privor	20,80		-1,20		1,19	
	Kupres	22,00					
Chest perimeter	Dub	103,25	9,74**	4,75	12,15**	1,47	4,40**
	Privor	91,10		-7,40*		2,22*	
	Kupres	98,50					
Shin perimeter	Dub	10,91	16,98**	1,58**	1,81**	4,05**	5,45**
	Privor	9,10		-0,23		0,57	
	Kupres	9,33					

*level of significant 0,05, **level of significant 0,01

For all measures results of F-test showed that there is statistically significant difference between Pramenka strain rams, except for hip width. Dub Pramenka rams have pronounced almost all measures in relation to the rams of Privor and Kupres Pramenka, except for shoulder width, which was highest in Kupres Pramenka rams. According the morphometric measurements Kupres Pramenka rams are larger than Privor Pramenka rams. Compared with the rams of Croatian autochthonous breeds, especially at whither height, it can be concluded that Dub Pramenka rams, which is not case with Privor and Kupres Pramenka, have height values, even from Istrian Pramenka *(Mikulec et al, 2007).* Privor and Kupres Pramenka rams have greater whither height than the Lika, Rab, Paški and Cres rams *(Mioč et al., 1998; Mioč et al., 2006; Pavić et al., 2005; Pavić et al., 2006).*

The results of morphometric variability indicate significant differentiation between three Pramenka strains from Central Bosnia. Despite the significant differences in phenotype, the results of genetic differentiations using modern methods indicate a low differentiation between the genotypes. *Ćurković et al. (2016)* report that the minimum genetic differentiation was observed between the seven Pramenka strains, which are in conformity with the results of *Ćinkulova et al. (2008)* and *Važić et al. (2015).* The explanation in the low genetic differentiation between Pramenka strains can be found in similar agro-ecological conditions in which they are bred, in the geographical nearby as well as the mixing populations through a long history of seasonal migration. On the other hand, *Ćurković et al. (2016)* also reported that seven Pramenka strains from Croatia and Bosnia and Herzegovina, including Dub, Privor and Kupres, displayed the highest allelic and genetic diversity.

Initiated public interest in the early nineties, encouraged the responsible authorities to accede to the inventory of genetic resources and their inclusions in the system of support and sustainability *(Caput et al., 2010).* In this sense Pramenka as autochthonous sheep breed from Bosnia and Herzegovina has a significant place. In support of this is the conclusion of *Ćurković et al. (2016),* who recommends that preserve of Pramenka strains should be conserved with a high global priority to ensure sustainable sheep breeding in the future. According to numbers in Central Bosnia is the most common Dub Pramenka, which is rapidly expanding in the Kupres and Privor breeding area. Farmers from the Privor and Kupres area go to Vlašić and buying Dub Pramenka rams, and they are used for breeding in their own flock. In addition, a number of farmers from the municipality of Travnik over the summer graze their sheep in the field of breeding Privor and Kupres Pramenka, where there is an exchange between the local sheep flock and flock received from Travnik. Long term, the application of unplanned animal crossing well lead to Privor and Kupres Pramenka strains should be converted to Dub strain. For fear that this way will lead to disappearance of origin genome of Kupres and Privor Pramenka strain it is necessary to take certain conservation measures in order to preserve this population from extinction.

Conclusion

Based on the results for the eight most important body measurements, it can be concluded statistically significant difference between three Pramenka strains from Central Bosnia, especially for ewes. Also the application of unplanned animal crossing long-term leading to disappearance of origin genome of Kupres and Privor Pramenka strain. According the importance of the total genetic variability of Pramenka sheep it is necessary as soon as possible to take certain conservation measures in order to preserve this population from extinction.

Sličnost i razlike morfometrije tri genotipa pramenke centralne Bosne

Božo Važić, Biljana Rogić, Milanka Drinić, Nebojša Savić

Rezime

U cilju genetičke karakterizacije autohtonih sojeva pramenki srednje Bosne urađena je morfometrijska karakterizacija tri soja: dupskog, privorskog i kupreškog. U radu je ukupno izmereno 205 ovaca i ovnova sa ciljem utvrđivanja sličnosti i razlika između istih. Uzimane su osnovne morfometrijske mere koje se najčešće koriste pri naučnim istraživanjima: visina grebena, visina krsta, dužina trupa, širina grudi iza lopatica, dubina grudi, širina kukova, obim grudi i obim cevanice. Analizirane morfometrijske mere naglašenije su kod ovaca dupske pramenke u odnosu na ovce privorske i kupreške pramenke, a utvrđene razlike statistički su značajne i visoko značajne. Između ovnova istraživanih sojeva zabeležena je statistički visoko značajna razlika za sve mere, osim za širinu kukova. Dobijeni rezultati ukazuju na značajnu razliku u morfometriji između tri soja pramenke srednje Bosne.

Ključne reči: ovce, autohtoni sojevi pramenke, Centralna bosna, morfometrijska karakterizacija, razlike

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PREDICTION OF TEST DAY MILK YIELD BY AC METHOD IN INDIGENOUS BALKAN GOATS IN MACEDONIA

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Abstract. Accurate and precise milk recording is one of the most important moments for a successful selection of milking goats. In this context, breeders are constantly making efforts to find the most suitable and cheapest methods for conducting of tests for milk production. The goal of this research was to compare the A4 method (as referent method) with AC method (as alternative method), for determination of milk production, on the day of recording of the indigenous Balkan goat, in the period of 2014-2016 with milking of goats in the morning and evening. It was determined that the difference between the predicted daily milk yield with one milking (in the morning or evening) and the measured milk yield using the A4 method is too low and almost negligible. With the analysis of all factors (year, lactation and number of milk tests), it was determined that the prediction of total daily milk yield, based on the evening milking, provides more accurate result, in relation to the prediction during morning milking, in cases when using the AC method.

Key words: Balkan goat, daily lactation, method A4, method AC, predicted lactation

Introduction

Goat breeding in Republic of Macedonia, for the last two decades (2000-2017), although with small oscillations, is stable, especially if we take into consideration the number of goats (70.000-80.000 goats). This is due to the interest of dairies for purchase of goat milk for processing as well as the interest of certain slaughter houses for purchase of lambs, as raw material for the production of goat meat. This purchase, although difficult, still maintains the branch in some way on a relatively stable basis.

In the past period there have also been certain attempts by the state for genetic improvement of the goat population (reproduction), with the import of more productive goat breeds from abroad (import of French Alpine in 1999). According to the data of the Food and Veterinary Agency, the Department for identification and registering of domestic animals, there are 6 genotypes of goats in R. Macedonia: Domestic Balkan goat, Alpine, Saanen, Alpine crossbreed, crossbreeds with Saanen goat and population registered under the term of Other.
According to this agency, the most represented goat breed in the country is Domestic Balkan goat, with a number of around 38.378 goats, goats registered as *Other* with a number of 21.772 goats, the number of crossbreeds with Alpine is 6330, Saanen with 6256 goats, Alpine is represented with 4193 and crossbreeds with Saanen is represented with 2735 goats *(Pacinovski et al., 2012).*
Balkan goat is well adopted to the existing climate conditions in the country as well as to the existing nutritional resources especially in the hilly-mountainous areas of Republic of Macedonia, which are not suitable for other domestic animals.
It is the shrubbery vegetation which is especially attractive to goats. The excellent adaptability of the breed is due to the excellent health condition of goats manifested during the whole year. Compared to the other breeds (Alpine, Saanen and crossbreeds between the same with other breeds), Balkan goat is extremely resistant to many diseases (chronic, bacterial etc.) They are especially resistant to emergent climate changes that affect the goat health.

As an indigenous goat breed, Domestic Balkan goat has been protected in order to preserve the breed, having in mind the growing pressure for its crossbreeding with more productive breeds such as Alpine and Saanen breeds of goats. This is the reason why some of the Balkan goats are tested in milk production, hence it is logical to search for the most accurate and cheapest methods.

The aim of the study was to determine the accuracy of prediction of the actual daily milk yield in the Balkan goats, measured twice a day (method A4), in the morning and evening and predicted based on only one milking on the day of testing (method AC).

Such test are being conducted mostly in sheep, almost never in goat. Therefore the comparison of results was performed with data and analysis of milk tests conducted in sheep.

For example, the results obtained in Bulgaria, show that the total amount of milking milk is slightly decreasing with AC method for 120 days *(Ivanova, 2013).* Also some results from limited number of Awassi sheep in Macedonia show that the correlation between the two methods is high, with maximal variations in prediction from 1.9 to 3.4 L *(Gievski et al., 2006).*

Material and methods

We used a flock of Balkan goats as basic experimental material located on south-east Macedonia. The number of goats included in these tests was around 242 goats per year, in the period of three years (2014-2016). A total number of 726 goats (lactations) were monitored for three years of testing. According to the age range, goats were from first to ninth lactation (Table 1).

Table 1. Age range of tested goats per year

Year	Number of lactation									Total
	I	II	III	IV	V	VI	VII	VIII	IX	
2014	84	53	70	26	4	3	2	0	0	242
2015	0	84	53	70	26	4	3	2	0	242
2016	0	0	84	53	70	26	4	3	2	242
Total	84	137	207	149	100	33	9	5	2	726

During the first three years of production, total number of 4598 individual lactation tests were conducted or according to the age: 588 tests in the first, 882 tests in second, 1318 in third, 919 in fourth, 598 in fifth, 195 in sixth, 56 in seventh, 30 in eighth and 12 in ninth lactation (Table 2).

Table 2. Individual lactation tests in goats

Year	Number of lactation									Total
	I	II	III	IV	V	VI	VII	VIII	IX	
2014-2016	588	882	1318	919	598	195	56	30	12	4598

Mainly combined (barn-pasture) system of breeding is used on the farm, which is using of available vegetation during almost the entire year, whereas in a certain period of the year, especially in winter, goats are fed additionally with meadow hay (November - February) and concentrate (January - April). Kids stay with their mothers depending on the purpose. Kids intended for slaughter stay with their mothers until they are sold for meat (2-2.5 months old), whereas those intended for reproduction, stay little longer, or up to 3 months.

The production of milk in goats was monitored according to the standard A4 method *(ICAR, 2009, ICAR, 2012)*, which means measuring of daily production of milk per goat, in the interval of 28 to 34 days.
The recording of milk commenced after the weaning of kids (60 days) and lasted until the moment of drying in milk (end of October or mid-November). The total number of conducted milk recordings of milk was 7 tests in 2014, 6 tests in 2015 and 6 tests in 2016.

The data used were monthly test day yields of the goats in three consecutive years, 2014, 2015 and 2016. The actual yield per day is the sum of the morning and evening milk yield of each goat. The predicted milk yield per day is based either on the morning measurement or evening measurement. These measurements were weighted by the relative total amount of the milk in the flock, for the corresponding milking: morning or evening to the total milk yield for the day of test in the separate year-test days:
Predicted daily yield on morning milking = morning milk yield * K1,
Where K1 is the ratio of total daily milk yield for the flock to total milk yield in the morning.
Predicted daily yield on evening milking = evening milk yield * K2,
Where K2 is the ratio of total daily milk yield for the flock to total milk yield in the evening.
For the available in the study 20 year-test day yields these coefficients are presented in the Table 3:

Table 3. Total flock milk yield for the day of test and appropriate coefficients for prediction of test day yield based on the morning (K1) and evening (K2) milking

Year / month	Morning, litres	Evening, litres	Total, litres	K1	K2
2014/1 Total	140.00	164.00	304.00	2.17	1.85
2014/2 Total	139.80	148.25	288.05	2.06	1.94
2014/3 Total	99.00	126.55	225.55	2.28	1.78
2014/4 Total	79.85	103.55	183.40	2.30	1.77
2014/5 Total	61.4	81.5	142.9	2.33	1.75
2014/6 Total	47	64.05	111.05	2.36	1.73
2014/7 Total	35.15	47.5	82.65	2.35	1.74
2015/1 Total	162.77	181.81	344.58	2.12	1.90
2015/2 Total	140.25	164.45	304.7	2.17	1.85
2015/3 Total	113.65	137.85	251.5	2.21	1.82
2015/4 Total	79	90.15	169.15	2.14	1.88
2015/5 Total	59.05	64.77	123.82	2.10	1.91
2015/6 Total	43.65	44.71	88.36	2.02	1.98
2016/1 Total	152.53	200.93	353.46	2.32	1.76
2016/2 Total	100.89	128.05	228.94	2.27	1.79
2016/3 Total	92.67	124.13	216.8	2.34	1.75
2016/4 Total	64.72	85.41	150.13	2.32	1.76
2016/5 Total	41.61	59.45	101.06	2.43	1.70
2016/6 Total	32.35	41.05	73.4	2.27	1.79

Actual yield, predicted yield and differences between the predicted and actual milk yield were analyzed for the effects of year, lactation and day of test effects by a fixed LS-model.
Regarding the statistic analyses, the characteristics of daily milk production (morning, evening and total amount of milk) were analyzed using the following model:

$$Y_{jklm} = \mu + Y_j + L_k + TD_l + e_{jklm}$$

where:
Y - is an individual observation of each trait during a test (daily) test (morning, evening and total amount of milk) of the m-th individual-test day measurement;
μ - is general mutual average for tested characteristics;
Y_j - effect of j-th year with (j=2014, 2015 and 2016);
L_k - effect of k-th lactation with (k=1,2,3,4,5,6,7,8,9);
TD_l - effect of l-th test day with (l=1,2,3,4,5,6,7);
e_{jklm} - residual influence

The influence of certain effects (year, lactation and number of test) was studied using the F-test, whereas the analyses were performed using the package programs SPSS *(SPSS, 1994).*

Results and Discussion

In order to minimize the error of predicting of daily milk yield, the influence on variations of certain factors was analyzed in predicting the morning and evening milking (Table 4).

Table 4. Influence of the certain factors on the measured and predicted daily milk yield

Factor	df	Morning	Evening	Total	Expected (morning)	Expected (evening)	Difference (morning)	Difference (evening)
Year	2	46.33***	18.24***	29.30***	23.23***	33.24***	0.78	0.71
Lactation	8	7.31***	9.12***	8.50***	7.43***	9.10***	2.50*	2.47*
Number of test	6	427.76***	535.29***	500.21***	406.28***	558.17***	0.00	0.00
R^2, %		37.3	41.9	40.5	35.7	43.2	0.4	0.4

During the analyses, it was determined that all three analyzed factors (year, lactation and number of milk test), had highly significant influence (P<0.001) on morning, evening and total amount of milk. The same factors also had highly significant influence (P<0.001) on the expected total amount milk, using morning and evening milk.

The lactation had significant influence (P<0.05) on the difference in the morning and evening milking, whereas the year and number of milk test had no significant influence (P>0.05), on this two traits.

The coefficient of determination, R^2, showed that the complex of the studied factors determine or explain from 35.7% to 41.9% of the variation of the measured milk yield, and of 0.4% of variation of the difference between the predicted and total TD milk yield.

Significant influence of the year on the determined and expected daily milk yield (using A4 and AC methods) has been determined in East-Frisian breed of sheep in Macedonia, but not on the differences determined between them *(Pacinovski et al., 2015)*.

During the analyses of the data for the three years of testing (2014-2016), it was determined that the average milk yield during morning, evening and total milk yield was 0.314, 0.391 and 0.705 liters, consequently (Table 5).

Table 5. Average milk yield L/day

Trait	Mean (L)	±SE
Morning	0.314	0.010
Evening	0.391	0.010
Total	0.705	0.019
Predicted morning	0.698	0.021
Predicted evening	0.710	0.018
Difference-morning	-0.007	0.005
Difference-evening	0.005	0.004

The average milk yield expected during morning milking is 0.698 liters, which is a difference or underestimation of – 0.007 liters. The same milk yield determined using the production of milk obtained during evening milking is 0.710 liters, which is overestimation for 0.005 liters.

The standard error ranges from 0.004 for the difference in total amount of milk determined by evening milking, to 0.021 for predicted total amount of milk by morning milking.

While predicting the daily milk yield of East-Frisian breed of sheep using milk obtained at morning, using the AC method, an underestimation of 0.3% of daily milk yield has been reached as well as overestimation of 0.3% using milk obtained at evening.

The influence of each year on the analyzed characteristics of daily milk production separately is presented in Table 6.

Table 6. Effect of the year on the measured and predicted milk yield, L

Year	N	Morning	Evening	Total	Predicted - morning	Predicted-evening	Difference-morning	Difference-evening
2014	1694	0.338	0.421	0.760	0.750	0.768	-0.0103	0.0076
2015	1452	0.342	0.392	0.735	0.730	0.738	-0.0047	0.0034
2016	1452	0.260	0.358	0.618	0.611	0.622	-0.0061	0.0047
SE		0.011	0.011	0.022	0.025	0.020	0.005	0.004

According to the same table, the predicted morning, evening and total amount of milk during 2014 was 0.34 liters, 0.42 liters and 0.76 liters, consequently. These values, in 2015 and 2016 were 0.26 liters, 0.36 liters and 0.62 liters, consequently. The prediction of total production of milk per day using milk yield obtained during morning and evening milking was 0.75 or 0.77 liters, which was underestimation for -0.0103 liters during morning milking, or overestimation for 0.0076 liters during evening milking.

The standard error ranged from 0.004 for the difference in total amount of milk determined by evening milking, to 0.025 for predicted total amount of milk by morning milking.

The influence of age i.e. lactation on tested characteristics of daily production of milk are presented in Table 7 separately.

Table 7. Effect of the lactation on the measured and predicted milk yield, L

Lactation	N	Morning	Evening	Total	Predicted-morning	Predicted-evening	Difference-morning	Difference-evening
1	588	0.30	0.35	0.65	0.66	0.64	0.01	-0.01
2	882	0.35	0.43	0.79	0.79	0.79	0.00	0.00
3	1318	0.35	0.43	0.78	0.79	0.78	0.00	0.00
4	919	0.34	0.42	0.76	0.75	0.77	-0.01	0.01
5	598	0.37	0.46	0.83	0.83	0.83	0.00	0.00
6	195	0.37	0.44	0.81	0.82	0.80	0.01	-0.01
7	56	0.23	0.32	0.55	0.52	0.57	-0.03	0.02
8	30	0.25	0.32	0.56	0.54	0.58	-0.02	0.02
9	12	0.27	0.35	0.61	0.59	0.63	-0.03	0.02
SE		0.006-0.06	0.006-0.06	0.01-0.13	0.01-0.14	0.01-0.12	0.003-0.03	0.002-0.02

The highest value of total milk production per day was determined in goats in fifth lactation (0.83 liters), whereas the lowest value was determined in goats in seventh lactation (0.55 liters). During morning milking, the highest value of total milk production per day was determined in goats in fifth and sixth lactation (0.37 liters), whereas the lowest value in goats in seventh (0.23 liters). During evening milking, the condition is almost the same i.e. the highest value of milk production was determined in goats in fifth lactation and the lowest value was determined in goats in seventh and eighth lactation. It is interesting to be mentioned that after the

seventh lactation, an increase of total milk production per day was determined in goats in eighth and ninth lactation, which generally is a fact that goats of this breed have longer period of milk production. However we consider this small increase as a result of the selection carried out, than due to biological features.

The results obtained during the prediction of total milk yield per day in the morning or evening were almost identical. More precisely, the most accurate prediction of total milk production per day in the morning and evening was obtained in goats in second, third and fifth lactation. In goats at other ages, there was deviation performed during the prediction. For example, during the prediction of total milk production per day in the morning, the biggest deviation was performed in goats in seventh lactation (underestimation for -0.03 liters), whereas during the prediction of total milk production per day in the evening, the biggest deviation was performed in goats in seventh, eighth and ninth lactation (overestimation for 0.02 liters).

