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POULTRY WELFARE IN TERMS OF POULTRY RED MITE (*DERMANYSSUS GALLINAE*) IMPACT AND CONTROL

Aleksandar Pavličević[1], Ivan Pavlović[2], Radomir Ratajac[3], Danica Popović[4], Branislav Davidović[5], Dejan Krnjajić[6]

[1]AVES MIT" DOO, Petefi Šandora 3c, 24210 Bajmok, Srbija
[2] Scientific veterinary institute Serbia, Vojvode Toze 14, 11000 Belgrade, Serbia
[3] Scientific veterinary institute „Novi Sad“, Rumenički put 20, 21000 Novi Sad, Serbia
[4]“Pullos” d.o.o., Dušana Vukasovića 45/15, 11070 Novi Beograd, Srbija
[5]“Nutrivet” d.o.o., Vojvođanska 22, 11271 Surčin, Srbija
[6] Faculty of Veterinary Maedicine, Bulevar Oslobođenja 18, 11000 Belgrade, Serbia
Corresponding author: dripavlovic58@gmail.com
Review paper

Abstract: Technological solutions and environmental conditions have a significant impact on infestation intensity and the problems around *D. gallinae* control. Changes in keeping laying hens in EU, in terms of *D. gallinae* influence, have not led to the welfare of the layers. On the contrary, they have contributed to the spreading of disease, have worsened conditions for control and accentuated harmful consequences. Apart from the poultry, these changes have also had a negative impact on the welfare of humans, through a toxicological and zootonic risk, and economic damages. Conventional cages so far provide the most appropriate environment for *D. gallinae* control. Opportunities for improving, even solving the problem of *D. gallinae* control in egg production do exist, however they require a changing the entire approach hitherto.

Key words: Poultry welfare, *Dermanyssus gallinae* control

Introduction

EU has laid down the poultry welfare as a necessary condition in consumer egg production. By adopting the Directive 1999/74/EC a ban on using conventional cages has been put in place, which came into effect on 01.01.2012. Since then, keeping laying hens in EU is allowed only in alternative systems: enriched cages, aviaries, barns, free range and organic. The 2012-2015 EU Strategy is based on scientific indicators, transparency, reference centers and competencies of those handling the poultry (*Van Emous, 2017*).

Poultry red mite (*Dermanyssus gallinae,* De Geer) is a cosmopolitan, hematophagous ectoparasite. *Dermanyssosis* is considered as one of the most

important health and economic problem in egg production. *D. gallinae* is a temporary parasite, which stays on the poultry only during feeding time, and otherwise remains in appropriate hiding places in the housing system. This is why *D. gallinae* is a problem of both the flock and the environment. The new changes in rearing systems have also had an impact on *Dermanyssosis* manifestation.

The aim of our paper is to consider poultry welfare in terms of *D. gallinae* impact and control in different technological conditions.

Red mite control through legislative

In 1979 the UK Farm Animal Welfare Council defined animal welfare in terms of 5 freedoms: 1. from hunger and thirst; 2. from discomfort; 3. from pain, injury and disease; 4. to express normal behaviour; 5. from fear and distress. The European consortium defined welfare in terms of 4 categories and 12 subcategories. These are: 1. good feeding (absence of prolonged hunger and thirst); 2. good housing (comfort around resting, thermal comfort, ease of movement); 3. good health (absence of injuries, absence of disease, absence of pain induced by management procedures); 4. appropriate behaviour (expression of social behaviors, expression of other behaviors', good human-animal relationship, positive emotional state). There is a great number of varying interpretations of farm animal welfare, incompleteness, but also opposing claims. According to the aforementioned definitions, health in reference to the specific case of *Dermanyssosis* control, is an important factor in achieving animal welfare.

According to EU's commitment that each new legislation is to be based on the latest scientific knowledge and advice, the European Food Safety Authority (EFSA) was requested to provide an opinion for the purpose of assessing health and welfare effects on laying hens. EFSA Report (2004) has confirmed the scientific and economic foundation and justification for changing cage systems, claiming that there is crucial evidence which show that the ban on conventional battery cages for laying hens can make considerable improvements for the health and welfare of these birds. In addition to this, a research program called 'LayWel' was financed by the EU, which confirmed the accuracy of EFSA research results (IP/08/19). Economic foundation for technological changes was motivated by the fact that egg producers in the EU, based on the costs of production, can hardly be considered competitive. Since market research (CEAS) established a potential in increasing the price of eggs, the introduction of higher poultry welfare standards is also a way of creating economic prosperity of poultry keeping in the EU (preserving its competitiveness).