Although in sheep, similar results about the influence of lactation on daily milk production were obtained in Awassi and East-Friesian sheep in Macedonia *Dimov et al. (2005) and Djabirski et al. (2006).*

The influence of the milk test on analyzed characteristics of daily production of milk is presented in Table 8, separately.

Table 8. Effect of the test day on the measured and predicted milk yield, L

Test day	N	Morning	Evening	Total	Predicted-morning	Predicted-evening	Difference-morning	Difference-evening
1	726	0.60	0.72	1.32	1.31	1.32	-0.0071	0.0053
2	726	0.49	0.58	1.07	1.06	1.08	-0.0071	0.0053
3	726	0.39	0.50	0.89	0.89	0.90	-0.0071	0.0053
4	726	0.28	0.35	0.63	0.62	0.64	-0.0071	0.0053
5	726	0.19	0.25	0.45	0.44	0.45	-0.0071	0.0053
6	726	0.14	0.18	0.31	0.31	0.32	-0.0071	0.0053
7	242	0.10	0.15	0.26	0.25	0.26	-0.0071	0.0053
SE		0.01	0.01	0.02-0.04	0.03-0.04	0.02-0.03	0.006-0.008	0.005-0.007

The highest value of milk yield during morning, evening and total milk production was determined during the first milk test (0.60 liters, 0.72 and 1.32 liters consequently), whereas the lowest value was determined during the last test (0.10, 0.15 and 0.26 liters consequently). The general conclusion is that, the production of milk decreases continuously from the first to the last milk test and during morning and evening milking, which, on the other hand shows the necessity of paying special attention to the beginning of the lactation in order to obtain greater amount of milk (commercial) thus bigger financial effect.

During the prediction of total milk production in all milk tests, almost identical results were obtained during morning i.e. evening milking. More precisely, during the prediction of total milk production per day in the morning, in all seven milk

test, there is an underestimation for 0.007, whereas during the prediction of total milk production in the evening, there was an overestimation for 0.0053 liters in all milk tests.

Conclusions

Based on the conducted tests, the following can be concluded:

- Evening milking predicted TD milk yield slightly more accurate, but for the breeding purposes both measurements are reliable enough in the breeding programs,
- The difference of predicted and measured TD milk yield is less than 10 ml for different lactations and test days,
- Such results indicate the fact that the quite cheap AC method as well as the referent A4 method can be used, with significant precision of prediction.

Predviđanje prinosa mleka domaćih balkanskih koza u Makedoniji korišćenjem dnevne test AC metode

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Rezime

Precizna i tačna kontrola mlečnosti je jedan od najvažnijih trenutaka za uspešan izbor koza za mužu. U ovom kontekstu, uzgajivači konstantno pokušavaju da pronađu najprikladnije i najjeftinije metode za sprovođenje testova za proizvodnju mlijeka. Cilj ovog istraživanja bio je upoređivanje metoda A4 (kao referentnog metoda) metode i AC (kao alternativnom metodom), za određivanje proizvodnje mleka, na dan evidentiranja, kod autohtone balkanske koze, u periodu 2014-2016. godine, sa mužom koza koja se izvodila ujutru i veče. Utvrđeno je da je razlika između predviđenog dnevnog prinosa mlijeka sa jednom mužom (ujutro ili uveče) i izmerenim prinosom mlijeka metodom A4 bila mala i skoro zanemarljiva. Analizom svih faktora (godina, laktacija i broj ispitivanja testova) utvrđeno je da predviđanje ukupnog dnevnog prinosa mlijeka, zasnovano na večernjoj muži, daje tačniji rezultat, u odnosu na predviđanje tokom jutarnje muže, u slučajevima kada se koristi AC metod.

Ključne reči: balkanska koza, dnevna laktacija, metod A4, metod AC, predviđena laktacija

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PROTEINS SEQUENCE ANALYSIS OF CONTAGIOUS CAPRINE PLEUROPNEUMONIA

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Abstract. A total of twenty (20) contagious bovine pleuropneumonia (CCPP) proteins were retrieved from the GenBank (www.ncbi.nlm.nih.gov). The proteins sequences were used to investigate the molecular identity of various CCPP proteins. The physico-chemical properties of CCPP proteins were performed using protparam tool. Isoelectric point (pI), molecular weight (MW), extinction coefficient (EC); instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY) were computed. The study revealed that the pI of CCPP proteins were acidic and basic in nature. The EC and II of CCPP proteins indicate better stability which is an indication of resistant to mutation and thermally stable. The GRAVY of CCPP proteins revealed some are positive while some are negative. The positive value indicates solubility (hydrophilic) in water while negative is not soluble (hydrophobic) in water. The amino acid composition of CCPP proteins indicates that they are rich in isoleucine, leucine and lysine. The three dimensional structures (3D) of the CCPP proteins were determine using Phyre2 server. The amino acid sequences of CCPP proteins were subjected to secondary structure prediction using ExPASy's SOPMA tool. The proteins are more of alpha helix structure. The genetic information eminating from this study may bring insight into mutagenesis and pharmacogenetic.

Keywords: protein, caprine pleuropneumonia, Sequence

Introduction

Contagious Caprine Pleuropneumonia (CCPP) is a devastating disease of goats included in the list of notifiable diseases of the Organization for Animal Health (OIE).The first description of the disease dates back to 1873, in Algeria *(Thomas, 1873).*CCPP is a contagious disease of goats, which occurs in per acute,

acute or chronic forms and is characterized by fibrinous pneumonia, pleurisy and profuse pleural exudates *(Edelsten et al., 1990).* Mortality rates of 60–100% are common *(Edelsten et al., 1990).* The disease is reported to occur in many countries in West and Eastern Africa and in Pakistan and India *(OIE, 2001)*. The infectious agent *Mycoplasma capricoleum* subspecies *capripneumoniae*, formerly known as the F38-like group, is difficult to isolate and has only been identified in a few of the countries where the disease has been reported *(Bolske et al., 1995a).*

Materials and Methods

A total of twenty (20) CCPP proteins of goat were retrieved from the GenBank (www.ncbi.nlm.nih.gov). The Genbank accession numbers of the sequences and sequence variations are shown in Table 1. ProtParam Tool was used for the computation of various physical and chemical properties of the CCPP proteins using amino acid sequences. The computated parameters were molecular weight, theoretical pI (isoelectric point), amino acid composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) *(Gasteiger, 2005).* The amino acid sequences of CCPP proteins were subjected to secondary structure prediction using ExPASy's SOPMA tool. It predicts 69.5% of amino acids for a 3 state description of the secondary structure (a helix, b sheets and coil). The Phyre2 server was used to predict the 3D structure of CCPP proteins. These servers predict the three-dimensional structure of a protein sequence using the principles and techniques of homology modeling *(Kelley and Sternberg, 2009).* Currently, the most powerful and accurate methods for detecting and aligning remotely related sequences rely on profiles or Hidden Markov Models (HMMs). 3DligandSite was used to predict the binding site of the 3D structure of the CCPP proteins. Phyre2 is coupled to the 3DligandSite server for protein binding site prediction *(Wass et al., 2010).*

Results

Physico-chemical characteristics of CCPP proteins predicted by protparam are shown in Table 2. The computed isoelectric point (pI) values of CCPP proteins in the study revealed Phosphoglycerate kinase, Glycyl-tRNA Synthetase, ATP-dependent protease La, GTP-Binding protein, tRNA Modification GTpass, Lysine-tRNA ligase and Chromosome segregation ATPase are acidic which have (pI<7) while the rest appeared to be basic in nature with (pI>7). The net charge of CCPP protein revealed only Phosphoglycerate kinase is neutral (no charge). Glycyl-tRNA Synthetase, ATP-dependent protease La, GTP-Binding protein, tRNA

Modification, Lysine-tRNA ligase and Chromosome segregation ATPase are negatively (-) charge while the rest of the protein are positively (+) charge. The extinction coefficient of a protein at 280 nm depends almost exclusively on the number of aromatic residues, particularly tryptophan *(Gill et al., 1989).* Extinction coefficient values for CCPP proteins at 280 nm ranged from 8940 to 143950 (Signal recognition particle protein is lowest and Prolipoprotein diacylglyceryl tranferase is highest) respectively.

Table 1: Protein name, accession, and number of goat CCPP

S/N	NAME OF PROTEIN	ACCESSION No	AMINO ACID No
1	Phosphoglycerate kinase	KEY8461	404
2	Chaperone protein Dnaj	KEY84219	372
3	Amino acid permease	KEY84758	515
4	Glycyl-tRNA Synthetase	KEY84567	456
5	GTP pyrophosphokinase	KEY84560	754
6	ATP-dependent protease La	KEY84622	779
7	DNA-primase	KEY84568	604
8	Histidy-tRNA Synthetase	KEY84179	414
9	GTP-Binding protein	KEY84763	364
10	Excinuclease ABC Subunit B	KEY84755	665
11	tRNA Modification GTpass	KEY84779	452
12	Tyrosine-TRNA ligase	KEY84654	414
13	PTS system-IIBC component	KEY84580	602
14	Hypothetical Protein Mccp 3340	KEY84577	820
15	Dihydro folate-foly poly glutamate synthase	KEY84753	369
16	Lysine-tRNA ligase	KEY84440	500
17	Prolipoprotein diacylglyceryl tranferase	KEY84751	526
18	Chromosome segregation ATPase	KEY84561	988
19	Cell division protein FtsY	KEY84539	424
20	Signal recognition particle protein	KEY84596	447

The half life of protein is the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell of the proteins. In this study the half life of all the CCPP proteins is 30 hours.The instability index provides an estimate of the stability of the protein in a test tube. A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein will be unstable *(Guruprasad et al., 1990).* The result from this study shown that ATP-dependent protease La, Excinuclease ABC Subunit B, Prolipoprotein diacylglyceryl tranferase and Cell division protein FtsY protein have value >40 while the rest of protein are have<40.The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). The result revealed that Amino acid permease, DNA-primase, GTP-Binding protein, tRNA Modification GTpass, PTS system-

IIBC component, Hypothetical Protein Mccp 3340, Dihydro folate-foly poly glutamate synthase and Prolipoprotein diacylglyceryl tranferase proteins from this study have AI>100 while the rest of the CCPP protein have AI<100. The grand average hydropathicity **(**GRAVY) of the CCPP protein revealed that Amino acid permease, tRNA Modification GTpass and PTS system-IIBC component have positive while the rest of CCPP protein have negative value.

Table 2: Physico-chemical characteristic of proteins of CCPP predicted by protoparam

Protein	AA	Mol Wt	PI	Q	EC	Half Life	II	AI	GRAVY
Phosphoglycerate kinase	404	44636.4	6.94	Neu	38055	30hrs	25.67	97.97	-0.188
Chaperone protein Dnaj	370	42107.8	8.75	+	22850	30hrs	34.01	83.31	-0.545
Amino acid permease	515	57092.7	9.54	+	55725	30hrs	31.24	118.72	0.685
Glycyl-tRNA Synthetase	456	53446.9	6.58	-	76110	30hrs	35.91	84.65	-0.510
GTP pyrophosphokinase	754	87111.5	9.11	+	90900	30hrs	30.95	98.41	-0.409
ATP-dependent protease La	779	88439.6	5.62	-	65210	30hrs	47.76	98.70	-0.400
DNA-primase	604	69930.8	8.84	+	44155	30hrs	30.70	106.71	-0.404
Histidy-tRNA Synthetase	414	48625	8.61	+	41510	30hrs	38.56	93.94	-0.526
GTP-Binding protein	364	40671	5.23	-	27765	30hrs	28.80	105.27	-0.079
Excinuclease ABC Subunit B	665	76544.7	7.61	+	44030	30hrs	47.91	99.71	-0.395
tRNA Modification GTpass	452	50443.1	4.81	-	19035	30hrs	33.55	124.65	0.001
Tyrosine-TRNA ligase	414	47159.2	8.58	+	42650	30hrs	33.25	96.11	-0.252
PTS system-IIBC component	602	65666.3	9.43	+	47120	30hrs	22.68	134.07	0.715
Hypothetical Protein Mccp 3340	820	95588.3	8.95	+	105785	30hrs	31.37	100.11	-0.436
Dihydro folate-foly poly glutamate synthase	369	43100.3	8.78	+	45060	30hrs	18.78	112.74	-0.097
Lysine-tRNA ligase	500	58160.4	5.49	-	40925	30hrs	36.18	90.42	-0.465
Prolipoprotein diacylglyceryl tranferase	526	63020.6	9.43	+	143950	30hrs	40.46	104.89	-0.025
Chromosome segregation ATPase	988	112835.3	5.71	-	52845	30hrs	39.46	96.13	-0.514
Cell division protein FtsY	424	48485.7	8.80	+	29910	30hrs	87.87	85.54	-0.720
Signal recognition particle protein	447	50114.3	9.39	+	8940	30hrs	39.54	96.62	-0.364

AA=amino acid; pI=isoelectric point; Q=net charge; II=instability index; AI=alphatic index; GRAVY= grand average of hydropathicity ; EC= extinction coefficient; Mol wt=molecular weight, +=amino acid resides that positively charge, -= amino acid resides that negatively charge, Neut= amino acid resides that are neutral

The prediction of secondary structure of CCPP proteins is shown in Table 3. The result revealed that Signal recognition particle protein showed the highest alpha helix (53.91%) and the lowest is Chaperone protein Dnaj (26.88%). The extended strand prediction, Dihydro folate-foly poly glutamate synthase gives

highest value (28.18%) and the lowest is Signal recognition particle protein (13.87%). The beta turn prediction of secondary structure revealed that Chaperone protein Dnaj gives the highest value (14.25%) and Chromosome segregation ATPase is the lowest (5.67%). The random coil prediction of secondary structure revealed that Chaperone protein Dnaj gives the highest value (35.22%) and Chromosome segregation ATPase showed the lowest value (20.04%). All the CCPP proteins are having higher value in alpha helix structure. The amino acid composition percentage of CCPP protein is shown in Table 4. All the CCPP proteins used for this study have similar amino acid composition of all the CCPP protein with higher percentage in isoleucine, leucine and lysine. Isoleucine and leucine are aliphatic amino acid and lysine is polar amino amino acid. All the CCPP proteins have zero percentage composition of selenocystein and pyrrolysine amino acids.

Table 3: Prediction of secondary structures of CCPP proteins

Protein	Alpha Helix (%)	Extended Strand (%)	Beta Turn (%)	Random Coil (%)
Phosphoglycerate kinase	42.57	21.78	12.62	23.02
Chaperone protein Dnaj	26.88	23.66	14.25	35.22
Amino acid permease	39.03	27.57	10.29	23.11
Glycyl-tRNA Synthetase	37.06	20.61	10.53	31.80
GTP pyrophosphokinase	50.13	20.56	7.43	21.88
ATP-dependent protease La	46.21	17.33	8.34	28.11
DNA-primase	47.02	21.36	7.78	23.84
Histidy-tRNA Synthetase	37.68	20.77	8.94	32.61
GTP-Binding protein	49.45	19.23	10.16	21.15
Excinuclease ABC Subunit Bs	51.13	17.14	10.23	21.50
tRNA Modification GTpass	45.80	22.35	7.74	24.12
Tyrosine-TRNA ligase	48.79	18.60	10.87	21.74
PTS system-IIBC component	40.37	26.08	10.63	22.92
Hypothetical Protein Mccp 3340	51.59	16.22	7.44	24.76
Dihydro folate-foly poly glutamate synthase	41.19	28.18	8.67	21.95
Lysine-tRNA ligase	40.60	22.40	7.60	29.40
Prolipoprotein diacylglyceryl tranferase	38.40	24.52	10.08	27.00
Chromosome segregation ATPase	58.20	16.09	5.67	20.04
Cell division protein FtsY	52.83	15.09	9.20	22.88
Signal recognition particle protein	53.91	13.87	8.41	24.16

Parameters:
Window width: 17
Similarity threshold: 8
Number of states: 4

Table 4: Amino acid composition (%) of CCPP proteins

Protein	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V	O	U
Phosphoglycerate kinase	7.2	2.0	5.9	6.2	0.5	2.7	7.4	7.7	1.0	8.7	8.9	11.6	1.5	4.7	2.5	5.7	5.2	1.2	1.7	7.7	0.0	0.0
Chaperone protein Dnaj	3.0	2.4	9.9	6.5	2.2	4.6	5.6	6.7	1.3	10.2	7.0	11.6	1.3	5.1	1.9	9.1	3.0	0.0	4.0	4.6	0.0	0.0
Amino acid permease	7.6	2.7	4.7	3.3	1.2	3.3	1.7	7.6	1.6	11.7	11.7	5.6	2.9	9.1	1.9	8.5	3.9	1.2	2.9	7.0	0.0	0.0
Glycyl-tRNA Synthetase	3.3	4.2	8.1	6.1	1.1	5.9	7.2	5.0	1.1	8.3	9.4	9.0	1.8	7.0	2.9	6.4	3.7	2.2	3.1	4.2	0.0	0.0
GTP pyrophosphokinase	5.0	3.6	6.5	5.3	0.7	4.1	7.7	3.7	1.5	12.6	7.7	11.8	1.6	3.3	2.1	6.8	5.6	0.9	4.6	4.9	0.0	0.0
ATP-dependent protease La	4.7	3.2	4.5	5.8	0.0	3.0	10.3	5.1	1.8	9.2	9.9	10.8	1.9	2.8	4.0	6.8	5.3	0.5	3.7	6.7	0.0	0.0
DNA-primase	3.1	1.3	10.9	6.1	1.0	3.6	6.5	2.3	2.2	13.6	10.6	12.9	0.8	4.1	2.5	6.3	4.8	0.3	3.6	3.1	0.0	0.0
Histidy-tRNA Synthetase	2.4	4.3	9.7	6.0	1.2	4.6	7.2	4.1	0.5	10.4	10.4	10.1	1.7	4.8	3.4	5.3	5.1	0.2	5.8	3.6	0.0	0.0
GTP-Binding protein	6.9	2.5	5.5	6.9	1.6	4.1	7.1	6.3	0.5	8.8	11.5	9.6	1.1	4.9	2.5	5.2	4.7	0.5	3.0	6.6	0.0	0.0
Excinuclease ABC Subunit B	5.6	5.6	7.4	5.7	0.8	5.6	7.4	3.5	1.7	9.3	11.1	7.7	1.8	3.8	2.6	5.4	6.8	0.3	3.3	5.0	0.0	0.0
tRNA Modification GTpass	5.3	2.7	10.2	6.6	0.7	3.3	7.7	5.1	0.7	13.3	12.4	7.7	1.5	2.7	1.1	6.2	4.0	0.2	2.0	6.6	0.0	0.0
Tyrosine-TRNA ligase	5.6	1.9	7.0	6.5	1.0	6.3	4.8	5.1	1.7	8.2	11.6	10.4	1.7	6.3	1.4	6.3	6.0	1.2	2.4	4.6	0.0	0.0
PTS system-IIBC component	6.1	1.3	6.5	3.2	0.8	3.0	2.2	8.6	1.5	14.1	13.1	6.6	1.8	6.6	3.0	6.1	4.8	0.8	2.2	7.5	0.0	0.0
Hypothetical Protein Mccp 3340	2.7	0.2	12.6	5.9	0.2	5.7	5.1	1.7	0.2	9.4	13.4	12.3	0.6	6.0	1.3	8.4	5.9	1.2	4.1	2.9	0.0	0.0
Dihydro folate-foly poly glutamate synthase	1.6	1.4	7.9	6.0	1.1	3.5	5.4	4.1	2.2	13.3	11.4	11.7	1.1	6.1	2.4	6.0	4.1	0.8	5.1	5.1	0.0	0.0
Lysine-tRNA ligase	4.4	6.0	5.6	7.2	0.4	3.2	9.4	5.0	2.6	7.6	9.4	7.6	3.0	5.0	3.4	4.6	4.4	0.4	4.0	6.8	0.0	0.0
Prolipoprotein diacylglyceryl tranferase	2.5	2.7	9.7	2.1	1.0	4.6	4.8	3.8	2.3	12.9	10.6	8.0	1.1	8.6	3.8	6.1	2.9	3.4	5.7	3.6	0.0	0.0
Chromosome segregation ATPase	5.7	4.4	7.9	6.1	0.2	4.7	9.4	3.8	0.8	9.8	9.6	9.9	1.5	3.5	1.5	6.5	6.5	0.2	2.8	5.1	0.0	0.0
Cell division protein FtsY	6.4	1.9	6.4	7.1	0.2	5.9	9.7	4.2	0.2	6.1	9.2	16.3	2.8	3.1	0.9	4.0	6.1	0.7	2.1	6.4	0.0	0.0
Signal recognition particle protein	5.6	4.3	6.9	4.7	0.0	5.1	8.1	7.4	0.4	7.4	11.6	11.2	4.5	3.6	2.2	5.4	4.5	0.0	1.3	5.8	0.0	0.0