Already in 2000, the great problems around the complex situation of rearing systems conditions and the available options for *D. gallinae* control were evident (*Nordenfors, 2000*). *Sparagano et al. (2009)* have pointed out a high global prevalence of *D. gallinae*, but also the unfavorable expectations regarding the

manifestation of *Dermanyssosis* in alternative rearing systems of layers in the EU. Eight years later, *Flochlay et al. (2017)* have found this was precisely the case. A harmful effect of *D. gallinae* in Europe has increased in the last decades, with a tendency of the situation to get worse. The authors consider Directive 1999/74/EC and changes in poultry housing as the first factors of this negative development of events. Changes which were meant to improve poultry welfare, have created a more complex environment, which provides more favourable conditions for *D. gallinae.* Technological changes have also had a negative impact on farm staff, highlighting the zoonotic aspect of *Dermanyssosis* (COREMI, 2016). The report from 2016 suggested an infestation level of farms in Europe of 83%. The Netherlands, Germany and Belgium had a prevalence of 94%. *Nicole et al. (2017)* propose that the prevalence is lower in cage systems and that the complexity of housing in alternative solutions is unfavorable, as well as that *D. gallinae* control has not been resolved, and requires urgency in finding a solution. Along with prevalence and difficulty in *D. gallinae* control, economic losses have also increased. In the period between 2005 and 2017, economic losses have increased to 40% per hen, and at EU level have been estimated at 231 million annually (V*an Emous, 2005; 2017*).

D. gallinae control has additionally been placed in public focus due to the toxicological affair of consumer egg production in 2017 (*Pavličević et al.,2017c)*. The situation is further made difficult by the lack of appropriate solution and generally a small number of efficient products and methods of *D. gallinae* control available. More recent reports find high levels of *D. gallinae* resistance (*Abbas et al., 2014; Pavlicevic et al., 2016a*). The foundation of all these problems (high prevalence; health effect on poultry; spreading of communicable diseases; toxicological risk for humans, poultry and environment; accentuated zoonotic impact; losses in productivity and high material damages; intense development of chemoresistance) is an incorrect approach to *D. gallinae* control across several decades, which is now further challenged by these new changes in technological conditions.

Challenges in red mite control in different rearing systems

The conditions of the rearing environment have a key influence on the inaccessibility and distribution of *D. gallinae,* but at the same time, also the distribution, accessibility and efficiency of products and methods used in its control. This means that environment and technology in egg production, have a significant role in determining the effects of *D. gallinae* control (*Pavlicevic et al., 2016b*). In earlier periods, intensive egg production was based exclusively on conventional cage systems. Quality and conditionality of certain types depended on the model that is cage manufacturer (*Pavlicevic et al., 2016c*). In relation to *D. gallinae* control, the development of conventional cages can in general be traced to

the stages described below.

Cages with static manure collection ("California type", cages with scrapers and plates) were the most challenging hygienic conditions for *D. gallinae* control in caging systems, which were, apart from greater presence of static impurities, often followed by unfavorable constructions, for example, cylindrical constructions for adjoining sides.

The next were cages with mobile litter belts and a more complex construction. In this phase, hygienic conditions in the cages themselves were ensured, but were regularly followed by unfavorable construction. However, possibilities for *D. gallinae* control became much more favorable.

Cage manufacturers have gradually simplified cage construction for rearing layers and have thus improved the overall conditions. This way, these cages have ensured the best conditions for efficient *D. gallinae* control hitherto. At one point, the situation became more complicated by installing manure drying tunnels. These have to an extent challenged the conditions for *D. gallinae* control. For the rest of the cage and equipment there was a general tendency of simplifying constructions, and the general conditions were still good.

Isolated attempts to create cages for warming layers in intensive poultry farming which would control *D. gallinae.* The first documented idea comes from the USA, 1928 (*Van Emous, 2006*). We consider that the approach to solving is not rational in terms of the general problem of *D. gallinae* control in poultry farming. The efficiency of the thus far implemented models is questionable. Instead of the optimisation of conventional cages, the technological development of cages for layers was directed at alternative rearing methods, with the aim of poultry welfare.

At the moment, most intensive poultry farming in the EU is done in enriched cages. The construction of enriched cages has greatly diminished the efficacy of existing measures and products for *D. gallinae* control. The most harmful in this respect, has been the existence of appropriate hiding places in the immediate proximity of the hens, which are inaccessible or hardly accessible to external application. This situation requires more work in terms of application. This is due to the furnishings in the cages, constructions and perches, depending on the model of the cage. These environmental conditions (inconveniences) can have an immense capacity for a big *D. gallinae* infestation. Apart from providing hiding places, these areas cannot be protected though the residual effect of the product on external surfaces, due to immediate proximity and contact with the hens. Another negative effect is the blocking of surfaces for distribution, with external application (as the dominant application method) of the product. The third consideration is merely the increase of surface unit per hen, which has considerably raised the expenditure of materials and cost of control. Then, there was a tendency to minimize the number of hiding places by redesigning existing additions: new type of slatted floor, new type of laying nest floor (*Van Emous, 2006*). Creating unfavourable conditions has been somewhat masked by physical extermination or

disruptions created by the change in caging systems.

In aviaries, barn, free range and organic systems litter mats have been introduced, which provide a protective environment for *D. gallinae*; the construction of perches, nests, and equipment disrupts or prevents machine work; application by hand means bigger expenditures per hen of total capacity and greater risk from application mistakes; the distribution of infestation of the same intensity is greater; hiding places depend on the model of the cage and there are models which are difficult to control technically. We have thus far not been able to assess the importance of free range systems for *D. gallinae* control.

For comparison, the main characteristics of conventional cages, relate do *D. gallinae* control are the following:

- ideal hygienic conditions
- simple and efficient detection of even a small number of *D. gallinae* (included in the regular working process, without additional costs) with floor dust (*Pavlicević et al., 2007; 2017a,b*);
- greatest applicability of products for external use;
- greatest efficacy;
- greatest rationality of costs;
- the possibility of additional optimization of the environment, which would greatly facilitate control measures and increase efficacy. These innovations are applicable even in alternative systems, but will be much more apparent in conventional systems.
- available innovative technology (P 547/17) which eliminates safety risks and offers efficient and economically advanced *D. gallinae* control, and if appropriate conditions are met, also the solution to the problem. It has a physical mode of action, by creating a long-lasting, inert layer with a prolonged effect on non-absorbent surfaces. Although it is possible to apply this technology in alternative systems, the maximal effect is provided in conventional systems.

Conventional cages with a simple construction and good hygienic conditions have so far ensured the best conditions for *D. gallinae* control in intensive poultry farming. Apart from that, there is also the possibility to further optimise conditions in cage systems for *D. gallinae* control. Alternative poultry rearing methods provide possibilities for improvement which could contribute to *D. gallinae* control, but the challenges of these conditions by far outweigh this.

However, the problem of environmental conditions can be approached by adapting the type of application. The concept of the new veterinary medicine, based on the insecticide fluralaner (isoxazolinic) is application though drinking water (*per os*), which means it can be effective in these conditions (*Heckeroth et al., 2015; Thomas and Flochlay-Sigognault, 2017*). However, it is to be expected that the circumstances (infestation distribution and intensity) will decrease the efficacy of the medicine and contribute to development of quicker chemoresitance in alternative systems. Subsequent clinical experience will determine the possible

contribution of this veterinary medicine, the application of which is based on a curative approach.

Preventive veterinary medicine holds multiple advantages over the curative approach: safety, efficiency, rationality and longevity. Preventive veterinary medicine is the foundation of the program control of *D. gallinae*, which is focused on implementing measures for control before the new flock is housed. An example where this preventive mode of action was missing was the change of cage systems in the EU.

In regular technological conditions, the most complex and most problematic part of the environment, in terms of *D. gallinae* control are cages and equipment. In a situation when cages and equipment are disassembled and removed, the environment is simple and accessible for *D. gallinae* control. The scientific plan of the EU was obliged to prepare measures for changing rearing systems, which would be used in a planned and systematic manner to allow farmers a simple and economic way of conducting eradication and introducing security measures. The change in cages and equipment was a remarkable opportunity which, on its own, could improve welfare and change the prevalence of *D. gallinae* in EU (*Pavlicević et al., 2016a*). Instead, quite the opposite occurred. The changes in rearing systems have contributed to the spreading of disease, which had a negative effect on the neighbouring countries as well. What happened was that conditions have not been scientifically assessed and met with adequate measures of changing caging systems in the EU. With the export of second-hand cages and equipment, poultry red mite was also widely spread. In newly built facilities with new equipment, in most cases, proper biosafety measures for preventing *D. gallinae* entering were not introduced.

Red mite infestation impact on poultry and humans

Poultry in flocks highly infested with *D. gallinae* is exposed to stress, anemia and a disrupted immune response (*Kaoud and El-Dahshan, 2010*). It is more susceptible to infections and more exposed to communicable diseases, more susceptible to cannibalism, with a disrupted general health status. Stress is clinically visible through the distress of poultry, which can also resemble symptoms of mental illness if *D. gallinae* enters the outer ear canal (*Simić and Živković, 1958*). Somatic and psychogenic stress have also been diagnosed. Stress is also haematologically diagnosed. Corticosterones are also increased 1.5 times, and the level of adrenaline as much as doubled (*Kowalski and Sokol, 2005*). The manifestation of stress and anemia depends on the intensity of infestation of *D. gallinae.* It has been established that with medium infestation the number of mites per hen ranges from 25,000 to 50,000, but can reach as many as 500,000 (*Kilpinen et al., 2005; Van Emous et al., 2005; Mul et al., 2013*). In these situations, poultry is constantly exposed to *D. gallinae* at night, but also during daytime. A hen