A=Alanine, Arginine=R, Asparagini=N, Aspartic acid=D, cysteine=C, Glutamic acid=E, Glutamin=Q, Glycine=G, Histidine=H, Isoleucine=I, Leucine=L, Lysine=K, Methionine=M, Phenylalanine=F, Proline=P, Serine=S, Theonine=T, Tryptophan=W, Tyrosi

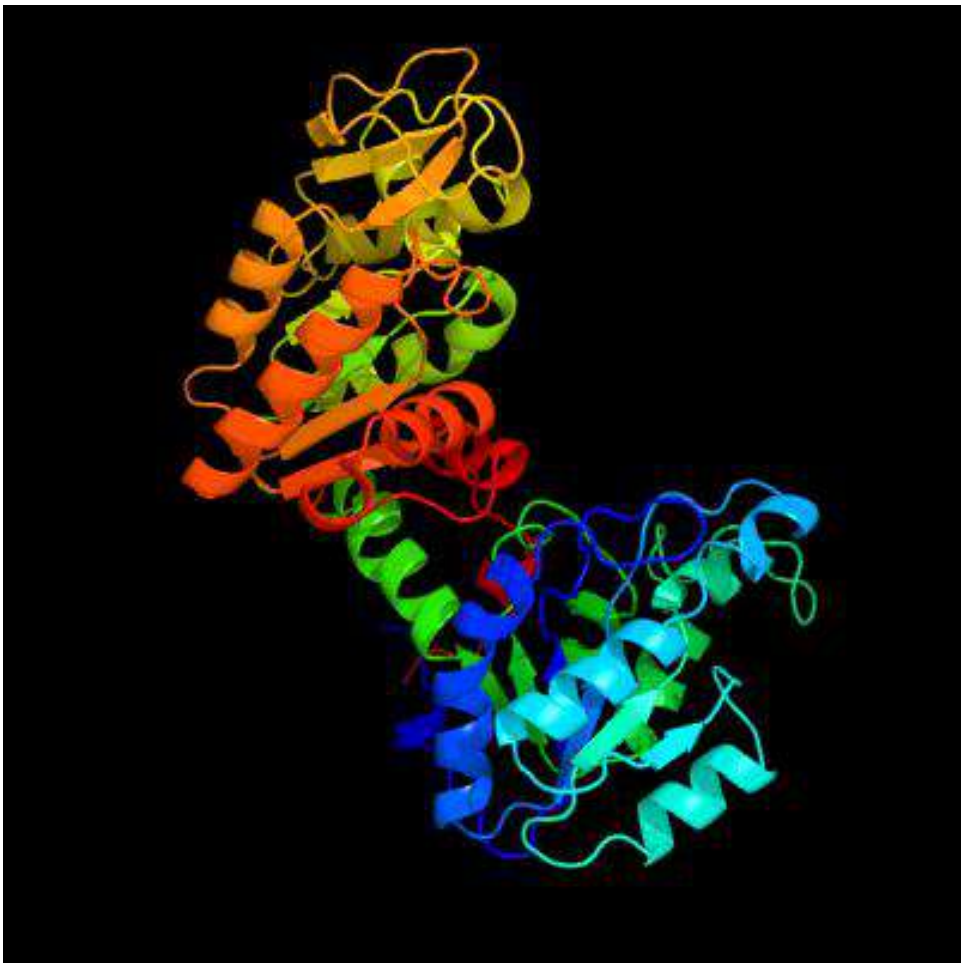

Image coloured by rainbow N → C terminus
Model dimensions (Å): X:69.827 Y:47.914 Z:70.606
Figure 1: Schematic 3D structure of goat Phosphoglycerate_kinase-caprine protein

Discussion

CCPP diseases disease notifiable to the World Organization for Animal Health (OIE) since it has a major impact on livestock production and a potential for rapid spread across national borders. As a result, CCPP-infected countries are excluded from international trade. At present, the disease causes vast problems in

Africa with severe socio-economical consequences. The computed isoelectric points (pI) for both CCPP will be useful for developing buffer system for purification by isoelectric focusing method. The isoelectric point is of significance in protein purification because it is the pH at which solubility is always minimal and at which mobility in an electro focusing system is zero and therefore the point at which the protein will accumulate *(Fennema, 2008).*

The extinction coefficient of a protein at 280 nm depends almost exclusively on the number of aromatic residues, particularly tryptophan *(Gill and Von-Hippel, 1989).* This indicates that the higher the EC value of the CCPP proteins, the higher the number of aromatic residues *(Gasteiger 2003; Munduganore et al., 2012)*. In particular, hydrophobic amino acids can be involved in binding/recognition of hydrophobic ligands such as lipids *(Betts et al., 2003*). All the CCPP proteins have zero selenocystein and pyrrolysine which is interpret as stop codons (protein cannot conclusively determine the identity of a residue) *(Suchanek et al., 2005).*

Many important biological processes such as cell signaling, transport of membrane-impermeable molecules, cell–cell communication, cell recognition and cell adhesion are mediated by membrane proteins *(Jones, 2007).* Although there has been some recent progress in predicting the full 3-D structure of transmembrane proteins (e.g. *Yarov-Yarovoy et al., 2006),* the most widely applied prediction technique for these proteins is to determine the transmembrane topology, i.e. the inside–outside location of the N and C termini relative to the cytoplasm, along with the number and sequence locations of the membrane spanning regions. This will facilitate the understanding of the structure and function of CCPP proteins.

Determining the structure and function of a novel protein is a cornerstone of many aspects of modern biology. The accuracy of protein structure prediction depends critically on sequence similarity between the query and template as observed in the present study. If a template is detected with >30% sequence identity to the query, then usually most or all of the alignment will be accurate and the resulting relative positions of structural elements in the model will be reliable *(Kelley et al., 2015).* The practical applications of CCPP protein structure prediction include guiding the development of functional hypotheses about hypothetical proteins, improving phasing signals in crystallography and selecting sites for mutagenesis *(Qian et al., 2007; Rava and Hussain, 2007).*

Conclusion

The physico-chemical properties, amino acid composition, and secondary structure of CCPP proteins indicated physical, chemical and thermal stability of the protein molecules. These indicated that the proteins are resistant to mutation and can withstand wide range of temperature. Genetic data revealed from this study

will bring new insights into epidemiological questions. Molecular typing has been instrumental in determining the population structure and evolution of pathogens. Since CCPP has both economical and nutritional consequences, efforts should be intensified towards finding sustainable genomic solutions to these deadly diseases which continue to ravage the livestock industry. New typing tool may help improve the surveillance and control of the disease, as well as to trace new epidemics.

Analiza sekvence proteina zarazne pleuropneumonije koza

Ayuba Dauda, Abdulmojeed Yakubu, Ihe Ndu Dim, Deeve Sebastian Gwaza

Rezime

Ukupno od dvadeset (20) proteina zarazne pleuropneumonije pluća goveda (contagious bovine pleuropneumonia - CCPP) je preuzeto iz GenBank-a (www.ncbi.nlm.nih.gov). Sekvence proteina korišćene su za ispitivanje molekularnog identiteta različitih CCPP proteina. Fizičko-hemijske osobine CCPP proteina su analizirane korišćenjem protparam alata. Isoelektrična tačka (pI), molekularna masa (MW), koeficijent ekstinkcije (EK); indeks nestabilnosti (II), alifatski indeks (AI) i veliki prosek hidropatičnosti (GRAVY). Studija je otkrila da su pI CCPP proteina bili kiseli i bazni po svojoj prirodi. EC i II proteina CCPP ukazuju na bolju stabilnost koja je indikacija otpornosti na mutaciju i toplotnu stabilnost. GRAVY CCPP proteina je otkrio da su neki pozitivni, dok su neki negativni. Pozitivna vrednost ukazuje na rastvorljivost (hidrofilni) u vodi, dok negativni nije rastvorljiv (hidrofobni) u vodi. Sastav amino kiselina proteina CCPP-a ukazuje na to da su bogati isoleucinom, leucinom i lizinom. Trodimenzionalne strukture (3D) proteina CCPP su određene pomoću Phyre2 servera. Aminokislinske sekvence CCPP proteina su podvrgnute predviđanju sekundarne strukture korišćenjem ExPASy's SOPMA alata. Proteini su više strukture alfa heliksa. Genetske informacije koje su rezultat ove studije mogu doneti uvid u mutagenezu i farmakogenetiku.

Ključne reči: protein, pleuropneumonija koza, sekvenca

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GENETIC VARIATION OF THE JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA)* BASED ON BIOCHEMICAL POLYMORPHISM

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Abstract. The study aimed at characterizing the Japanese quail using biochemical markers. Blood protein polymorphism of one hundred and sixty-six (166) Japanese quails of both sexes comprising of 83 each of mottled brown and white quails were analysed using cellulose acetate paper electrophoresis. Six loci which includes hemoglobin (Hb), transferrin (Tf), albumin (Alb), carbonic anhydrase (CA), alkaline phosphatase (Alp) and esterase-1 (Es-1) were tested. All the loci tested were polymorphic with each locus having two co-dominant alleles controlling three genotypes. Allele B was predominant at Hb, Tf and Es-1 locus with frequencies 0.90, 0.55, and 0.77, respectively while Allele A was predominant at Alb and Alp locus with frequencies 0.83 and 0.58 respectively. The Allele A had generally lower frequencies than B at the CA loci having values of 0.43 - Brown, 0.38 - White and 0.40 – overall. The mean observed heterozygosity (H_o) was 0.48 with brown and white quails having H_o values of 0.47 and 0.49 respectively, and the expected heterozygosity was observed to be higher in white quails (0.39) than in the mottled brown (0.31). The genetic distance (0.0534) between white and brown quails in this study showed little genetic differentiation between the brown and the white quails. Dendogram generated from the genetic distance values indicated that the two strains had common origin.

Key words: dendogram, Japanese quail, polymorphism, characterization, electrophoresis

Introduction

Livestock populations have evolved unique adaptation to their agricultural production systems and agro-ecological environments. The knowledge of their genetic diversity is important as it forms the basis for designing breeding programs and making rational decisions on sustainable utilization of animal genetic resources

(Mwacharo et al., 2005). Genetic characterisation through the use of molecular markers is providing new avenues for decision making choices for the conservation and rational management of Animal Genetic Resources (AnGR) *(Ajmone-Marsan et al., 2010; Groeneveld et al., 2010; FAO, 2011).* The order *Galliformes* includes many wild bird species and the entire row of domestic species and breeds, the majority of which is well known by their morpho-physiological and productive qualities. This group includes the Japanese quail (*Coturnix coturnix japonica)* which has a very wide natural distribution. Since the 1th century, the Japanese quail has been known as a meat and egg-type bird, but it has never been as popular as chicken because of its small body size *(Cheng and Kimura, 1990).* However, it has occupied an equivalent position with other popular experimental animals. The wide distribution of the Japanese quail as an experimental animal began two decades ago, during which time no particular attention was paid to the birds themselves other than being used in comparison in scientific research *(Cheng and Kimura, 1990).* A major factor contributing to the variations in the results of experiments on animals is their genetic backgrounds *(Van Zupthen et al., 1993).* This is a major reason for the study of the genetic variability of quail. Another reason why the Japanese quail is so interesting in respect to the protein polymorphism is its wide natural distribution in comparison with other birds of the order *Galliformes (Cheng et al., 1992).* The Japanese quail is highly adaptable to an extensive range of ecological conditions due to an unusually high frequency of polymorphic loci and average individual heterozygosity *(Baker, 1967).* In spite of the wide natural distribution of this galliform bird and its adaptability to a wide range of ecological conditions *(Cheng et al, 1992),* it is worth mentioning that the population of the Japanese quail have been observed dwindling in the last 3 decades *(Kimura, 1991).* At present, there are two strains of Japanese quail in Nigeria classified according to plumage colour. The most common strain is the mottled brown quail while the least common is the white quail and popularly known among farmers as 'albino' for its characteristic white plumage.

Genetic diversity studies are undertaken to classify individuals or populations; and has been accessed in farm animals through morphological, molecular or biochemical methods *(Mohammadi and Prasanna, 2003; Goncalves et al., 2009).* Several experiments have described various molecular genetic markers used for evaluation of genetic variability in different poultry species and breeds. The utilization of DNA as genetic marker to evaluate genetic variability of poultry breeds and lines was reported by *Semionova et al. (1996).* The use of biochemical markers is also significant *(Cywa-Benko et al., 1994; Inafukuk et al., 1998).* The characteristic features of biochemical markers are high stability and conservativity. The allelic variants of protein visualized after electrophoresis are the products of certain genes. Studies of such polymorphic proteins may provide additional information on the genetic differences among separate individuals, populations, breeds or species and on the influence of natural or artificial selection on genetic

processes - gene drift, gene flow and others, which occur in populations and breeds *(Kuznetsov, 1995).* The present study was aimed at describing the genetic variability of the Japanese quail using biochemical markers.

Materials and Methods

Blood samples were randomly collected from a total of one hundred and sixty-six (166) birds of both sexes and 12-24 months of age comprising eighty-three (83) each of mottled brown quails and white quails sourced from reputable farms in Ibadan. Blood samples were collected by jugular venipuncture into tubes containing heparin as anticoagulant and kept refrigerated during transportation. Plasma and erythrocyte samples were separated from the heparinized whole blood by centrifugation. The electrophoresis of blood proteins and enzyme systems of Hemoglobin (Hb), Transferrin (Tf), Albumin (Alb), Carbonic anhydrase (CA), Alkaline phosphatase (Alp), Esterase-1 (Es-1) were performed on cellulose acetate membrane following the procedure described by RIKEN (2006) with slight modifications.

Allele frequencies for each locus in each sample were computed by direct gene counting method and tested to fit Hardy-Weinberg ratios using Chi square (χ^2) goodness of fit test. The observed (H_o) and expected heterozygosity (H_e) were calculated according to *Nei (1972)* with the correction for small samples *(Levene, 1949).* The genetic identity (I) and genetic distance (D) were calculated according to *Nei's (1978).* The matrix of the distances was used to construct a dendogram of relationships according to Unweighted pair-group with arithmetic mean (UMPGA) *(Sneath and Sokal, 1973).* F-statistics (fixation indices Fis, Fst, Fit) was calculated. All computations were performed using Popgene *(Yeh et al., 1997)* and Tools for Population Genetic Analyses *(TFPGA; Miller, 1997).*

Results

Allele frequencies of the analyzed loci were as presented in Table 1. All loci investigated polymorphic with two co-dominant alleles A and B. Hb^B (0.89, 0.92), CA^B (0.57, 0.62) and Es-1^B (0.75, 0.78) had the most frequent occurrence in the brown and white quails respectively. Alb^A (0.88) was more frequent in white quails, while Alb^B (0.79) was more in brown. Tf^A (0.52) and Alp^A (0.79) were more in brown quails whereas Tf^B (0.62) and Alp^B (0.62) were most frequent in white quails. The result observed from this present study shows that the frequency of Alp^A was higher in brown quail than white quail while Alp^B was observed to be higher in white quail than brown quail.

Heterozygosity estimates were as presented in Table 2. The observed heterozygosity (H_o) was 0.47 and 0.49 for brown and white quail birds, respectively with an overall average of 0.48. The value of expected heterozygosity (H_e) was observed to be 0.33 and 0.30 for brown and white quails, respectively. The mean value of H_e for all population was 0.32.

Population differentiation examined by fixation indices Fis, Fst, Fit for each of the 6 loci studied across population were as shown in Table 3. Fit was estimated at 0.16 with Hb (-0.106) being the only locus with negative value. The heterozygote deficit within population evaluated by Fis was negative (-0.03 and -0.17) for Alb and Tf, respectively. The global breed differentiation (Fst) evaluated as 0.0439 with a range of 0.0017 (Hb) and 0.1727 (Alp). The gene flow values for each of the six loci studies ranged from 1.197 (Alp) to 149.75 (Hb). The mean gene flow for all loci was recorded as 5.45.

Table 1. Allele frequency of polymorphic loci

Locus	Allele	Observed number of alleles	POPULATIONS		
			Brown	White	Overall
Hb	A	32	0.11	0.08	0.10
	B	300	0.89	0.92	0.90
CA	A	134	0.43	0.38	0.40
	B	198	0.57	0.62	0.600
Alb	A	55	0.21	0.88	0.83
	B	277	0.78	0.12	0.17
Tf	A	149	0.51	0.38	0.45
	B	183	0.48	0.62	0.55
Es-1	A	78	0.25	0.22	0.24
	B	254	0.75	0.78	0.77
Alp	A	194	0.79	0.38	0.58
	B	138	0.21	0.62	0.42

Hb- Haemoglobin, CA Carbonic Anhydrase, Alb- Albumin, Tf- Transferrin, Es-1 - Esterase 1, Alp- Alkaline Phosphatase

Table 2. Heterozygosity (Ho, He) and Deviation from Hardy-Weinberg Equilibrium (DHWE) per strain across allozyme loci

Strain/Population	No	Heterozygosity*		DHWE
		Ho	*He*	
Brown	83	0.4687(0.1335)	0.3313(0.1335)	0
White	83	0.4928(0.1286)	0.3072(0.1286)	0
Overall	166	0.4807	0.3193	
St. Dev.		0.1233	0.1233	

N= number of samples; St. Dev = Standard deviation; *Standard deviation in parenthesis

The genetic distance is a measure of genetic difference between population and genetic variability within a population. The distance between the two population was 0.0517 and genetic identity of 0.95 which show a close similarity between the two quail population. According to Wright's values of genetic distance, a dendogram (Fig. 1) was drawn using the unweighted pair-group clustering analysis (UPGMA). The dendogram indicated the genetic processes.