infested with *D. gallinae* can lose 3% of its total blood every night, and as much as 5% at an extremely high number of *D. gallinae* (*Van Emous, 2005*). The blood analysis of infested poultry has established a dramatic decrease of erythrocytes, from 3.1 million to 1.2 million (*Babić et al., 1956*), as well as the damage to humoral immunity (*Kowalski and Sokol, 2015*). The role of *D. gallinae* vector is complex: mechanic, transstadial and transovarian (*Moro et al, 2005*), and relates to multiple causes of diseases: viruses, bacteria, protozoans and filarias (*Moro et al., 2007*). We highlight *Sallmonella gallinarum* and *S. enteritidis* (*Moro et al., 2009*) and *A. influence* virus (*Sommer, 2011*). Besides the basic definition of welfare, establishing the importance of categories is of vital importance. It is clear that animals which are under stress, with a disturbed general health status and increased mortality cannot fully make use of the benefits at their disposal in alternative housing. A complete elimination of all harmful consequences on poultry in intensive poultry farming is possible through *D. gallinae* eradication from production systems and introduction of biosafety measures (*Pavlicević et al., 2017b*).

Apart from this, poultry welfare ought to be coordinated with human welfare. Burdening *D. gallinae* control, the challenges of an otherwise problematic *D. gallinae* control have been even further multiplied and raised, which has also increased the toxicological risk (level) to which consumers, poultry and the environment are exposed to (*Giangaspero et al., 2011; Marangi et al., 2012*). Here it is also important to consider that *D. gallinae* control is based on chemical synthetic neurotoxic compounds (acaricides, insecticides in a wider sense), which are often used in conjunction with illegal and products not registered for these particular purposes (*Giangaspero et al., 2017*). The situation is especially worrying if we take into account the rate of frequency and concentration in application, which are motivated by the absence of expected effects and resistance of *D. gallinae.* Prohibitions and legal framework are necessary, but alone they are not sufficient to completely eliminate toxicological risk. To do this, it is necessary to eliminate the need for farmers to use poisons. Primarily, toxicological risk has come about as a consequence of the absence of a solution. Therefore, in order for the risk to be minimized, it is necessary to ensure a safe, efficient and rational control. To eliminate the toxicological risk completely, it is necessary to eliminate *D. gallinae* from production facilities. *George et al. (2016)* point to the zoonotic importance of *D. gallinae*. This way, the zoonotic influence of *D. gallinae* on farms will be also eliminated. Depending on the intensity of *D. gallinae* infestation, mortality is increased, egg laying and weight of eggs are reduced (Kaoud and El-Dahshan, 2010). Lowering of production results alongside additional costs affect the economic competitiveness of farmers. We have proved that eradication is possible and that the problem of poultry red mites, along with all its consequences should not exist in poultry farming (*Pavlicević et al., 2017a*).

Environmental conditions have a manifold impact on poultry welfare. For

industrial poultry keeping they are alone not the solution to *D. gallinae* control. However, the role of environmental conditions in *D. gallinae* control is extremely significant and requires much greater scientific attention than it is given at present.

Conclusion

Technological changes in rearing methods for layer hens in the EU have not brought poultry welfare in terms of *D. gallinae* control, but have rather had a negative impact, even to human welfare. Possibilities of improving, even solving *D. gallinae* control in egg production do exist, but they require chaining the entire approach hitherto.

Prilog razmatranju dobrobiti živine sa aspekta uticaja i kontrole crvene kokošije grinje (*Dermanyssus gallinae*)

Aleksandar Pavličević, Ivan Pavlović, Radomir Ratajac, Danica Popović, Branislav Davidović, Dejan Krnjajić

Rezime

Tehnološka rešenja i ambijentalne prilike bitno utiču na intezitet infestacije i problematičnost kontrole *D. gallinae*. Promene u načinu držanja kokošaka nosilja u EU, sa aspekta uticaja *D. gallinae*, nisu dovele do dobrobiti nosilja. Naprotiv, doprinele su širenju bolesti, pogoršale uslove kontrole i naglasile štetne posledice. Osim na živinu, promene su nepovoljno uticale na dobrobit čoveka kroz toksikološki i zoonotski rizik, i ekonomske štete. Konvencionalni kavezi obezbeđuju do sad najprikladniji ambijent za kontrolu *D. gallinae*. Mogućnosti za unapređenje, pa i rešenje kontrole *D. gallinae* u proizvodnji jaja postoje, ali ona zahteva promenu celokupnog dosadašnjeg pristupa.