Table 3. F-Statistics and Gene Flow for all Loci Studied

LOCUS	Sample Size	Fis	Fit	Fst	Nm*
Hb	332	0.1085	-0.1067	0.0017	149.750
CA	332	0.0969	0.0991	0.0024	103.390
Tf	332	-0.1723	0.1550	0.0148	16.6778
Alb	332	-0.0306	0.0106	0.0194	12.6361
Es-1	332	0.3285	0.3297	0.0018	137.333
Alp	332	0.4304	0.5288	0.1727	1.1974
Mean	332	0.1182	0.1569	0.0439	5.4479

*Nm= gene flow estimated from F_{ST} =0.25(1-F_{ST})/F_{ST}; Hb- Haemoglobin, CA- Carbonic Anhydrase, Alb- Albumin, Tf- Transferrin, Es-1 - Esterase 1, Alp- Alkaline Phosphatase

Discussion

In this study two hemoglobin alleles; Hb^A and Hb^B were observed. *Dimiri (1981)* in a study of the effect of haemoglobin genotypes on growth and some physiological parameters in Japanese quails reported, three genotypes of hemoglobin (AA, AB and BB) which were controlled by two autosomal alleles A and B. *Mazumder et al. (1989)* reported frequencies of 0.96 (Hb^A) and 0.04 (Hb^B) for white leghorn chickens, and 1.00 (Hb^A) for broiler chickens which contradicted the results of this study as frequency of Hb^A were 0.108 and 0.08 in brown and white Japanese quail, respectively, while frequency of Hb^B were 0.89 and 0.92 respectively. Frequency of Hb^B was predominant in both populations. *Yakubu and Aya (2012)* reported Hb^A frequencies that were higher for all genetic groups of Nigerian indigenous chickens

in their study. In a study of blood protein polymorphism and genetic diversity in locally adapted Muscovy ducks in Nigeria, *Oguntunji and Ayorinde (2015)* reported the predominance of Hb^A over Hb^B in all the ecotypes studied. *Mazumder et al. (1989)* reported the presence of gene fixation as only genotype Hb^{AA} was identified in their study including broiler chickens. The preponderance of the B allele observed is similar to those obtained by *Okamoto et al. (2003)* in Asian native fowl. *Okamoto et al. (2003)* reported in general that Asian fowl was being fixed in Hb^B while Hb^A was detected at extremely low frequencies in some chickens. *Washburn et al. (1971)* related hemoglobin types with Marek disease and concluded that chickens with homozygous mutant hemoglobin genotypes were approximately 20% less susceptible to Marek disease. In the same way, *Dimri (1981)* reported that hemoglobin polymorphism affects growth rate and hatchability, with the highest in Hb^{AA} (62.20%) followed by Hb^{AB} (48.20%) and Hb^{BB} (31.50%). *Akinyemi et al. (2014)* reported that frequency of Hb^A was higher than Hb^B in Muscovy ducks while Hb^B was higher in Mallard ducks. Polymorphism at this locus was also observed by *Ismoyowati (2008)* in Tegal ducks and in Mallard ducks by *Oates and Principato (1994)* and in Nigerian local fowls *(Ajayi et al., 2013).*

Table 4. Nei's Original Measures of Genetic Identity and Genetic distance Nei (1972)

Population	Brown	White
Brown	****	0.9496
White	0.0517	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Ismoyowati (2008) reported that Tegal Duck with Hb^{AA} genotype on all loci had higher egg production than Hb^{BB} and Hb^{CC} homozygote. *Ordas (2004)* reported that Hb^A has a higher affinity for molecular oxygen than Hb^{BB} because of differences in oxygen dissociation rates. Frequency of CA^B was higher in both mottled brown and white Japanese quail populations. This observation suggests a close relationship between the two populations. *Ige et al. (2013)* reported the predominance of CA^f in both Yoruba and Fulani ecotype population. *Oguntunji and Ayorinde (2015)* reported the predominance of CA^F over CA^S in various Muscovy ecotypes in Nigeria. *Akinyemi et al. (2014)* reported predominance of CA^A in both Muscovy and Mallard ducks in Nigeria. However, the activity of CA has been positively correlated with egg shell thickness *(Das and Deb, 2008).* This may aid in selection for increased shell thickness in the breeds to guide against cracks and breakages in egg production. The allele frequency of Alb^A was higher in brown quail, while for white quail it was Alb^B. Similar result has been reported for Japanese quails and their hybrids *(Vaida et al., 2000).* They reported higher frequency of Alb^A for brown (0.820), white (0.729) and hybrid (0.500) Japanese quail respectively. They also reported lower frequency of Alb^B (0.461). *Butkauskas*

et al. (2000) reported similar result with Alb^A of 0.5356 in Japanese quail. *Akinyemi et al. (2014)* reported higher frequencies for Alb^B in Muscovy ducks and higher values of Alb^A for Mallard ducks. The authors also reported the presence of Alb^C in Mallard ducks. All samples tested were polymorphic at the Tf locus with two Alleles (A and B). *Butkauskas et al. (2000)* reported the presence of Tf^C in quails, while Tf^D was reported to be present in turkey and Tf^E in chicken. *Okomato et al. (1999)* reported gene fixation as only genotype Tf^B and Tf^C was identified in their study. *Akinyemi et al. (2014)* reported the presence of only Tf^A and Tf^B with Tf^A being predominant in both Muscovy and Mallard ducks. *Oguntunji and Ayorinde (2015)* reported the presence of $Tf^{A, B, C \text{ and } D}$ with Tf^B (0.475) and Tf^C (0.419) being the most predominant in the populations of Muscovy ducks locally adapted in Nigeria. *Okamoto et al. (1999)* reported high frequency of Alp^B for the three breeds of chicken studied. Two co-dominant alleles F and S were reported for indigenous turkeys in Nigeria *(Fatai et al., 2017).* The authors reported Alp^F to be more predominant then Alp^S in the studied population. The presence of two alleles at the Alp locus has also been reported by other authors. *Singh and Nordskog (1981)* reported the prevalence of the slow allele Alp^S in some lines of Leghorn chickens. *Das and Deb (2008)* reported that for sexual maturity, birds with the fast type allele Alp^F mature about 13 days before those with Alp^S. *Singh et al. (1983)* reported higher levels of Alp in pullets selected for higher production, suggesting that the enzyme likely plays a significant role in sexual maturity.

The observed heterozygosity is the proportion of heterozygotes observed at a locus while expected heterozygosity or gene diversity is the proportion of expected heterozygotes under random mating. The values were high for both mottled brown quail and white quail with an overall Ho of 0.48 with a value of 0.47±0.13 and 0.49±0.13 for brown quail and white quail respectively while the overall He was lower with values of 0.33 ±0.13 and 0.31±0.13) in brown and white quails. The result of gene diversity from this study was higher than those reported by *Maedal (1999)* and *Vaida et al. (2000).* This could be a contributing factor to better adaptability of the Japanese quail to the prevailing tropical conditions. However, *Meadal et al. (1980; 1999)* reported that there was no clear difference in heterozygosity in quail lines selected for large and small body weight and also between the selected and random bred lines. Heterozygosity of blood proteins and enzymes of brown and white quails showed no significant differences ($P>0.05$). *Maeda et al. (1999)* reported that increasing the number of loci studied helps to detect small differences of heterozygosity, and it can be achieved now more easily through molecular markers

Chi-square test of Hardy-Weinberg proportions showed no significant differences in the observed and the expected frequencies of brown and white Japanese quails. This revealed that the gene and genotype frequencies of the two populations were in Hardy-Weinberg proportions as they were not affected by nonrandom mating, genetic drift, mutation, genetic migration and selection.

F-statistic values of Fst and Fit are measures of deviation from Hardy-Weinberg proportions and total populations, respectively. Positive values indicate a deficiency in heterozygotes and negative values an excess of heterozygotes. Fɪs can be interpreted as a measure of inbreeding (the measure of allelic fixation of individuals relative to the sub-populations). Thus, the negative value (-0.1067) of Fit observed at three loci out of the 6 loci that were studied for the quail populations indicates a deficiency of homozygotes in the populations and that mates were less related in comparison with the average relationship of the population. The negative values of Fɪs observed at two out of the six loci also indicate a deficiency of homozygotes in the populations. The observed excess of heterozygotes could be due to the non-random mating and genetic exchange between populations.

The estimated Fsт value, which corresponds to the proportion of genetic variability accounted for by the differences among breeds, was 0.0439. Thus, a larger part of the total genetic diversity can be explained by the variation within breeds (0.9561) and to a smaller extent by the variation among breeds (0.0439). This result indicated that the genetic diversity quantified by allozyme markers shows little genetic differentiation among the quail population studied. The degree of differentiation observed between the two quail population studied could be due to source, geographic proximity, similarities in environment and breeding practices.The standard genetic distance *(Nei, 1972)* obtained in this study between white and brown quails were 0.0534 indicating little genetic differentiation between the brown and the white quail. Similar trend was observed with *Nei (1976)* for the two strains under investigation (0.0517). *Vaida et al. (2000)* reported D value 0.0226 between white and motley quails using data from 10 loci. The estimated Nei's genetic identity (0.9480) obtained in this study was similar to 0.991 reported by *Vaida et al. (2000)* between white and brown quails.

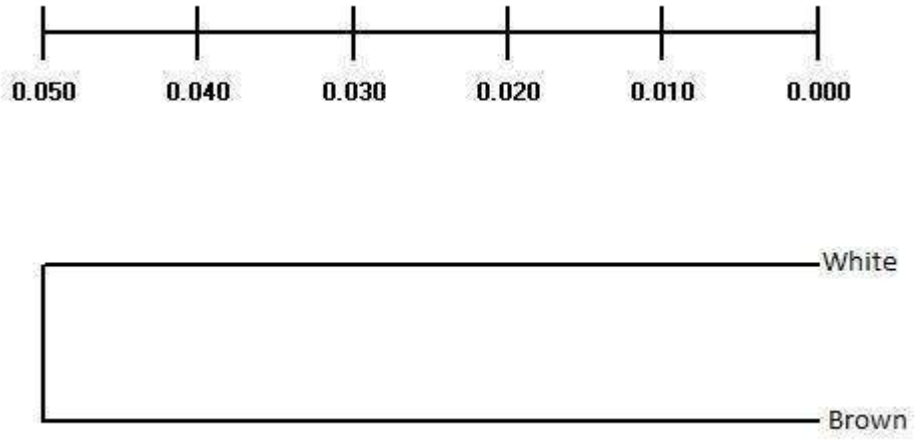

Figure 1. Dendogram of genetic distance between two strains of Japanese quail based on six allozyme loci

Conclusion

The present study demonstrated the usefulness of protein markers to characterize Japanese quail. Genetic similarity as measured by dendogram supported high gene flow between the two strains of Japanese quails; thus, indicating that the two populations were genetically related. Further studies should focus on other protein markers and DNA related studies on polymorphism. This may be useful as an initial guide in defining objectives for designing future investigations of genetic integrity and developing strategies for sustainable use of the Japanese quail genetic resource of in Nigeria.

Genetska varijacija japanske prepelice (*Coturnix coturnix Japonica*) bazirana na biohemijskom polimorfizmu

Adebukola Abiola Akintan, Osamede Henry Osaiyuwu and Mabel Omolara Akinyemi

Rezime

Cilj istraživanja je karakterizacija japanske prepelica koristeći biohemijske markere. Polimorfizam krvnih proteina od sto šezdeset šest (166) japanskih šarenih smeđih i belih prepelica, 83 svakog pola, analizirani su pomoću elektroforeze

celuloznog acetat papira. Ispitivano je šest lokusa koji uključuju hemoglobin (Hb), transferin (Tf), albumin (Alb), karbonatnu anhidrazu (CA), alkalne fosfataze (Alp) i esteraze-1 (Es-1). Svi testirani lokusi su polimorfni sa svakim lokusom koji ima dva ko-dominantna alela koja kontrolišu tri genotipa. Alel B je dominantan za Hb, Tf i Es-1 lokus sa frekvencijama 0,90, 0,55 i 0,77, dok alel A preovlađuje na Alb i Alpu lokusima sa frekvencijama 0,83 i 0,58 respektivno. Alel A imao je generalno niže frekvencije od B u CA lokusima sa vrednostima od 0,43 - Braon, 0,38 - Bela i 0,40 - ukupno. Prosečna opažena heterozigotnost (H_o) bila je 0,48 sa smeđim i belim prepelicama sa vrednostima H_o 0,47 i 0,49 respektivno, a očekivana heterozigotnost je bila viša kod belih prepelica (0,39) nego u šareno smeđim (0,31). Genetička distanca (0,0534) između bele i smeđe prepelice u ovoj studiji pokazala je malo genetske diferencijacije između. Dendogram koji je generisan iz vrednosti genetičke distance pokazuje da su dva tipa imala zajedničko poreklo.

Ključne reči: dendogram, japanska prepelica, polimorfizam, karakterizacija, elektroforeza

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EFFECTS OF SUPPLEMENTATION OF DIFFERENT LEVELS OF GARLIC (*Allium sativum*) ON SELECTED BLOOD PROFILE AND IMMUNITY OF WHITE LEGHORN CHICKEN

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Abstract. The study was conducted to evaluate the effect of feeding different levels of garlic powder inclusion on selected blood profile and immunity of white leghorn chicken. A total of 180 chickens (156 layers and 24 cocks) were randomly distributed in to 12 pens and assigned to 4 treatments. Treatments were rations containing 0, 1, 2, and 3% garlic powder for T1, T2, T3 and T4, respectively. The CP and ME content of treatment rations were 16-16.6% and 3021.31-3244.4 kcal/kg DM, respectively. Blood profile parameters were determined using established laboratory methods. The value of hemoglobin (Hb) increased insignificantly due to supplementation of different levels of garlic powder. Total white blood cell count (TWBC), basophile, lymphocytes, heterophils and monocytes were not affected ($P > 0.05$) by treatments. But, slight rise in lymphocyte and heterophil counts were observed in garlic supplemented groups which may be due to immuno-stimulatory effects of garlic. Packed cell volume (PCV) and eosinophils were affected ($P < 0.05$) by treatments, PCV (38.1, 45,2,41.5 and 39. 2), eosinophils (4.9, 3.2, 3 and 2.8), for T1, T2, T3, and T4, respectively. Mean values of total protein (g/dl) was not affected ($P > 0.05$) by treatment. The mean values of total immunoglobulin (gm/dl) (3.53, 4.09, 5.58, 3.04) was significantly ($P<0.05$) higher in T_3 compared to other treatments. Generally, inclusion of 2% garlic powder has significantly improved total immunoglobulin. But it significantly lowered eosinophils compared to control group. The present study revealed that mixing layer diets with 1-3% garlic powder could be used in practical layer diets to improve some haematological and immunoglobulin values which might consequently improve blood circulation and immunity of White Leghorns Chickens.

Keywords: garlic powder, layers, haematological parameters, immunoglobulins

Introduction

Antimicrobial compounds produced by microorganisms have been used in animal rations as growth promoters for many years (*Church, 1998*). Antibiotics affect birds' gut microflora and they have been used widely to prevent poultry diseases for the improvement of egg and meat production (*Botsoglou, 2002*). However, the use of antibiotics in animal feeds has been linked to antibiotic-resistant bacteria (*Glynn, 1998*). Consequently, many countries have banned the use of sub therapeutic levels of antibiotics in production of animal rations. To prevent a potential economic hardship and alleviate problems associated with antibiotic resistance photogenic feed additives have been developed as alternatives to antibiotics. As a consequence varieties of substances are used in conjunction with, or as alternatives to, antibiotics in poultry diets. Probiotics, prebiotics, organic acids, and plant extracts have all shown promising results for use in organic poultry production (*Griggs, 2005*).

Garlic (*Allium sativum*) is one of the most recognized plant species used for organic poultry production. Garlic is a bulbous perennial herb, closely related to the onion. It has anti-bacterial, anti-viral, anti-fungal, and anti-parasitic properties (*Puvaca et a., 2014*) and has been used traditionally for ages to treat a wide array of diseases, namely, respiratory infections, ulcers, and diarrhea and skin infections (*Fenwick and Hanley, 1985*). *Reuter et al. (1996)* also reported garlic as a plant possessing antibiotic, anticancer, antioxidant, immune modulator, anti-inflammatory, hypoglycemic and cardiovascular- protecting effects. Garlic (*Allium sativum*) gained the trust of many scientists and cultural remedies all over the world for the prevention and treatment of many diseases and is broadly dispersed and consumed as a spice and herbal medicine for thousands of years.

According to reports of *Sonaiya and Swan (2004),* traditional treatment and control of poultry disease is important for Ethiopia as most developing countries. These countries cannot afford to import veterinary medicine and vaccination for chickens. In Ethiopia, farmers were trying to treat their birds traditionally. A survey conducted by *Mengesha et al. (2011)* on the use of garlic as traditional treatment for birds indicated that 48.5% of the respondents were feeding garlic-onion and alcohol with soften injera to sick birds.

The major phytogenic compound obtained from garlic is allicin. This compound is derived from naturally occurring amino acid allin which is transformed into allicin (dially-thiosulphanate) by the enzyme allinase. This enzyme is inactivated by heat, oxygen and water *(Mantis et al., 1978)* leading to

reduction in both odour and medicinal properties of garlic. In pursuit of improved broilers health and in order to fulfill consumer expectation in relation to food quality, poultry producers commonly apply natural feeding supplements, mainly herbs (*Gardzielewska et al., 2003*). Garlic extract and/or garlic components were able to prevent chemically induced tumors or acute toxic effects of chemicals due to its attribute of containing several bioactive organosulfur compounds. Recent research works on herbal formulations as feed additive have shown encouraging results with regards to weight gain, feed efficiency, lowered mortality and increased liveability in poultry birds (*Kumar, 1991; Babu et al., 1992; Mishra and Singh, 2000; Deepak et al., 2002; Jahan et al., 2008; Puvača et al., 2014*). Furthermore, garlic has been found to have antimicrobial effect (*Shalaby et al., 2006; Durak et al., 2002*; *Weber et al., 1992),* and anti-cancer *(Durak et al., 2002)* activities, and lower cholesterol levels (*Jimoh et al., 2012*).

In general, today many pharmacological properties are attributed to garlic. Garlic has been shown to improve cardiovascular health through improving blood circulation (*Ernst, 1987*), minimizing atherosclerosis (*Yamasaki et al., 1994; Brodia, 1981*), inhibition of blood coagulation because of its effect on platelet aggregation and platelet growth (*Rahman, 2007; Srivastava and Tyagi, 1993; Harenbery et al., 1988; Apitz-Castro et al., 1983*), significantly reducing the level of serum cholesterol and triglyceride (*Streiner et al., 1996; Berthold et al., 1998; Yeh and Liu, 2001; Yamasaki et al., 1994*) and stimulates phagocytotic function of macrophage and lymphocyte proliferation (*Tidy et al., 1990*). However, its impact on blood profile and immunity is poorly investigated and there is dearth of scientific information available. Therefore, this paper seeks to evaluate the effect of different levels of inclusion of garlic (*Allium sativum*) on selected haematological and biochemical parameters of blood of White Leghorn layers.

Materials and Methods

The experiment was conducted at Haramaya University Poultry Farm located 505 km east of Addis Ababa, at an altitude of 1980 m.a.s.l, 9°26ˈN latitude and 42º3ˈE longitude. The mean annual rainfall is 780 mm. The mean annual minimum and maximum temperatures are 8ºC and 24, respectively (*AUA, 1998).*

The feed ingredients used in the formulation of the different experimental rations of this study were maize grain, noug seed cake, soybean meal, wheat short, limestone, salt (NaCl) and vitamin premix (Table 1). The newly harvested or fresh garlic bulbs were purchased from local market. The age of the bulbs, when supplemented was less than 4 months after harvesting. Peeled fresh garlic bulbs (cloves) were grinded and subsequently the garlic paste was thinly spread on a mat and air dried. The drying process which took 5 days on average continued until the garlic paste gets dried to the level it can be mixed thoroughly with the ration. The

prepared garlic powder was mixed with the diet of laying hens based on the specified levels. The diet has been stored at room temperature until it is fed according to the specified levels for layer hens. The layer treatment ration was formulated on an isocaloric and isonitrogenous basis to meet the nutrient requirements of 2800-2900 Kcal ME/Kg DM and 16-17 % CP *(NRC, 1994)*, respectively and water was always available to the animal.