Ključne reči*:* dobrobit živine, kontrola *Dermanyssus gallinae*

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WELFARE ASSESSMENT ON DAIRY CATTLE FARMS IN EASTERN CROATIA

Nicole L. Eberhart[1], Peter D. Krawczel[1], Pero Mijić[2], Vesna Gantner[2], Maja Gregić[2], Tina Bobić[2]

[1]Department of Animal Science, University of Tennessee, Knoxville, Tennessee, USA
[2]Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia
Corresponding author: Peter D. Krawczel, krawczel@utk.edu
Original scientific paper

Abstract: The objective of this study was to evaluate the welfare status of high-producing Holstein dairy cows on commercial Croatian farms. Lying behavior data was collected from 278 dairy cows across four farms with varying milking parlors and housing systems in eastern Croatia for at least 3 days. Data loggers recording at 1-min intervals recorded behaviors: lying time (min/d), lying bout duration (min/bout), lying bouts (n/d) and laterality of lying. Acceleration data was summarized into lying behaviors for each individual cow. Health scores (udder cleanliness, locomotion, and hock injuries) were also assessed. The univariate procedure was used to generate mean lying behaviors and health scores by farm with a 95% CI. Mean lying time per farm ranged from 11.7 ± 2.7 to 10.4 ± 2.7 h/d. Prevalence of lame cows ranged from 28% to 50%. Heavily soiled udders ranged from 2% to 12%. Prevalence of left hocks with minor to major swelling ranged from 50% to 100%; prevalence of right hocks with minor to major swelling ranged from 45% to 100%. In conclusion, all farms assessed have opportunities to improve overall welfare through increasing udder cleanliness and reducing hock injuries.

Key words: dairy cows, assessment, welfare, hygiene, lameness

Introduction

The European Safety Food Authority (EFSA) published a series of scientific opinions on the state of welfare in dairy cows and assessments of risk associated with cow management and practices (EFSA, 2009). The opinions offer a science-based set of suggestions to further define how to protect the “Five Freedoms” of animal welfare including access to adequate stall space in order for cows to be able to rise and lie without any restrictions and regular monitoring of dairy herds for lameness. Apart from legislative and scientific incentive to improve and maintain adequate welfare standards on dairy farms, worldwide public perception of the industry provides additional motivation. In a recent study, 68% of surveyed

consumers in the United Kingdom reported wanting to know how their food was produced and 55 % had avoided purchasing some food products over welfare concerns (*Ellis et al., 2009*). In order to ensure that consumers have access to products from animals raised and maintained in adequate welfare conditions, effective systems of assessment need to be in place.

Animal-based assessments (such as evaluating udder hygiene, lameness, and hock injuries) are important in determining overall cow comfort and well-being on dairy farms and the impact of cow welfare on production. Poor udder hygiene negatively affects milk production by increased Somatic Cell Score (SCS) (*Schreiner and Ruegg, 2003; Seegers et al., 2003*). Cows with hock injuries are more likely to become lame (*Klaas et al., 2003*) which causes alteration in normal lying behaviors (*Ito et al., 2010*). Therefore, it is likely that cows with higher hock injuries and locomotion scores will have abnormal lying behaviors. Assessment of cow well-being on farms can benefit the cow as well as the producer. Lameness and hock injuries decrease on farms previously assessed when a second evaluation was requested by the farmer (*Chapinal et al., 2014*). This suggests that information collected on farms can be a useful tool for producers and managers to make changes to facilities and practices in order to improve overall cow welfare.

The objective of this study was to assess the welfare status of high-producing Holstein dairy cows on commercial Croatian farms by collecting lying behavior, udder hygiene, lameness, and hock health data.

Materials and methods

The University of Tennessee Institutional Animal Care and Use Committee approved this project (approval number 2118-0812). Four commercial dairy farms across eastern Croatia were used for this study. Lying behavior data was collected from 81 cows on farm 1, 93 cows from farm 2, 42 cows from farm 3, and 62 cows from farm 4. Health scores were collected from 381 cows on farm 1, 213 cows from farm 2, 82 cows from farm 3, and 116 cows from farm 4 (representative of 30% of the cows housed with the farm defined “high” production pens). Cows in all stages of lactation were included in the study. Farms 1, 2 and 3 used free stall housing with mattresses while cows on farm 4 were loosely housed. All farms used straw as bedding, but quantity of straw used varied greatly. Parlor types varied across farms: farm 1 had 40 cow rotary parlor, farm 2 had a 24 double sided herringbone parlor, farm 3 used 6 automatic milking systems (4 were used to milk the “high” production cows), and farm 4 had a 20 double sided parallel parlor. Farms 1 and 4 milked twice daily, farm 2 milked three times daily except for late lactation cows, which were milked twice daily, and farm 3 cows had free choice for number of daily milkings. Farms 1, 2 and 3 had stocking densities below 100% stocking density while farm 4 was over 100%. All farms used DeLaval milking equipment (Tumba, Sweden) and fed total mixed ration two times per day.

Lying behaviors were collected with Hobo Pendant G data loggers (Onset Computer Corp., Bourne, MA) as previously validated (*Ledgerwood et al., 2010*) for a minimum of 3 days and summarized with a SAS code (AWP, 2013). Udder hygiene was assessed using a 4-pt scale with 0 indicating that fresh manure splashes covered <50 % of the udder and a score of 3 representing the entire udder covered in manure (*Schreiner and Ruegg, 2003*). Locomotion was evaluated using the NAMHS scoring system (NAHMS) with a score of 1 representing a sound cow, a score of 2 representing a moderately lame cow, and a score of 3 representing a severely lame cow. Hocks were scored on a 0-3 scale where 0 indicated no visible injury and a score of 3 indicated major swelling (*Fulwider et al., 2007*). Both right and left hocks were scored separately.