Table 1. Proportion of ingredients used in formulating the experimental diet

Ingredients	%
Maize	42.7
Wheat short	18
Noug seed cake	23
Soybean meal	8
Limestone	7
Salt (NaCl)	0.5
Vitamin premix	0.8
Total	**100**

The experiment was conducted in a completely randomized design (CRD), with 4 treatments each with 3 replications. A total of 156 White Leghorn pullets and 24 cockerels at 8 months of age were obtained from Haramaya University Poultry Farm. They were randomly distributed to each replication making up 13 White Leghorn layers per pen and 2 cockerels per replicate and a total of 45 birds per treatment. The garlic-free diet was used as the control diet. The treatment ration was formulated as indicated in Table 2.

Table 2. Layout of the experiment

Treatment	No of replication	No of birds / replication	No of birds / treatment
T1- Ration containing 0% garlic	3	15	45
T2- Ration containing 1% garlic	3	15	45
T3-Ration containing 2% garlic	3	15	45
T4-Ration containing 3% garlic	3	15	45
Total			**180**

Birds were adapted to experimental diets for 7 days before the actual data collection started. The experimental houses have wire-mesh partitioned pens with teff straw litter material of approximately 10 cm depth. Before the placement of the birds into the experimental house the experimental pens, watering and feeding troughs, and laying nests were thoroughly cleaned, disinfected and sprayed against external parasites. The feed was offered in a group per pen or replication twice a day at 08.00 and 17.00 hours throughout the experimental period. Feed were offered in hanging tubular feeders, which were suspended approximately at a

height of the backs of the birds and water was provided in a plastic fountains placed on a flat wood at the center of the pen. The feeding and watering troughs were cleaned every morning before the daily meal was offered. Water was available all the time and the experiment lasted for 90 day (three months). Vitamins were given to the birds, turning the litter and changing of extremely wet litter with clean and dry was carried out whenever required.

The chemical analysis was carried out at Haramaya University nutrition laboratory. Chemical analysis of the feed was made in duplicate. When the mean result of duplicates was not similar, the mean value of the two duplicates was taken, provided that the percentage error was not greater than 5%. For chemical analysis of the feed ingredients representative samples were taken from each of the feed ingredients used in formulation of the experimental diet, and chemical analysis was done before formulation of the treatment diets. The samples were subjected to proximate (Weende) method of *A.O.A.C (1990)* to determine dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE) and total ash content. Nitrogen was determined by using Kjeldhal procedure and CP was computed by multiplying the N content by 6.25. The metabolisable energy (ME) value was determined indirectly based on previously published method *(Wiseman 1987).*

$$ME\ (Kcal/\ kg\ DM) = 3951 + 54.4EE - 88.7CF - 40.8Ash$$

The calcium, phosphorus and other mineral contents analysis were determined by atomic absorption spectrophotometer and UV (ultra violet).

For the determination of hematological parameters, blood was collected via the jugular vein-puncture using sterile syringes and needles (25G). Accordingly, four birds were randomly selected from each replications and 2 ml blood was collected. Then the blood was transferred immediately into a set of sterile tubes containing anticoagulant, disodium-salt of ethylene diamine tetraacetic acid (EDTA). The values for the hemoglobin (HB) were obtained using the whole blood. The percentage Packed Cell Volume (PCV) was determined by centrifugation of capillary tubes for 5 minute at 1200 rpm and the hemoglobin content was determined by Actin hematin method. Total white blood cell count was conducted by using haemocytometer by the method described by *Campbell (1980),* and differential white blood cell count was determined on blood smear prepared by Wright's stain. The hematological parameters were determined by the methods described by *Davice and Lewis (1991).*

For serum biochemistry analysis four birds were randomly selected from each replications and three ml of blood samples in the set of bottles containing no anti-coagulant was kept in the refrigerator at about 4°C for about 3 hours to aid sedimentation. The samples were later spun in a centrifuge at 3,000 rpm for 10 minutes and the serum was separated, stored and frozen at -20°C for analysis. Refractometer was used to assess the total serum protein. Zinc sulfate turbidity test was used to estimate total immunoglobulin concentration from serum (*Mcevan et al., 1969).* Zinc sulfate solution was prepared by adding 250 mg of zinc sulfate in 1 liter of distilled water. Then 0.1 ml serum sample was added to 6 ml of zinc sulfate solution to make the test solution. To prepare the control, 6 ml of distilled water was added to the serum instead of zinc sulfate solution. Both test and control mixtures were shaken and kept at room temperature for 60 minute. The optical density (OD) of the test and the control solutions were recorded separately at 545 nm using spectrophotometer.

Total immunoglobulin (gm/d1), was calculated using the following formula

- Zinc sulfate turbidity (ZST) units = (optical density of test – optical density of control × 10)
- Total immunoglobulin (gm/d1) = 0 .04 + 0.98(ZST) units.

The data was subjected to statistical analysis using *Statistical Analysis Software (SAS) (2008)* version. Least significance difference (LSD) was used to locate the treatment means that were significantly different (*Gomez and Gomez, 1984*). The model used for statistical analysis was $\boldsymbol{Yij = \mu + Ti + eij}$, where: **Yij** = the response variable; $\boldsymbol{\mu}$ = over all mean; **Ti** = treatment effect and **eij** = random error

Results and Discussion

The chemical composition of feed ingredients used and the four compound rations are shown in Table 3 and Table 4, respectively. The garlic powder has good nutrient contents including minerals.

Table 3. Chemical composition of feed ingredients used to formulate experimental ration

Chemical composition	Feed Ingredients				
	MG	WS	SBM	NSC	GP
DM (%)	89.6	90.3	93.0	92.2	91.4
CP (DM %)	8.46	14.7	39.0	29.6	11.96
EE (DM %)	6.2	3.3	9.2	8.1	1.59
Ash (DM %)	5.9	5.53	5.75	9.1	3.169
CF (DM %)	2.8	9.9	5.7	18.3	0.9
Ca (DM %)	0.02	0.19	0.35	0.35	0.28
P (DM %)	0.09	0.78	0.83	0.32	0.698
Fe(ppm)	-	-	-	-	0.08
Mg(% DM)	-	-	-	-	0.66
K(% DM)	-	-	-	-	0.75
ME (Kcal/Kg)	3799.2	3030.7	3711.0	2401.8	3828.09

MG= Maize grain; WS= Wheat short; SBM =Soybean meal; GP = Garlic powder; NSC= Noug seed cake; DM=Dry matter; CP = Crude protein; EE = Ether extract; CF = Crude fiber; Ca = Calcium; Fe = Iron; Mg = Magnesium; K= Potassium; P= Phosphorus; ME = Metabolizable energy; Kcal= Kilo calorie and kg = Kilogram.

From the analysis result, it can be seen that soybean meal (SBM) and noug seed cake (NSC) are rich in crude protein (CP) content that make these ingredients to be a good source of protein supplement for poultry. The CP content of the NSC used in the current experiment is comparable to previous findings of *SDDP* (1997) and *Fantie and Solomon* (*2008*) which is 29.6% and 28.9% CP, respectively. Values for the CP and ME content of maize grain used in the current experiment were 8.46% and 3799.2 kcal/kg DM, respectively. The DM and CP content of wheat short used in this study were similar to *Meseret* (*2006*) which was 90.3% DM and 14.7% CP values.

Table 4. Chemical composition of experimental treatment diets containing different proportions of garlic powder

Chemical Components	Treatments			
	T_1	T_2	T_3	T_4
DM (%)	92.0	90.2	91.2	91.8
CP (% DM)	16.0	16.3	16.6	16.0
EE (% DM)	6.4	7.5	6.1	6.9
Ash (% DM)	13.4	12.0	13.4	13.1
CF (% DM)	7.0	7.0	8.0	8.0
P (% DM)	1.10	1.16	1.28	1.02
Ca (% DM)	2.81	3.15	2.86	3.38
ME kcal/kg	3127.15	3244.48	3021.31	3083.0

DM = Dry matter, CP = Crude protein, EE = Ether extract. CF = Crude fiber, Ca = Calcium, P= Phosphorus, ME = Metabolizable energy, kcal= Kilo calorie and kg = Kilogram, T1 = Ration containing no garlic powder, T2 =Ration containing 1% garlic powder, T3 =Ration containing 2% garlic powder, T4 =Ration containing 3% garlic powder.

The CP content of the treatment diets varied between 16.0% to 16.6% which was within the range of CP requirement (14-19%) as suggested by *Leeson and Summers* (*2001*) for layers. Similarly, *Tadelle (1997)* noted that the protein requirement of high producing laying hens should be between 16-18% of the diet to meet the needs of egg production, maintenance and growth of body tissues. The ME content of treatment diets were slightly decreased in T3 and T4 in comparison to T1 and T2 level of garlic powder and the results of the whole treatment s were slightly greater than the anticipated 2800 kcal/kg. The Ca content of treatment diets were within the range of 2.5-3.5 % DM needed for layers (*Eekeren et al., 2006*).

Hematological parameters of layers are presented in Table 5. In the present study, dietary supplementation of garlic powder in feed was found to cause insignificant increase ($P>0.05$) in the mean values of hemoglobin (Hb) as compared to control group. But, there has been report of significant rise in Hb concentration due to garlic supplementation in rats (*Iranloye, 2002)* which might be due to species variation. Garlic extract is an active oxygen scavenger. It is thus possible that garlic components compete with Hb in the RBC for oxygen resulting in hypoxia which then stimulates Hb synthesis and RBC production.

The one test offering more information than any other procedure about anemia and dehydration is packed cell volume. The normal PCV in avian species is 35% - 50%. The result from this study showed that PCV of T2 diet consumed hens was significantly higher ($P<0.01$) than that of layers fed T3, T4 and the control. In agreement with our results, the findings reported by *Oluwole* (*2001*) that the mean values of RBC and PCV in rats given 200 mg/day garlic for 30 days did not significantly differ from the control ($P > 0.05$), however, there were significant increase in RBC and PCV for animals given low dose (100 mg/day) of garlic for 30 days (*Iranloye, 2002*) observed a significant increase in RBC, PCV, WBC and total Hb concentration in garlic fed rats. The numerical increase observed in the Hb and PCV of birds fed garlic supplemented diets suggest that the diets were better utilized and assimilated into the blood stream for use by the birds. The normal PCV, Hb and other haematological values portray the nutritional status of the broiler chicken and thus indicating adequate nourishment of the birds (*Church et al., 1984*).

There was no significant difference in lymphocytes, basophils, monocytes and neutrophil in birds fed with garlic containing diet as compared to control birds. The mean values of eosinophils decreased ($P < 0.01$) with increasing levels of garlic powder (Table 3). The mean value of eosinophils was decreased in layer chicken fed garlic supplemented diets which suggests that garlic complemented the antiparasitic action of eosinophils. Garlic (*Allium sativum*) has antihelmintic action in vitro against *Heterakis gallinae* and *Ascaridia galli*, *Heamomonchus contortus*, a free- living nematode of *Rhabditis* sp (*Nagaich, 2000; Zafar-iqbal et al., 2001; Chybowski, 1997*). The present study also revealed slightly increased lymphocytes in the garlic fed groups in layer chickens. In agreement with the findings reported

by *Prasad et al.* (*2009*) slight rise in lymphocyte and heterophil count was observed in garlic supplemented groups, which may be due to immuno-stimulatory effects of garlic. *Yan et al*. (*2010*) noted that fermented garlic powder supplementation increased the lymphocyte count compared with the control group. *Chen et al*. (*2008*) suggested that dietary garlic powder (1 g/kg) increased the lymphocyte concentration in pig. *Tadi et al*. (*1990*) had suggested that garlic can stimulate the phagocytotic function of macrophage and lymphocyte proliferation. *Koy et al*. (*1998*) also concluded that allium could promote the lymphocyte synthesis, cytokine release, phagocytosis and natural killer cell activity. In the present study, dietary supplementation of garlic powder in layer chicken showed no significant (P>0.05) difference in the mean values of total leucocyte count (TLC) as compared to control diet. However, mean values of total leucocyte count (TLC) of T2 and T4 held numerically higher than T1, T3 and the control. In agreement with the present results of the experiment, garlic supplementation does not affect total and differential leucocyte counts in broiler chicks (*Jafari et al., 2008*). Conversely, *Ademola* (*2004*) reported increase in total white blood cells and heterophils by about 18.7% and 20.4%, respectively, in garlic powder treated birds as compared to control birds.

Table 5. Effect of different levels of garlic powder on hematological parameters of white leghorn chicken

Parameters	Treatments				SEM	SL
	T1	T2	T3	T4		
PCV (%)	38.1[b]	45.2[a]	41.5[b]	39.2[b]	0.938	*
Hemoglobin (%)	9.8	10.5	11.2	11.0	0.234	NS
WBC (In thousand/ml)	31.1	32.2	31.5	32.6	0.5	NS
Eosinophils	4.9 [a]	3.2[b]	3.0[b]	2.8[b]	0.269	***
Basophile	2.4	2.1	2.6	2.3	0.074	NS
Lymphocytes	73.3	76.3	76.5	75.8	0.719	NS
Monocytes	6.8	6.3	5.4	6.2	0.251	NS
Heterophils	12.3	12.3	12.4	12.9	0.405	NS

Means with in a row with different superscripts are significantly different; *=Significant at (P<0.05);***= Significant at (P< 0.001); PVC= Packed cell volume, WBC= White blood cell count, ml=Milliliter, T1 = Ration containing 0% garlic powder, T2 =Ration containing 1% garlic powder, T3 =Ration containing 2% garlic powder, T4 =Ration containing 3% garlic powder.

Serum biochemistry of layers is presented in Table 6. The mean values of total immunoglobulin (gm\dl) was significantly (P<0.05) higher in T_3 compared to other treatments. There was no significant (P>0.05) difference in the mean values of total protein (g\dl). But, the total plasma protein decreased with increasing level of dietary garlic (control, 1, 2, and 3%) and approached the normal protein level (4.5 g/dl). More garlic level (3%) administrated group has nearly normal plasma

protein concentration (6.16) compared with control group (9.37).The normal plasma protein concentration in birds is less than in mammal, and it generally ranges from 2.5 to 4.5 g/dl. Hyperproteinemia in most birds is indicated by plasma protein concentration of greater than 4.5 g/dl. Hyperproteinemia usually is the result of dehydration, acute or chronic inflammation, or preovulatory condition in hens. Dehydrated birds subjected to chronic stress or other immunosuppressive condition may demonstrate this type of plasma protein profile (*Mary et al., 2004*).

Table 6. Effect of different levels of garlic powder on serum biochemistry and yolk cholesterol of white leghorn chicken fed rations containing different levels of garlic powder

	Levels of garlic powder (%)					
Parameters	T1	T2	T3	T4	SEM	SL
Total Protein (g\dl)	9.37	9.33	7.35	6.16	0.683	NS
Total immunoglobulin (gm\dl)	3.53^{b}	4.09^{b}	5.58^{a}	3.04^{b}	0.343	*
Total yolk cholesterol (mg/dl)	18.5	19.0	17.1	19.8	0.482	NS

Means with in a row with different superscripts are significantly different; *=Significant at (P<0.05); T1 = Ration containing 0% garlic powder, T2 =Ration containing 1% garlic powder, T3 =Ration containing 2% garlic powder, T4 =Ration containing 3% garlic powder.

Conclusions

The present study revealed significantly increased hemoglobin (Hb) due to supplementation of different levels of garlic powder. These effects are may be due to the presence of some bioactive constituents and/or their metabolites in garlic. Total white blood cell count (TWBC), basophile, lymphocytes, heterophils and monocytes were not affected (P > 0.05) by treatment. But, slight rise in lymphocyte and heterophil counts were observed in garlic supplemented groups which may be due to immuno-stimulatory effects of garlic. Packed cell volume and eosinophils were affected (P < 0.05) by treatment, PCV (38.1, 45,2,41.5 and 39. 2 (SEM=.0938)), eosinophils (4.9, 3.2,3 and 2.8 (SEM=.269)), for T1, T2, T3, and T4, respectively. The mean values of eosinophils decreased in layer chicken fed garlic supplemented diets suggests that the antiparasitic action of garlic. The numerical increase observed in the Hb and PCV of birds fed garlic supplemented diets suggest that the diets were better utilized and assimilated into the blood stream for use by the birds.). Mean values of total protein (g/dl) (9.37, 9.33, 7.35 and 6.16 (SEM= 0.683)) was not affected (P > 0.05) by treatment, the mean values of total immunoglobulin (gm\dl) (3.53, 4.09, 5.58, 3.04, (SEM= .343)) was significantly (P<0.05) higher in T_3 compared to other treatments. Generally, the inclusion of 2% garlic powder has significantly improved total immunoglobulin. Additionally, 2% garlic inclusion has significantly lower eosinophils compared to control group. On the basis of the results of the present study, it was concluded that mixing layer diets with 1-3% garlic powder can be used in practical layer diets

improved some haematological value and total immunoglobulin which could contribute to improved blood circulation and immunity of White Leghorns Chickens.

Efekat dodavanja različitih nivoa luka (*Allium Sativum*) na određeni profil krvi i imunitet White Leghorn pilića

Tesfaheywet Zeryehun, Meseret Asrat, Negassi Amha, Mengistu Urge

Rezime

Studija je sprovedena kako bi se procenio efekat uključivanja različitih nivoa praška od belog luka u ishrani na izabrani profil krvi i imunitet pilića White Leghorn. Ukupno 180 pilića (156 nosilja i 24 petlića) su nasumično raspoređeni u 12 bokseva i u 4 tretmana. Tretmani su bili obroci koji sadrže 0, 1, 2 i 3% praška belog luka - T1, T2, T3 i T4, respektivno. Sadržaj SP i ME tretmana iznosio je 16-16,6% i 3021,31 -3244,4 kcal/kg SM, respektivno. Parametri profila krvi određeni su korišćenjem utvrđenih laboratorijskih metoda. Vrednost hemoglobina (Hb) se povećala neznatno kao rezultat dodavanja različitih nivoa praška belog luka. Ukupni broj bijelih krvnih zrnaca (TWBC), bazofila, limfocita, heterofilia i monocita nisu bili pod uticajem (P>0,05) tretmana. Međutim, mali porast broja limfocita i heterofila je primećen u grupama hranjenih dodatkom belog luka, što može biti posledica imuno-stimulativnih efekata belog luka. Kombinovani volumen ćelija (PCV) i eozinofili su bili pod uticajem (P<0,05) tretmana, PCV (38,1; 45,2; 41,5 i 39,2 (SEM = 0,038)), eozinofila (4,9; 3,2; 3 i 2,8 = .269)), za T1, T2, T3 i T4, respektivno. Srednje vrednosti ukupnog proteina (g/dl) (9,37; 9,33; 7,35 i 6,16 (SEM = 0.683)) nisu bile pod uticajem tretmana (P> 0.05). Srednje vrednosti ukupnog imunoglobulina (gm/dl) (3;53; 4,09; 5,58; 3,04, (SEM = .343) su značajno (P <0.05) veće u T3 u poređenju sa drugim tretmanima. Uglavnom, uključivanje 2% značajno je poboljšalo ukupan imunoglobulin, ali je značajno smanjio eozinofil u poređenju sa kontrolnom grupom. Ovo istraživanje je pokazalo da se dopunjavanje obroka za nosilje sa 1-3% belog luka u prahu može koristiti u obrocima za nosilje u praksi, kako bi se poboljšale hematoloških i imunoglobulinske vrednosti što bi moglo dovesti do poboljšanja cirkulacije krvi i imuniteta pilića White Leghorn.