A proc univariate model was used (SAS 9.3, Cary, NC) to generate mean lying behaviors by farm with a 95% CI. Results are presented in means ± standard deviation. Frequencies of health scores were analyzed using chi square tables by farm and health score.

Results and Discussion

Mean total lying time on each farm was close to 11 h/d (Figure 1). Mean right side lying time on each farm was close to 5 h/d. Mean left side lying time on each farm was close to 6 h/d except on farm 3, which had a mean left side lying time of 4.9 h/d (Figure 1).

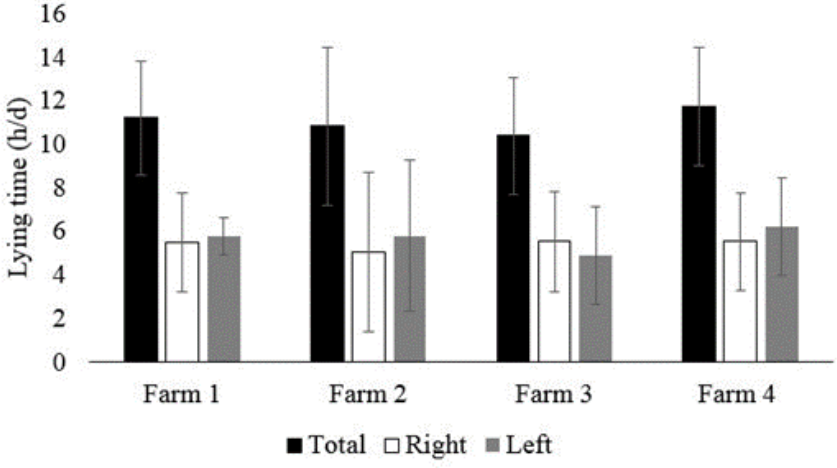

Figure 1. Mean daily lying time (h/d ± SD) by farm and cow side

Mean total lying bout duration ranged from 89.6 ± 67.2 (farm 2, Figure 2) to 58.5 ± 33.2 min/bout (farm 1, Figure 2). Mean total lying bouts ranged from 13.9 ± 6.4 (farm 1, Figure 3) to 9.1 ± 3.5 n/d (farm 2, Figure 3).

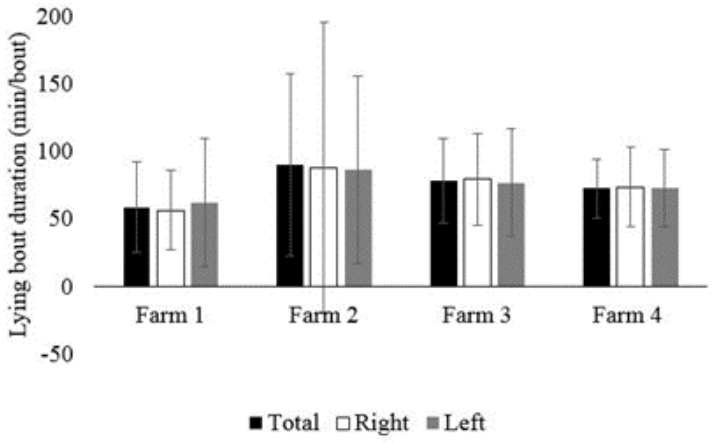

Figure 2. Mean lying bout duration (min/bout ± SD) by farm and by cow side

Farm 2 had the highest prevalence of cows with clean udders (89.7% clean) and farm 4 had the lowest prevalence (64.7 % clean; Figure 4) Farm 4 had more cows with heavily soiled udders (12.1%) compared to cows on farm 2 (1.88% heavily soiled; Figure 5).