Ključne reči: beli luk u prahu, nosilje, hematološki parametri, imunoglobulini

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THE PERFORMANCE OF PERENNIAL RYEGRASS IN BINARY MIXTURES WITH LUCERNE AND RED CLOVER UNDER N FERTILIZATION

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Abstract. Perennial ryegrass is a very important and widespread grass species used for livestock nutrition, in particularly ruminants. As a species that is most commonly used on grasslands, it is grown in mixtures with other types of grasses and legumes. The objective of the research was to investigate the performance of perennial ryegrass at various proportions in the mixtures with red clover and lucerne, and how different levels of N fertilization affect its competitiveness. Ryegrass achieved the highest yield with lucerne at seeding rate 50:50 and with red clover at seeding rate 70:30. Relative grass yield (RYg) of mixtures ranged from 1.01 to 1.55 respectively, which means that ryegrass in mixtures achieved 0.1-55% greater yield than pure ryegrass crop. N fertilization increased DMY and RYg, leaf : steam ratio, specific leaf area (SLA), leaf area ratio (LAR) and leaf area index (LAI) in both years thus increasing the competitive capability of perennial ryegrass.

Key words: perennial ryegrass, red clover, lucerne, performance, competitiveness

Introduction

Perennial ryegrass is a very important and widespread grass species used for livestock nutrition, particularly ruminants. As a plant of moderately warm conditions, with good supply of water, especially in conditions with irrigation, it realizes high yields of very good quality. However, due to the sensitivity to drought, it shows great yield reduction in summer months. As a species that is most commonly used on pastures, it is grown in mixtures with other types of grasses and legumes. Growing in the mixture is intended to achieve higher yields compared to

growing in pure crops. This phenomenon can be explained by the ability of individual species to use in a different way available resources in space and time (*Ergon et al., 2016*). Thus, a mixture of two grasses and two legumes gives higher yield by 57% compared to the examined most productive monoculture (*Nyfeler et al., 2009*). In studies by *Elgersma and Schlepers (1997),* pure crop of perennial ryegrass has achieved significantly lower yields of dry weight of 1.7-2.0 t ha^{-1}, compared to its mixtures with white clover of 8.7 to 12.2 t ha^{-1}. Also, cultivation of perennial ryegrass and other grasses in mixtures, improves intra-annual yield stability *(Sanderson, 2010),* while legume component increases the nutritional value of herbage by increasing of protein content, digestibility, reducing fibres concentration and contributing to a better balance of feed *(Fraser and Kunelius, 1995).*

However, when combining species in mixtures characteristics of species should be considered and their competitive ability as a very important determinant of yield and stability. Maintaining of well-balanced grass-legumes mixtures is very important, given that the grasses are generally more competitive than legumes, especially in regard to the nutrient uptake. Thus meadow fescue (*Festuca pratensis* Huds.) is the least competitive, while tall fescue (*Festuca arundinacea* Schreb.) and orchardgrass (*Dactylis glomerata* L.) are very aggressive *(Frame et al., 1998).* According to *Laidlaw and Teuber (2001),* grass of temperate climatic areas are less competitive so that they are more suitable for the cultivation with forage legumes.

The goal of this research is to investigate the performance of perennial ryegrass at various proportions in the mixtures with red clover and lucerne, and how different levels of N fertilization affect its competitiveness.

Material and Methods

The experiment was conducted during 2014-2016 at the experimental field of the Institute for Animal Husbandry Zemun, Belgrade (44°49′N, 20°17′E, and elevation 96 masl). The study was performed in three replications using randomised complete block system. The soil was silty clay loam with pH of 7.08. Climate of this area could be characterized as the temperate continental with cold winters and hot summers. Temperature and precipitation for study period are presented in Figure 1.

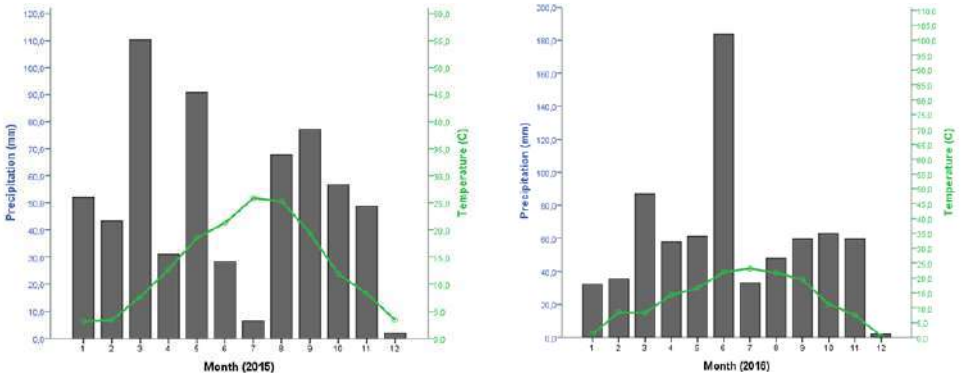

Figure 1. Average monthly temperature (°C) and sum of precipitation (mm) in two experimental year

In the spring of 2014., perennial ryegrass cultivar Calibra was sawn with lucerne cultivar NS Banat and red clover cultivar K-39 in grass-legumes mixtures with various proportions of species: 50% perennial ryegrass and 50% lucerne (50:50 PR+L); 50% perennial ryegrass and 50% red clover (50:50 PR+RC); 70% perennial ryegrass and 30% lucerne (70:30 PR+L); 70% perennial ryegrass and 30% red clover (70:30 PR+RC). Grass-legumes mixtures were sawn on field plots (2m x 5 m) with seed rate of 25 kg ha^{-1} for grass and 20 kg ha^{-1} for legumes. Nitrogen fertilizer in the form of ammonium nitrate was applied at the beginning of vegetation in amounts of 0, 50 and 100 kg N ha^{-1}.

Experimental measurements were done in the first and the second production year (2015-2016). All plots were cut three times in each studied year. Samples of herbage mass were collected from surface of 1 m^2, measured and after drying in the oven at 60 ° for 72 h, dry matter was determined. Also, another sample from 1m^2 surface, was collected and used for separation of species to determine botanical composition and for separation of leaves from steams to determine leaf : steam ratio. Leaf areas per plant were measured by using the software ImageJ and utilized for calculation of growth parameters: leaf area index (LAI), specific leaf area (SLA) and leaf area ratio (LAR).

LAI= leaf area per plant/No plant per m^2

SLA= leaf area per plant/leaf weight per plant (cm^2 g^{-1})

LAR= leaf area per plant/ weight per plant (cm^2 g^{-1})

Relative yields of the grass in the mixtures were calculated using the equation according to *De Wit (1960):*

RYg = DMYgl/DMYgg,

where DMYgl is the dry matter yield of the grass in the mixture and DMYgg is dry matter yield of the grass in the monoculture. RYg >1 indicated that grass in mixtures overyielded grass in monoculture which can be attributed to the level of nitrogen fertilization or efficiency of nitrogen fixation and transfer.
Data were analysed by the General linear model (SPSS 20.0) for ANOVA to detect differences of productive parameters of perennial ryegrass between mixtures at different level on nitrogen fertilization. Shapiro-Wilk test was used to determine whether or not the observations themselves are normally distributed and Levene's test for testing homogeneity of variances. Differences among means were detect with LSD at the probability level of 0.05.

Results and discussion

Dry matter yield and growth parameters of perennial ryegrass

Investigation of the effect of type of mixture and N fertilization on the dry matter yield and the relative yield of perennial ryegrass in the mixture, we have found that N fertilization had a significant impact on DMY and RYg in both experimental years, while the mixture, and the interaction of the two studied factors showed significant effect only in the first year of the study (Table 1 and Table 2). In the first production year, perennial ryegrass had significantly higher DM production in 70: 30 PR+RC mixture than in the other mixtures. In all mixtures perennial ryegrass yielded from 2.3 to 3.63 t ha^{-1} respectively. With lucerne ryegrass yielded more at seeding rate 50:50 and with red clover at seeding rate 70:30. In second production year, although there is no significance, red clover in mixtures reduced yield of ryegrass generally. The highest yield, ryegrass achieved in mixture with lucerne 50:50 PR+L. *Gierus et al. (2012)* concluded that ryegrass in mixtures with legumes achieved the highest yield in mixture with white clover, and the lowest in mixture with grazing-type of lucerne. Mixtures with red clover had yield of ryegrass greater then mixture with hay-type lucerne, but the differences were not statistically significance.

Table 1. Impact of N fertilization and mixture type on DMY and RYg of perennial ryegrass in 2015

Mixtures	DMY (t ha^{-1}) Level of N			Average mixtures	RYg Level of N			Average mixtures
	0	50	100		0	50	100	
50:50 PR+L	1.50	2.11	4.84	2.82^{b}	0.66	0.93	1.94	1.18^{b}
50:50 PR+RC	0.96	2.10	3.85	2.30^{b}	0.45	1.02	1.79	1.08^{b}
70:30 PR+L	1.43	2.78	3.28	2.50^{b}	0.35	1.24	1.33	0.98^{b}
70:30 PR+RC	2.92	4.12	3.86	3.63^{a}	1.29	1.86	1.49	1.55^{a}
Average fertilization	1.70^{c}	2.78^{b}	3.96^{a}		0.69^{c}	1.26^{b}	1.64^{a}	
level of significance								
N fertilization	**				**			
Mixture type	**				**			
N x mixture	*				**			

PR-perennial ryegrass, L-lucerne, RC-red clover, DMY-dry matter yield, RYg-relative grass yield; ns- non significant; *- significant at p≤0.05; **-significant at p≤0.01.

The mean RY of the grass component in the mixtures are presented in Table 1. And Table 2. The values of RYg in both years were greater than 1, indicating that DMY of ryegrass were higher than that in pure stand. Only mixture 70:30 PR+L showed less values than 1. RYg of all other mixtures ranged from 1.01 to 1.55 respectively, which means that in this mixtures ryegrass achieved 0.1-55% higher yield than pure ryegrass crop. Ryegrass in mixtures benefited from nitrogen fixation and mostly yielded more than ryegrass monoculture. Researching forage production of *Dactylis glomerata* and *Trifolium subterraneum* mixtures, *Kyriazopoulos et al. (2013)* founded that RY of orchard grass ranged between 0.47 and 1.17. Same as in our study mixture with grass-legume ratio, 75:25 had RY less than 1. Other mixtures with greater proportion of legume had RYg>1 which indicated better interspecific competition of grass as high presence of legume favours its productivity.

Table 2. Impact of N fertilization and mixture type on DMY and RYg of perennial ryegrass in 2016

Mixtures	DMY (t ha^{-1}) Level of N			Average mixtures	RYg Level of N			Average mixtures
	0	50	100		0	50	100	
50:50 PR+L	2.09	3.09	2.94	2.93	1.03	1.37	1.43	1.27^{a}
50:50 PR+RC	1.91	2.51	4.37	2.71	0.87	1.05	2.16	1.36^{a}
70:30 PR+L	2.00	0.93	2.89	2.09	0.89	0.37	1.40	0.89^{b}
70:30 PR+RC	1.51	2.35	2.36	1.94	0.69	1.03	1.31	1.01^{ab}
Average fertilization	1.88^{b}	2.23^{b}	3.14^{a}		0.87^{b}	0.95^{b}	1.57^{a}	
level of significance								
N fertilization	**				**			
Mixture type	ns				*			
N x mixture	ns				ns			

PR-perennial ryegrass, L-lucerne, RC-red clover, DMY-dry matter yield, RYg-relative yield grass; ns- non significant; *- significant at p≤0.05; **-significant at p≤0.01.

Regarding nitrogen fertilization, treatment with 100 kgN ha^{-1} had higher yield of ryegrass then other two treatments. In first production year mixture ryegrass with 100N achieved 3.96 t ha^{-1} and in second year 3.14 t ha^{-1}.Treatment with 50 kgN ha^{-1} also increase yield by 64% in first either 19% in second year compared to zero fertilization.

The average RYg values in both years were under production of pure stand for treatment without N fertilization. Yield of PR in mixtures in treatment with 50kgN ha^{-1}outyielded pure grass in first year by 26% and treatment of 100 kgN ha^{-1} by 64 and 57% respectively. This result indicate that N fertilization increased competitive ability of ryegrass what is in accordance with *Vasquez et al. (2008).*

The growth parameters of ryegrass plants in mixtures are presented In Table 3.

Table 3. LAR, leaf : steam ratio, LAI and SLA of perennial ryegrass in mixtures with legumes and fertilized with N in second investigation year

Mixture	LAR (cm^2 g^{-1})	Leaf : steam ratio	LAI	SLA (cm^2 g^{-1})
50:50 PR+L	95.26^{bc}	0.56^{c}	1.82	295.37^{b}
50:50 PR+RC	119.44^{ab}	0.78^{b}	1.89	322.57^{a}
70:30 PR+L	137.67^{a}	1.18^{a}	1.78	290.20^{b}
70:30 PR+RC	85.48^{c}	0.58^{c}	1.66	251.05^{c}
level of significance	**	**	ns	**
N fertilization				
0	103.74	0.61^{c}	0.83^{c}	265.65^{b}
50	117.90	0.77^{b}	2.07^{b}	277.30^{b}
100	106.75	0.96^{a}	2.46^{a}	326.45^{a}
Level of significance	ns	**	**	**

PR-perennial ryegrass; L-lucerne; RC-red clover;ns- non significant; *- significant at p≤0.05; **-significant at p≤0.01.

Mixture type significantly affected LAR, SLA and leaf : steam ratio. Mixture 70:30 PR+RC had the lowest value of all three parameters while leaf area ratio and leaf: steam ratio were the highest in 70:30 PR+L and SLA in 50:50 PR+RC.

N fertilization did not affect LAR. In research of *Lopes et al. (2011)* abundant N fertilization, significantly reduced LAR what they explained as the result of lower investment in the area of light capture with N fertilization at the expense of investment in the structural components of the plant, as the canopy becomes heavier. In our experiment low doses of N increased LAR, while higher level of N decreased LAR.

Added nitrogen increase proportion of leaves toward steam in range from 0.61 (0 kgN) to 0.96 (100kgN) or leaf proportion increase for 0.0035 for each kg of N, what is very desirable for better quality and digestibility of forages. In research of *Salvador et al. (2016),* N fertilization also increase leaf : steam ratio at the level of

100 kgN ha^{-1}. Further addition of N decline this ratio what authors explained as the result of change the use of this nutrient mostly for inflorescence formation and stem elongation.
Likewise, N fertilization impact on SLA, raising values from 265.65 cm^2 g^{-1} (0kgN) to 326.45 cm^2 g^{-1} (100kgN) respectively.
There was no significant difference in ryegrass LAI between mixtures. Only N fertilization showed significant impact on LAI value. Hence, LAI increase with N fertilization in the range from 0.83 (0 kgN) to 2.46 (100 kgN), respectively. Researching specific leaf area of three dominant perennial grass species in a long term nitrogen fertilization experiment, *Knops and Reinhart (2000)* also concluded that SLA and LAI increased with increasing levels of nitrogen fertilization.

Proportion of perennial ryegrass in mixtures

Botanical composition of the mixtures are presented in Figure 2. In both year proportion of perennial ryegrass was lower than the sown proportions. This fact could be explained with greater competitive ability of red clover and lucerne for light and nutrients in existing agro-climatic conditions.
In first production year proportion of ryegrass was higher in mixtures with lucerne in regard to red clover mixtures. Seeding rate of 50:50 had lower proportion of ryegrass, 22-40%, than seeding rate of 70:30, 42-48%. In second production year share of ryegrass in all mixtures were relatively constant from 22 to 29%. Nitrogen fertilization increased proportion of ryegrass in the mixtures, in the first place treatments with 50N. However, 50N increased ryegrass proportion to 45% in first and to 32% in second year. Increased within 100N is slightly less and varies from 43-26%. *Nyfeler et al. (2009)* also confirmed the fact that grass species proportion in mixtures are positively affected by N fertilization. In research of *Leto et al. (2008)*, N150 treatment increase 29% DM yield of *Dactylis glomerata* and *Poa pratensis* and 9% their contribution to total DM yield in comparison with N0.

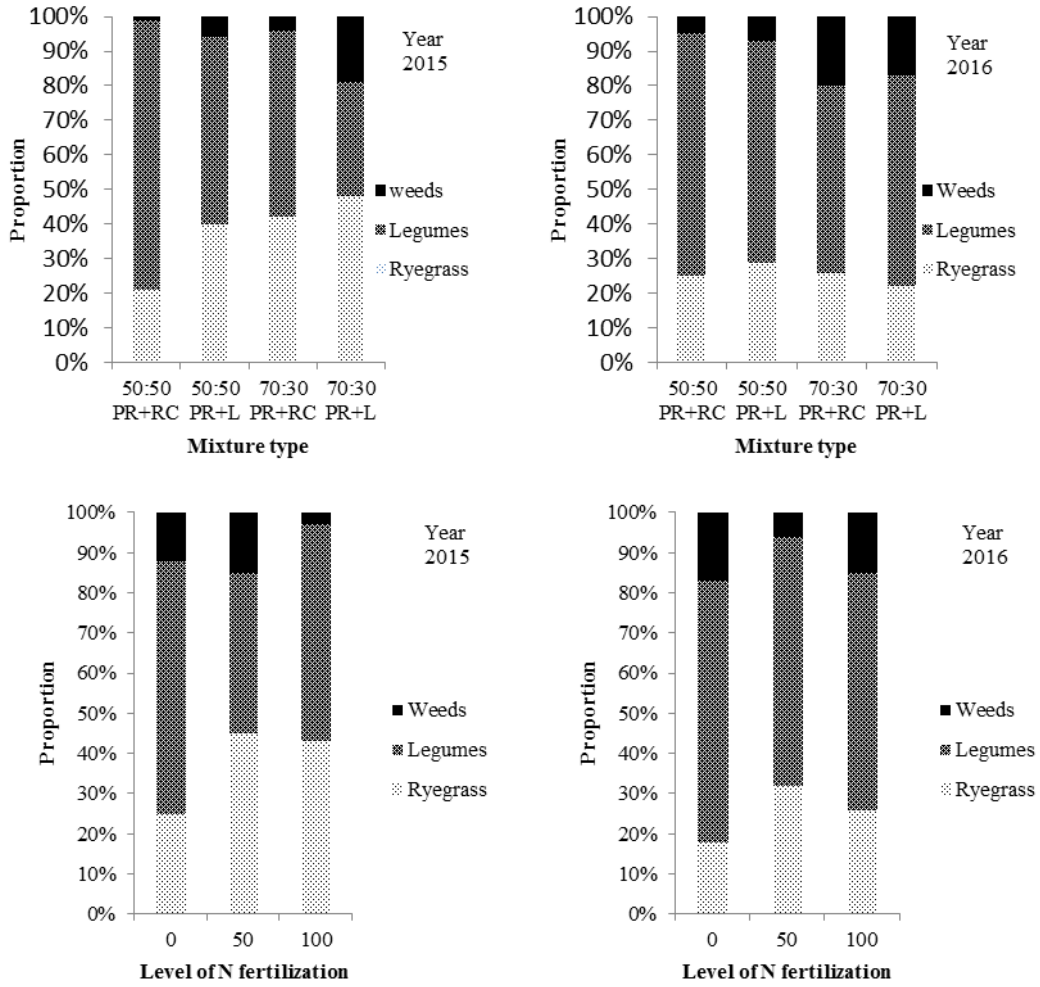

Figure 2. Proportional composition of mixtures of perennial ryegrass, red clover and lucerne, with different level of N fertilization, displayed for two cutting years

Dry matter yield of ryegrass in mixtures with legumes vs. legume proportion

In Figure 3. and Figure 4 were presented ryegrass DM yield relative to legume proportion in mixtures. Distinguishing between levels of N fertilization (Figure3) in 2015, the highest peak of the DMY line, shifts with increasing the proportion of legumes. Increased N fertilization reduced legume proportion to achieve the highest yield *(Nyfeler et al., 2009).* So, in treatment with 100 kgN ha^{-1} the highest yield of ryegrass were achieved when the proportion of legume were from 35-40%. Treatment with 50N gave highest yield with 40-50% legumes proportion and 0N

with 60-65% of legumes. In 2016 treatment N treatment gave highest yield when legume proportion were 40-50% and 0 treatment with 60% of legumes.

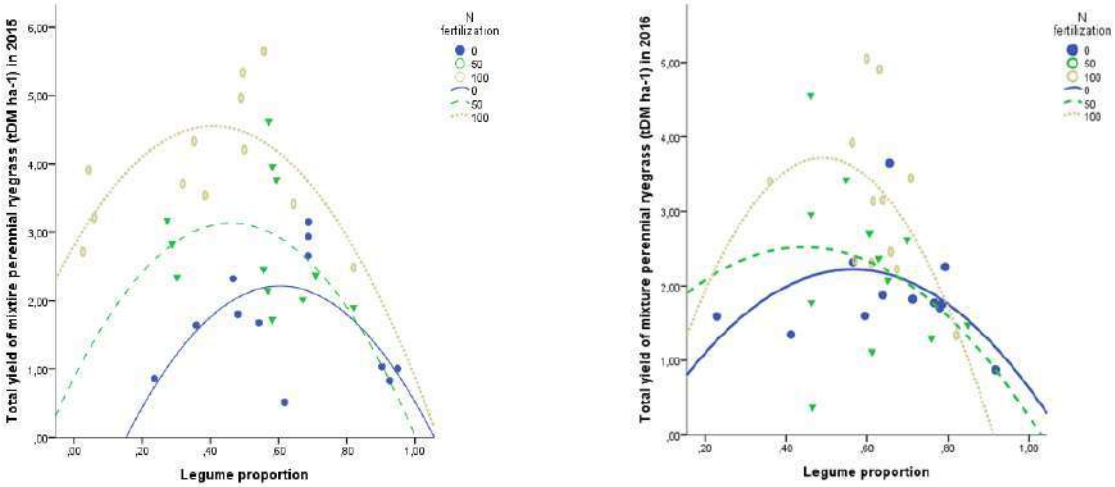

Figure 3. Dry matter yield of perennial ryegrass in mixtures fertilized with different levels on N vs. legume proportion

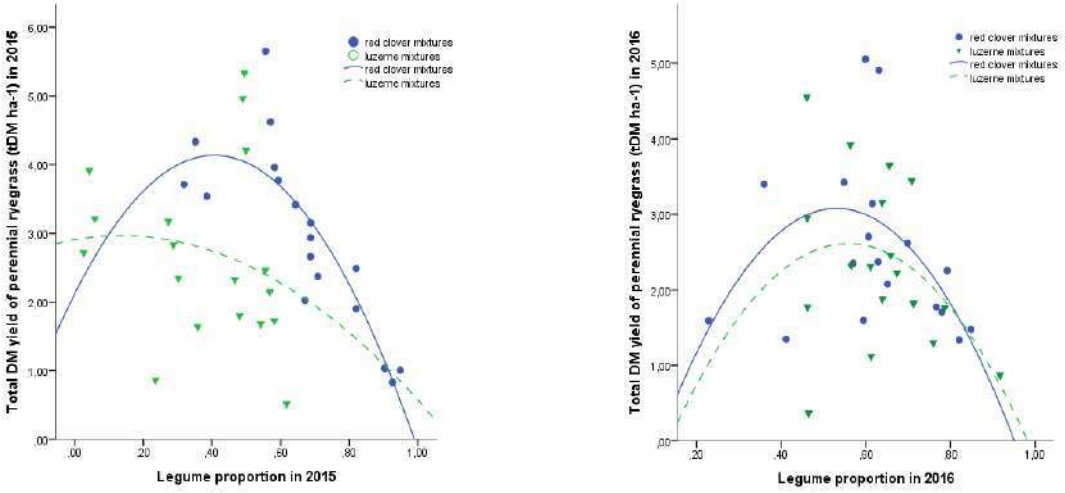

Figure 4. Dry matter yield of perennial ryegrass in mixtures with red clover and lucerne vs. legume proportion

In 2015 ryegrass in mixture with lucerne had highest DMY when the share of lucerne was lower and in 2016 the highest yield was achieved with lucerne proportion of 50-60%. Proportion of 45 and 55 % of red clover in both years effected the greatest DMY of ryegrass. Increase of legume share in mixture will

decrease ryegrass abundance, competitiveness and it will have implications in poor utilization of available resources.

Conclusion

In first production year ryegrass achieved the highest yield with lucerne at seeding rate 50:50 and with red clover at seeding rate 70:30. N fertilization increase DMY and RYg in both years thus increasing the competitive capability of perennial ryegrass. Added nitrogen also increase proportion of leaves toward steam, SLA, LAR and LAI. Even though the proportion of grass in the mixture were below the level of sowing rate, fertilization increased the proportion of grasses and particularly doses of 50N. Fertilization rate of 100N achieved the highest yield of ryegrass when the proportion of legume were from 35-40%, rate 50N with legume proportion 40-50% and 0N with 60-65% of legumes. Dry matter of ryegrass was higher in mixture with red clover than with lucerne particularly with red clover proportion of 45-55%. This grass can be grown successfully with red clover and lucerne with the addition of lower doses of nitrogen in order to maintain the legume component in the mixture.

Proizvodni rezultati višegodišnjeg ljulja u binarnim smešama sa lucerkom i crvenom detelinom u uslovima đubrenja azotom

Zorica Bijelić, Violeta Mandić, Vesna Krnjaja, Dragana Ružić-Muslić, Aleksandar Simić, Bogdan Cekić, Violeta Caro-Petrović

Rezime

Višegodišnji ljulj je veoma važna i rasprostranjena vrsta trava koja se koristi za ishranu stoke, naročito preživara. Kao vrsta koja se najčešće koristi na travnjacima, ona se uzgaja u mešavinama sa drugim vrstama trava i mahunarki. Cilj istraživanja bio je istražiti proizvodne rezultate višegodišnjeg ljulja u različitim proporcijama u smešama sa crvenom detelinom i lucerkom, kao i kako različiti nivoi N đubrenja utiču na njegovu konkurentnost. Ljulj je postigao najveći prinos sa lucerkom pri razmerisetvenoj of 50:50 i sa crvenom detelinom, 70:30. Relativni prinos trava (RYg) smeša se kretao od 1,01 do 1,55, što znači da je ljulj u smešama postigao 0.1-55% veći prinos od čistog useva. N đubrenje povećalo je DMY i

RYIg, odnos lista i stabljike, specifičnu lisnu površinu (SLA), odnos lisnih površina (LAR) i indeks lisnih površina (LAI) u obe godine, čime se povećava konkurentna sposobnost višegodišnjeg ljulja.

Ključne reči: višegodišnji ljulj, crvena detelina, lucerka, performanse, konkurentnost

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ACCUMULATION OF HEAVY METALS AND TRACE ELEMENTS IN *MEDICAGO SATIVA* L. GROWN ALONG THE E75 ROUTE SECTION BELGRADE-LESKOVAC

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Abstract. The contents of heavy metals and trace elements and their accumulation in *Medicago sativa* L., cultivated on Eutric cambisol along the E75 route section Belgrade-Leskovac, were examined in order to assess the health and safety of animal feed. The samples of soil and aerial parts of the plant material were collected from both sides of lanes at 10, 30, 50 and 400 m perpendicular to the direction of the highway. Soil and plant analyses of the metals content were done according to ICP methodology. The results showed that at the locality L 14, a distance of 30 and 50 meters away from the lanes, the content of total forms of Cr, Ni and Pb in soil was above the maximum permissible concentration. In the plant biomass it was determined the following: in a sample from the location L 14 at a distance of 50 meters from the lanes concentrations of Ni and Co were higher than normal values, and concentrations of Fe and Pb were above toxic levels or maximum tolerance levels for animal feed; determined Fe content in the sample of alfalfa at location L 11, 400 m away from the lanes, and Ni in the sample from the site D 12 at a distance of 50 m from the lanes, was above the normal values, while in the sample from D12 location, at a distance of 30 m from the lanes, the content of Pb was above the toxic levels or maximum tolerance levels for animal feed. The results suggest a caution in the use of alfalfa, grown near the highway route, for animal feed because of the potential entry of heavy metals into the food chain.

Key words: Eutric cambisol, highway, pollution, animal feed

Introduction

Risk assessment of threats to the environment caused by pollution of soil is particularly important in rural areas due to the fact that the metals potentially harmful to animal and human health exist in the soil and can be transferred into the

food chain in significant amounts *(Szinkovska et al., 2009)*. The rapid development of the industry, increased number of inhabitants and an intensification of road transport are one of the most significant causes of pollution of ecosystems in urban areas *(Jankievicz and Adamczyk, 2010)*. Heavy metals are found everywhere in the environment, whether as a result of natural or anthropogenic activities, to which a wildlife is exposed in different ways *(Wilson and Piatt, 2007)*. Urban roadside soils are the 'recipients' of large amounts of heavy metals from a variety of sources including vehicle emissions, coal burning waste and other activities *(Saeedi et al., 2009; Acosta et al., 2010)*. Heavy metals are found in fuels in the walls of fuel tanks, engines and other vehicle components, in catalytic converters, tires and brakes, as well as in the surface material on the roads *(Deska et al., 2011)* and as such represent the potential pollutants. The mobility and availability of heavy metals in the soil are generally low, especially when the soil is high in pH, clay and organic matter (*Petrotou et al., 2010)*. Heavy metal accumulation in plants depends upon plant species and the efficiency of different plants in absorbing metals, evaluated by either plant uptake or soil to plant transfer factors of the metals *(Rattan et al., 2005)*.

Some of the elements are necessary for growth and development of crops. Some of them have stimulating effect on plant growth, while a group of elements at high concentrations affects very toxically on the plants. An assessment of the environmental risk caused by soil contamination is especially important for agricultural as well as non-cultivated areas due the fact that metals potentially harmful to animal and human health persist in soils for a relatively long time and may transfer into the food chain in considerable amounts *(Szynkowska et al., 2009)*.

The highway presents the highest class of traffic routes. It is designed for fast motor traffic and consists of two physically separated lanes. In 2010, on Belgrade-Leskovac section of the highway E75, it was conducted the research of its impact on accumulation of heavy metals and trace elements in alfalfa cultivated on Eutric cambisol. The soil along the highway mostly belongs to the agricultural area. Thus, the study was aimed to determine whether there was a pollution of Eutric cambisol and cultivated crop (alfalfa) in the research area, and the level of pollution. A type of soil was determinated based on pedological map of Institute of Soil Science, Belgrade *(Mrvić et al., 2013)*.

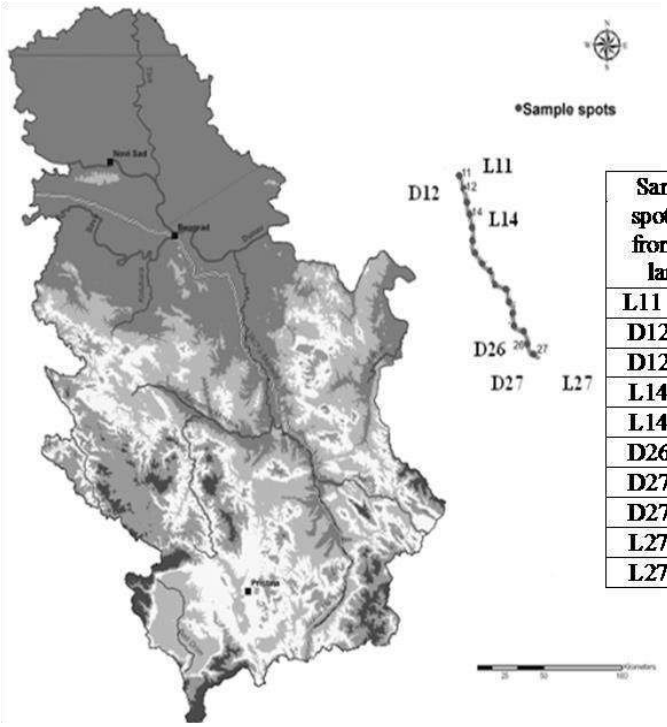
Material and Methods

Study area

The area of study included the highway route E75, section from Belgrade to Leskovac, where the soil and plant samples were taken from each side of the lane at a distance of 10, 30, 50 and 400 m perpendicular to the direction of the

highway (Figure 1). The sampling was carried out during August and September in 2010.

According to *WRB (2014)* classification studied soil was Eutric cambisol *(Mrvić et al., 2013)*. These are mainly medium-heavy soils, with a pronounced texture differentiation within the profile. Chemical properties vary depending on the intensity of use, degree of erosion, chemical properties of the parent material and level of development. The soil does not contain carbonates, belonging to the soils of high ecological and productive value.



Sample spots (m from the lane)	Coordinates	
	X	Y
L11 (400)	7501072	4931838
D12 (30)	7503516	4923959
D12 (50)	7503495	4923944
L14 (30)	7507509	4909703
L14 (50)	7507529	4909705
D26 (50)	7544419	4836338
D27 (30)	7548003	4830378
D27 (50)	7547990	4830558
L27 (30)	7548175	4830567
L27 (30)	7548193	4830583

Figure 1. Soil and plant sampling spots in the section of the study with corresponding distances and coordinates

In the framework of the Rule book of permissible concentrations of dangerous and hazardous materials in soil and in water for irrigation and methods for analysis *(Official Gazette of RS, 1994)* the maximum permissible levels of dangerous and hazardous matters were used in the interpretation of the results of the analyzed soil samples.

Soil sampling, preparation and analysis

Ten samples of soil were taken in disturbed state in three repetitions from the depth of 0-30 cm. Composite soil samples were carried to the laboratory, dried and passed through a 2-mm sieve *(SRPS ISO 11464:2004, 2004)*. Soil pH in H_2O and 1M KCl was analyzed potentiometrically with glass electrode *(SRPS ISO 10390:2007, 2007)*. The content of $CaCO_3$ was determined volumetrically, according to the standard *SRPS ISO 10693:2005 (2005)*. The contents of total N and C were determined using elemental CNS analyzer, Vario model EL III *(Nelson and Sommers, 1996)*, whereby on the basis of organic C content, the content of SOM (soil organic matter) was calculated using the formula: SOM content (%) = organic C content (%) * factor 1.724 *(Džamić et al., 1996)*. Available P_2O_5 and K_2O were analyzed by Al-method according to Egner-Riehm *(Riehm, 1958)*, where 0.1M lactate (pH 3.7), was used for extraction. After extraction, K_2O was determined by flame emission photometry and P_2O_5 by spectrophotometry after color development with ammonium molybdate and stannous chloride. Ca and Mg were extracted by ammonium acetate and determined with an atomic absorption analyzer SensAA Dual (GBC Scientific Equipment Pty Ltd, Victoria, Australia) according to *Wright and Stuczynski (1996)*. The total contents of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and As in soil samples were determined by inductively coupled plasma-atomic emission spectrometry - THERMO iCAP 6300 Duo (radial/axial view versions) ICP-OES, after the digestion of the samples with aqua regia *(ISO 11466:1995, 1995; ISO 22036:2008, 2008)*. Reference soil NCS ZC 73005, Soil Certificate of Certified Reference Materials approved by China National Analysis Center Beijing China, and reagent blanks were used as the quality assurance and quality control (QA/QC) samples during the analysis.

Collection, preparation and analyses of the plant material

Medicago sativa L. (alfalfa) is a perennial legume which is considered to be the leading and most important forage crop for the production of high quality feed, used in fresh and conserved state as hay, haylage, silage, meal, pellets and paste *(Vučković, 2004; Jakšić et al., 2013)*. The aerial parts of the alfalfa plant material, grown at selected locations, have been taken in the period August-September in 2010, in flowering stage during the third cutting. Average sample consisted of 15 to 20 individual plant samples, whereby the swath was carried out manually by cutting the plant at a height of 3-5 cm. Samples of the plant material were air-dried and milled using grinding mill. Then, they were dried at 105^{o}C for a period of 2 hours, using gravimetric method for determination of dry matter contents of plant tissues *(Miller, 1998)*. The contents of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and As in aerial parts were determined in triplicates with THERMO iCAP 6300 Duo (radial/axial view versions) ICP-OES after the digestion of the

samples with concentrated nitric acid and redox reaction with hydrogen peroxide for total forms extraction *(Soltanpour et al., 1996)*. Calibration standards were in the range of 0-10 ppm, except for iron (0-25 ppm).

Data analysis

The obtained data on trace elements and heavy metals concentration in the soil studied represent the arithmetic means of three replicates of each sampling, their ranges and standard deviations values. The data on trace alements and heavy metal concentrations in the alfalfa are presented by figures as the bar charts with standard deviation values.

Results and Discussion

Chemical characteristics of the studied soil

Chemical properties of the studied soil are shown in Table 1.The reaction of the analyzed soil samples ranged from acidic to slightly acidic. In relation to the content of available phosphorus, the values ranged from very low to very high, while the supply with available potassium goes from high to very high values. The humus content in the analyzed samples ranged from medium to high level of provision. Regarding the total content of heavy metals, in two tested soil samples from the L14 position at 30 m, and as well from the position L14 at 50 m distance from the highway route, the concentrations of Cr and Ni were higher than the maximum permissible concentration (MPC) in soil *(Official Gazette of RS, 1994)*.

Ni and Cr are mainly found in the basic and ultrabasic rocks, which is the main source of geochemical origin of these metals in the soil. Anthropogenically, they may enter the soil mostly through atmospheric deposition of the coal, oil and diesel combustion, which presumably could be the source of these metals high level in soil studied. For agricultural soils is typical the Ni pollution from the use of organic sludge, and to a lesser extent, it is contained in mineral fertilizers. Liming, as an ameliorative measure, could also be the source of Ni in the soil *(Kabata-Pendias and Mukherjee, 2007)*.

In addition, above the MPC was also the content of Pb in one soil sample from the position L14 at 50 m from the highway route. Unlike Ni and Cr, the soil contamination with Pb occurs in nature only anthropogenically. The most important anthropogenic sources of Pb are mines, smelters and the exhaust gases from vehicles, and in agriculture - industrial sludges and pesticides *(Kabata-Pendias, 2011)*.