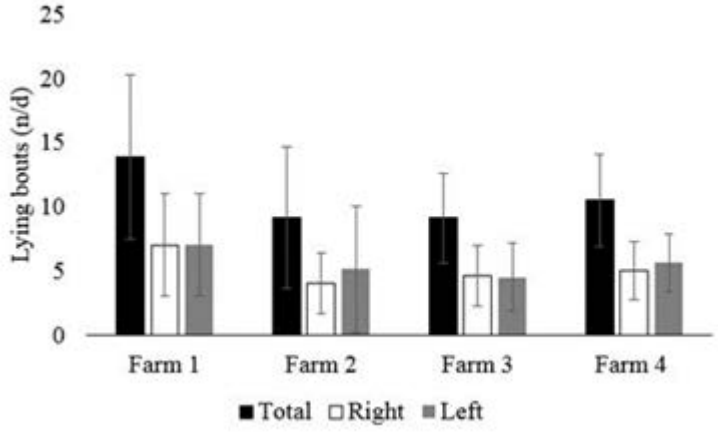

Figure 3. Mean daily lying bouts (n/d ± SD) by farm and cow side

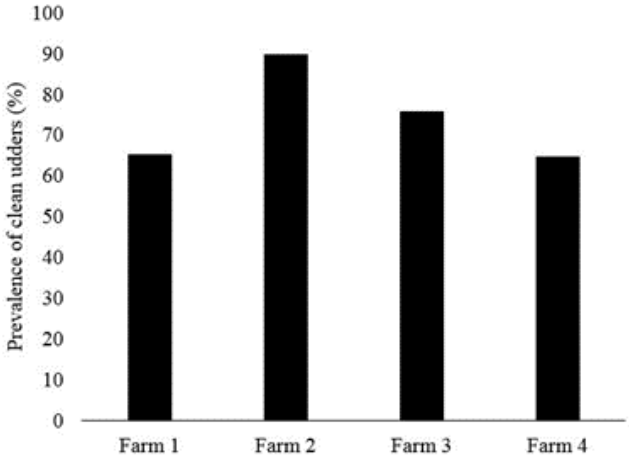

Figure 4. Prevalence of clean udders by farm

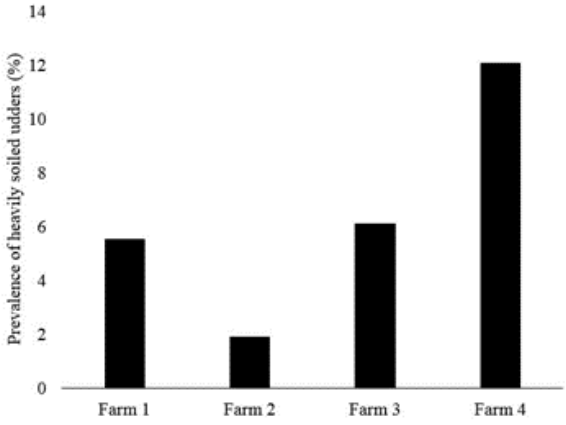

Figure 5. Prevalence of heavily soiled udders by farm

Lameness was most prevalent on farm 3 (50 % lame) and the least prevalent on farm 1 (28.4 % lame; Figure 6). Severely lame cows were most common on farm 3 (17.1 % severely lame) and the least common on farm 1 (5% severely lame; Figure 7).