Table 1. Chemical properties of the studied Eutric Cambisol (means ± standard deviation and intervals)

Property	Value		Property	Value
pH in 1M KCl	5.2±0.3 (4.7-5.6)		Total content of As (mg kg $^{-1}$)	6.6±2.6 (3.9-11.6)
Total content of $CaCO_3$ (%)	below the detection limit		Total content of Cr (mg kg $^{-1}$)	74.9±40.0 (37.4-141.6)
Available P_2O_5 (mg 100g $^{-1}$)	8.2±10.5 (0.6-35.2)		Total content of Ni (mg kg $^{-1}$)	49.7±25.3 (33.0-97.7)
Available K_2O (mg 100g^{-1})	27.5±3.2 (24.2-33.7)		Total content of Pb (mg kg $^{-1}$)	44.5±34.6 (25.0-122.8)
Total content of N (%)	0.2±0.1 (0.1-0.4)		Total content of Zn (mg kg $^{-1}$)	67.3±30.7 (40.9-130.1)
Total content of C (%)	2.1±0.7 (1.1-3.5)		Total content of Cd (mg kg $^{-1}$)	0.8±0.5 (0.3-1.5)
SOM (%)	3.6±1.8 (2.0-6.0)		Total content of Cu (mg kg $^{-1}$)	23.6±4.9 (17.5-31.6)

Trace elements and heavy metals content in alfalfa biomass

Figure 2 shows the mean and standard deviation values of the concentration of trace elements and heavy metals in the analyzed samples of the plant material. In addition, Table 2 displays the reference values for trace elements and heavy metals content in plants as compared to normal and toxic concentration.

Iron (Fe) can be accumulated in plants without any harmful effects *(Marić et al., 2013; Simić et al., 2015)*, so it is not uncommon that the contents of this element could be higher than the MPC. It is an essential element required in many physiological and biochemical processes. The concentration of Fe, assimilated by plant, besides the type and stage of its development, depends on the soil properties. High pH value and high concentrations of phosphate and calcium ions reduce its assimilation. Normal iron content in plant material, according to *Kabata-Pendias (2011)*, ranges from 18-1000 mg kg^{-1} of dry matter. In plants an excess of this element is rare, and its deficiency occurs when its content in the dry matter of leaves is less than 50 mg kg^{-1}. From the total of ten samples of alfalfa biomass, only in one sample from the location L14 at a distance of 30 m from highway lane it was identified iron content above the MPC for animal feed, which is 1250 mg kg^{-1} of dry matter. The mentioned indicates that, in addition to the possible impact of the highway traffic on the iron content, the reason of undesirable Fe content occurrence in plant material may be an increased content of this element that entered the soil otherwise than atmospheric (use of pesticides, fertilizers, etc.).

Manganese (Mn) is actively assimilated and quickly transported through the plants, in which process a passive absorption occupies a special place. Due to the rapid transfer through the plant, it mostly accumulates in the organs of young plants and less in the root. In plants organs Mn appears in excess when there is a

high concentration of this element in the soil, together with low pH values and high redox potential *(Misra and Mani, 1991; Kastori et al., 1997)*. In all tested samples of alfalfa a toxic content of this element (>400 mg kg^{-1}) was not registered, which indicates that the proximity of the road did not cause an increased concentration of this element in the plant material.

Copper (Cu) belongs to the category of micronutrients. The rate at which the plant assimilates the copper is largely dependent on the type of plant and the origin of the copper present. The species sensitive to the toxicity of copper are grains, legumes and spinach. For normal development, copper is required in the plants in small quantities (5-20 mg kg^{-1}), and less than 4 mg kg^{-1} is considered as a deficiency, while more than 20 mg kg^{-1} can cause the occurrence of toxicity *(Kloke et al. 1984*; *Kastori et al., 1997)*. In the tested samples of alfalfa a toxic value of copper was not registered.

Zinc (Zn) is an essential nutrient for plant growth and is involved in important metabolic processes. Soluble forms of zinc are easily available to the plants and assimilation of this element is in a linear relation with the content of this element in the nutrient solution or in soil. The composition of the nutrient solution, especially of calcium content, is of great importance to the assimilation of zinc *(Kastori et al., 1997)*. Registered zinc content in the analyzed samples of plant material was not above the toxic levels (>200 mg kg^{-1}).

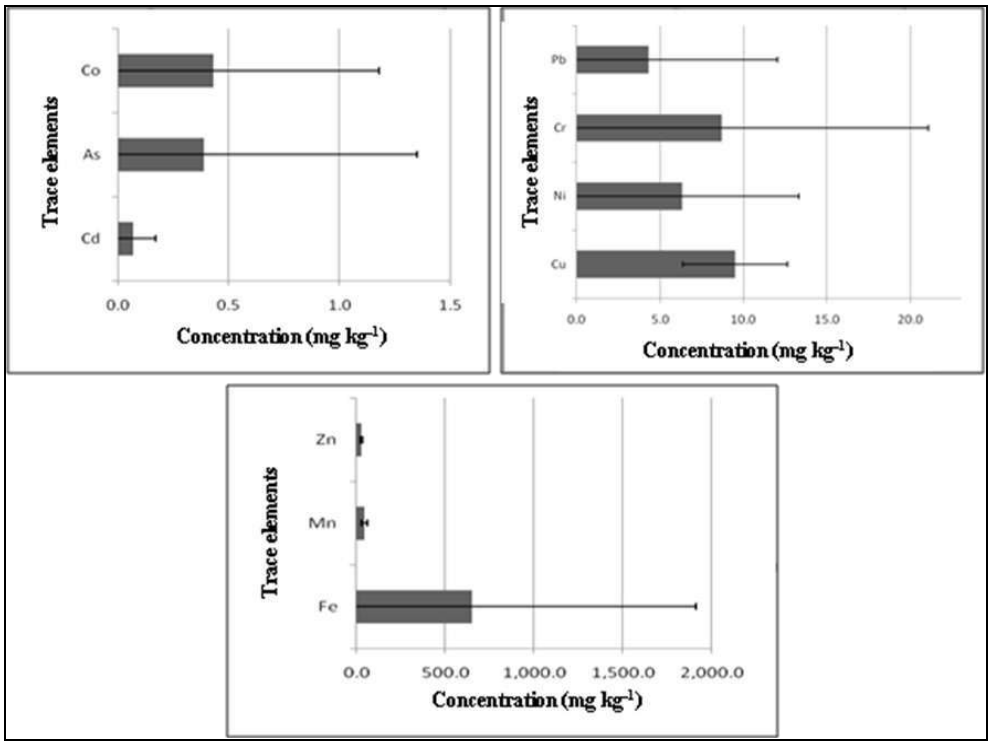


Figure 2. Average values of analyzed trace elements in the aboveground biomass of alfalfa (mg kg^{-1})

Table 2. Reference values for trace elements content in plants according to literature sources

Element	Normal concentrations	Toxic concentrations	Maximum tolerant level for fodder
	(mg kg^{-1})		
Cu	3-15[a]	20[b]	12-50[g]
Ni	0.1-5[a]	30[b]	50[g]
Pb	1-5[a]	20 [b]	40[g]
Cr	<0.1-1[a]	2[b]	-
Cd	<0.1-1[a]	10[b]	1[g]
Mn	15-100[e]	400[b]	-
Zn	15-150[a]	200[b]	2000[g]
Co	0.05-0.5[e]	30-40[d]	-
Fe	50-250[f]	(>500)[f]	1250[g]
As	10-60[c*]	<2[c]	4[g]

[*]μg kg^{-1}; reference values: [a]*Kloke et al. (1984)*; [b]*Kastori et al. (1997)*; [c]*Kabata-Pendias and Mukherjee (2007)*; [d]*Kabata-Pendias (2011)*; [e]*Misra and Mani (1991)*, [f]*Schulze et al. (2005)*; [g]*NRC* (2005), [g]*Adams (1975)*.

Assimilation of nickel (Ni) depends on soil properties and on properties of the plant itself. The most important factor is the pH value of the soil. Its origin is

also very important for assimilation of this element, because the studies indicate the occurrence that anthropogenically deposited nickel is more easily assimilated *(Kloke et al., 1984*; *Kastori et al., 1997).* Since nickel is readily mobile in plants, usually all parts of the plant have high concentrations of this element. In the analyzed samples of plant material the content of nickel was above the normal levels (>5 mg kg^{-1}) in two samples from the positions D12 and L14 at a distance of 50 m from the lanes. Partly, the zones of occurrence of this element within the specified range overlap with the zones where the total content of nickel in the soil was above the MPC (position L14, 50 m from the lane).

Chromium (Cr) content in plants is very different and depends largely on the geological substrate. It is almost always higher in root than in leaves or stems, while the lowest concentrations were registered in the fruits. Chromium content in the tested plant samples does not exceed toxic levels of this element for animal feed (50-3000 mg kg^{-1}).

Cadmium (Cd) is one of the most toxic and dangerous element, which has damaging effects on biological activity of the soil, plant metabolism and the health of humans and animals. It is easily assimilated through the root system and accumulated in the aboveground parts of plants. The pH value of the soil solution is considered to be a major factor in assimilation of cadmium. In addition to this, the contents of clay and carbonate in the soil are also very important. Origin of cadmium is also an important factor that affects the solubility and availability of this elements *(Adams, 1975; Kloke et al., 1984*; *Kastori et al., 1997; NRC, 2005).* Cadmium content in the analyzed samples of plant material is in the range of normal values (up to 10 mg kg^{-1}), which is a desirable outcome.

Available previous studies suggest that arsenic (As) from the soil into the plant gets through the passive way. Most studies indicate that an increased content of arsenic in the soil leads to the accumulation of arsenic in roots and old leaves. Legumes are sensitive to the effects of arsenic. The most common result of the high content of this element in the soil is a reduction in crops yield *(Kabata-Pendias and Mukherjee, 2007; NRC, 2005).* The content of arsenic in studied plant material is in critical concentrations only in one sample from the positions L14 at a distance of 50 m from the lanes (3.10 mg kg^{-1}), while in the other tested samples it is present in normal concentrations. The value of arsenic content in mentioned sample is below the maximum tolerable level for animal nutrition.

The studies on cobalt (Co) content in plants have become very important when it was observed that its deficiency in the soil and therefore in the plant biomass causes the diseases in sheep, goats, cattle and other livestock. In the soil this element is commonly found as a companion of iron, nickel and other heavy metals *(Misra and Mani, 1991; Kabata-Pendias, 2011).* In the tested samples of the plant material only in one sample from the position L14 at a distance of 30 m from the lanes it was determined a value of Co higher than the normal value (2.535

mg kg^{-1}). In the other analyzed samples of alfalfa the value of this element ranged from 0.08 to 0.285 mg kg^{-1}.

Lead (Pb) is a non-essential element for plants, although in lower concentrations has a stimulative effect. Lead could be accumulated through the food chain and become toxic to humans or animals. It is the least moveable element within trace elements in soil *(Kabata-Pendias, 2011)*; its assimilation and transfer to the aerial plant parts are low, except in acid soils. Plants can accumulate lead either from soil or from the air. Most of the lead from the soil is not available to plants. The inorganic forms of lead become available to plants only in acid soils *(Wiklander and Vahtras, 1977)*. Lead originating from the air is the main source of pollution from this element. According to some studies, about 95% of the total content of lead in the plant can be derived from the air *(Kloke et al., 1984*; *Kastori et al., 1997)*. It is evident that, on the location where the plant material with increased concentrations of this element was sampled, it was also determined the total content of lead in the soil above the MPC (position L14, 50 m from the lanes). Increased content of this element, which is in the range of critical concentrations for animal nutrition (10-30 mg kg^{-1}), was also recorded in the sample from the position D12 at a distance of 30 m from the lanes. This could result in contamination of animals through ingesting the polluted forage. Lead has a toxic effect and oncological action to animals, leading to hepatic, cutaneous and pulmonary cancer and changed haematological parameters *(Kochare and Tamir, 2015)*.

Conclusion

In addition to anthropogenic pollution, which is reflected in the excessive use of plant protection preparations and fertilizers, as well as the impact of air pollution from motor vehicles originating from the certain sections of study, the presence of geochemical pollution of soil cover is evident. The results showed that at the locality L 14, a distance of 30 and 50 meters away from the lanes, the content of total forms of Cr, Ni and Pb in soil was above the maximum permissible concentration. In the plant biomass it was determined the following: in a sample from the location L 14 at a distance of 50 meters from the lanes concentrations of Ni and Co were higher than normal values, and concentrations of Fe and Pb were above maximum tolerant level for animal feed; determined Fe content in the sample of alfalfa at location L 11 400 m away from the lanes, and Ni in the sample from the site D 12 at a distance of 50 m from the lanes, was above the normal values, while in the sample from D12 location, at a distance of 30 m from the lanes, the content of Pb was above the toxic levels or maximum tolerance levels for animal feed. The results suggest a caution in the use of alfalfa, grown near the

highway route, for animal feed because of the potential entry of heavy metals into the food chain.

Akumulacija teških metala i mikroelemenata u lucerki (*Medicago sativa* L.) gajenoj uz deonicu autoputa E75 Beograd-Leskovac

Radmila Pivić, Zoran Dinić, Aleksandar Stanojković, Jelena Maksimović, Dragana Jošić, Aleksandra Stanojković-Sebić

Rezime

U deset uzoraka zemljišnog i biljnog materijala duž trase autoputa E 75 kroz Republiku Srbiju, na deonici od Beograda do Leskovca, ispitan je sadržaj teških metala i mikroelemenata i njihova akumulacija u *Medicago sativa* L. gajenoj na eutričnom kambisolu, radi ocene zdravstvene ispravnosti stočne hrane. Uzorci zemljišta i nadzemnog dela biljnog materijala uzorkovani su sa obe strane kolovoznih traka i to na 10, 30, 50 i 400 m upravno na pravac autoputa. Analiza zemljišnih uzoraka pokazala je da je na lokalitetu L 14, na udaljenosti 30 i 50 metara od kolovoznih traka sadržaj ukupnih formi Cr, Ni i Pb bio iznad maksimalno dozvoljenih koncentracija. U biljnoj masi u uzorku sa lokacije L14 na udaljenosti 50 metara od kolovoznih traka koncentracije Ni i Co su bile više od normalnih vrednosti, a koncentracije Fe i Pb bile su iznad toksičnih vrednosti odnosno maksimalno tolerantnog nivoa za ishranu životinja. Utvrđeni sadržaj Fe u uzorku lucerke na lokaciji L11 udaljenoj 400 m od kolovoznih traka, kao i Ni u uzorku sa lokacije D12 na udaljenosti 50 m od kolovoznih traka, bio je iznad normalnih vrednosti, dok je u uzorku D12 na udaljenosti 30 m od kolovoznih traka sadržaj Pb, bio iznad toksičnih vrednosti, odnosno maksimalno tolerantnih nivoa za ishranu životinja. Dobijeni rezultati upućuju na oprez pri korišćenju lucerke gajene pored trase autoputa za ishranu životinja zbog mogućeg ulaska teških metala u lanac ishrane.

Ključne reči: Eutrični kambisol, autoput, zagađenje, stočna hrana

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Corresponding author: Milan M.Petrović, **e-mail address**
Review paper

Example 2

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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Original scientific paper

Original scientific paper should contain following paragraphs with single spacing (title of paragraphs should be in Times New Roman 14 **bold**, except for **Abstract** and **Key words** where font size is 11 **bold**):

Abstract: up to 250 words, Times New Roman, font size 11, justify. Abstract should contain a brief overview of the methods and the most important results of the work without giving reference. Abstract submitted in English language.

Key words: not more than 6. The selection carried out by relying on widely accepted international source such as a list of keywords Web of Science.

Introduction – present the review of previous research and objective of the paper.

Materials and Methods – state methods applied in the paper; experimental research design. Use SI system of measurement units.

Results and Discussion – present investigation results separately from discussion or together in one paragraph. Presentation of the results should be precise and without repetitions, and include the evaluation of significant differences and other parameters.

Text and titles of tables, figures and graphs, Times New Roman, font size 9, **bold**, in the following form:

Table 1. Least square means for the reproductive traits of cows

Tables and figures should be numbered and with adequate title and legend, width and height not exceeding 12 cm and 17 cm, respectively. Tables should be prepared according to instruction for forming of tables in Office Word. Each column in table must have heading and, when necessary, abbreviations should be explained in the legend/footnote.

Conclusion – containing the most important issues of the paper

After Conclusion the title of the paper in Serbian in Times New Roman 14 **bold**, is stated, followed by authors in Times New Roman 11 *italic*, example:

Potencijali srpske stočarske proizvodnje – izgledi i budućnost

Milan M. Petrović, Stevica Aleksić, Milan P.Petrović, Milica Petrović, Vlada Pantelić, Željko Novaković, Dragana Ružić-Muslić

Summary – in Serbian language, 250 max. words (non-Serbian authors should provide Summary in English language that will be translated to Serbian by Editor's office)

Key words: not more than 6 (in Serbian language)

Acknowledgment – for example:

Research was financed by the Ministry of Science and Technological Development, Republic of Serbia, project TR 6885.

References – should be in alphabetical order. Names of the authors must be given in capital letters followed by the year of publication in brackets, titles in the language of the original. Use only the full name of the journal.

In scientific journals:

PETROVIĆ M. M., SRETENOVIĆ LJ., BOGDANOVIĆ V., PERIŠIĆ P., ALEKSIĆ S., PANTELIĆ V., PETROVIĆ D. M., NOVAKOVIĆ Ž. (2009): Quantitative analysis of genetic improvement of milk production phenotypes in Simmental cows. Biotechnology in Animal Husbandry, 25,1-2, 45-51.

ŠKRBIĆ Z., PAVLOVSKI Z., LUKIĆ M. (2007): Uticaj dužine tova u različitim sistemima gajenja na klanične osobine brojlerskih pilića genotipa Redbro. Biotechnology in Animal Husbandry 23, 3-4, 67-74.

WEBB E., O'NEILL H. (2008): The animal fat paradox and meat quality. Meat Science, 80, 28-36.

PhD Thesis:

RUŽIĆ-MUSLIĆ D. (2006): Uticaj različitih izvora proteina u obroku na proizvodne rezultate jagnjadi u tovu. Doktorska disertacija. Univerzitet u Beogradu, Poljoprivredni fakultet.

CAETANO A.R. (1999): Comparative mapping of the horse (*Equss caballus*) genome by synteny assignment of type-I genes with a horse-mouse somatic cell hybrid panel. Ph.D. Dissertation, University of California, Davis.

In Scientific Books:

PETROVIĆ P.M (2000): Genetika i oplemenjivanje ovaca. Naučna knjiga, Beograd, pp365.

FITZGERALD M. (1994): Neurobiology of Fetal and Neonatal Pain. In: Textbook of Pain. 3rd edition. Eds Wall P. and Melzack R. Churchchill Livingstone, London, UK, 153-163.

At Scientific Meetings:

ŠKRBIĆ Z., LUKIĆ M., BOGOSAVLJEVIĆ-BOŠKOVIĆ S., RAKONJAC S., PETRIČEVIĆ V., DOSKOVIĆ V., STANOJKOVIĆ A. (2015): Importance of farm management in reducing broilers skin lesions. Proceedings of the 4th International Congress "New Perspectives and Challenges of Sustainable Livestock Production", October 7 – 9, Belgrade, 145-158.

Citations in the text are presented in italic form, examples: …results of *Petrović (2009)*; *Petrović et al. (2009)*; *Webb and O'Neill (2008)….*; *(Škrbić et al., 2015); (Ružić-Muslić, 2006); (Webb and O'Neill, 2008)*

Editor's office