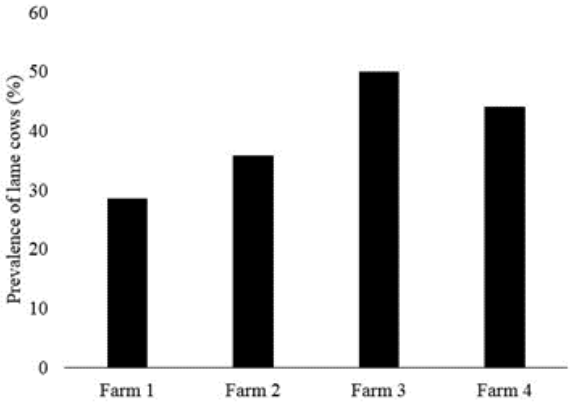

Figure 6. Prevalence of lame cows by farm

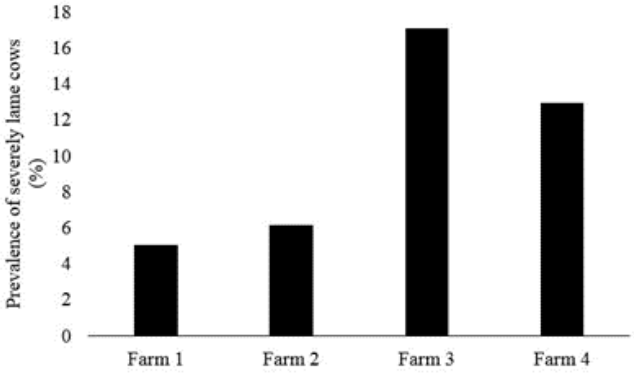

Figure 7. Prevalence of severely lame cows by farm

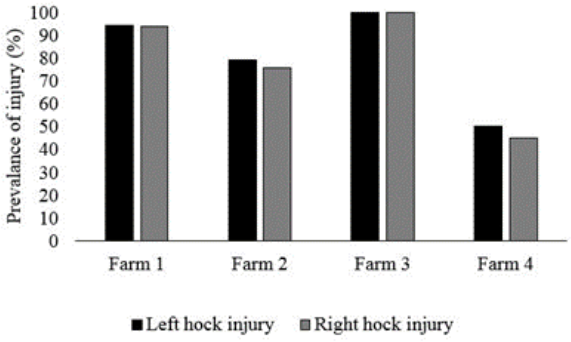

Figure 8. Prevalence of hock injuries by farm and side of injury

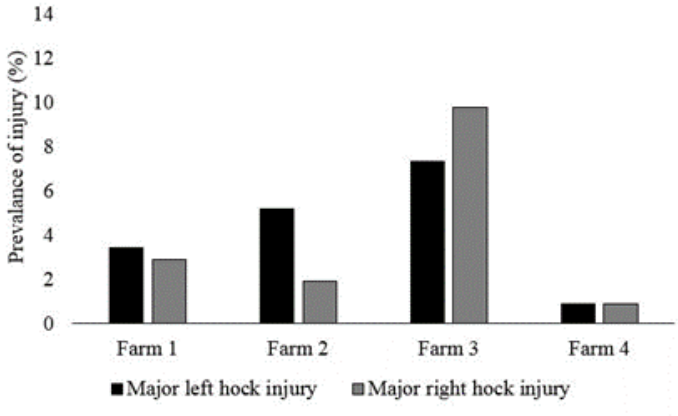

Figure 9. Prevalence of major hock injuries by farm and side of injury

Every cow scored on farm 3 had at least a minor left and right hock injury. Farm 4 had the lowest prevalence of left and right hock injuries (50% and 44.8 % injured, respectively; Figure 8). Farm 3 had the highest prevalence of cows with major hock swelling on both left and right hocks (9.8 % and 7.3 %, respectively). Farm 4 had the lowest prevalence of cows with major hock swelling on left and right hocks (Figure 9) with one cow having major swelling on both left and right hocks.

Cow welfare, by this approach, was assessed for the first time on Croatian dairy farms with free-stall or loosely housed systems. An assessment of the welfare state on Croatian dairy farms with tie-stall systems was previously conducted using visual assessments for cow behaviors as well as hygiene scoring (*Vučemilo et al.,*

2012). These authors found that cows in tie-stalls with rubber mattresses and partial access to pasture were more likely to be dirty than cows housed in tie stalls with straw bedding and without pasture access. A similar study was conducted in Macedonia on farms with tie-stall systems that found high prevalence of poor udder hygiene, hock injuries and moderate lameness (*Radeski et al., 2015*).

An observed daily lying time for commercial farms using free-stalls has been found to range from 9.5 to 12.9 h/d (*Ito et al., 2009*) and 10.5 to 11.9 h/d on free-stalls with mattresses among sound and lame cows (*Ito et al., 2010*). Lying behavior variation found on free-stalls farms can be a result of differing management practices and varying stall quality, in particular bedding quality and quantity (*Tucker et al., 2003; Fregonesi et al., 2007*). The farm with loosely housed cows (farm 4) averaged 11.7 ± 2.7 h/d of daily lying time. Average lying times found previously on bedded pack housing (which allows cows to freely move about) have ranged from 11.8 ± 0.5 h/d to 14.1 ± 0.3 h/d and indicate that cows will spend more time lying down in areas with larger open spaces (*Fregonesi and Leaver, 2001*). This is consistent with the behavioral response observed in the present study.

Cow averages for lying durations across farms are similar to average ranges of 65 to 112 min/bout previously reported across commercial dairy farms in Western Canada (*Ito et al., 2009*). Farm averages for lying bout are similar to the 7 to 10 bouts/d found on free-stalls (*Ito et al., 2009*) and 6.8-11.5 bouts/d on free-stalls and open yard facilities (Tolkamp et al., 2010). Comparing lying durations and lying bouts from the present study to previous work indicates while farm variation exists, there is little to suggest abnormal cow lying behaviors.

The prevalence of lameness assessed across all farms in this study (34.9 % of cows with locomotion scores of 2 or 3) was similar to the 36.8% (SE ± 1.3%) previously found on farms surveyed in England and Wales with both free-stall and deep stray yard housing types (*Barker et al., 2010*).

The prevalence of cows with dirty udders (27.3%) on farms in this study were lower than assessments conducted in Algeria on part time tie stall housing systems where 62.6% of cows had dirty udders and assessments in Macedonia on tie stall housing systems that found a prevalence of dirty udders to be 65.2% (*Benatallah et al., 2015, Radeski et al., 2015*). Part of this difference in udder hygiene differences could be due to the differences in housing types (tie stall vs. free-stall and loose housing). Dirty udders are a symptom of inadequate waste management, which results in an increased incidence of clinical mastitis (*Bartlett et al., 1992; Reneau et al., 2003*). Due to the relatively high prevalence of cows with dirty udders in the current study, cows from each assessed farm could be at a higher risk for developing clinical mastitis and diminished overall welfare.

Previous studies have found hock injury prevalence of 42% in British Columbia, 56% in California, and 81% in north-eastern United States (*von Keyserlingk et al., 2012*) on free-stall farms. The current study found that the farm with loosely

housed cows (50 % left side, 45% right side) had less prevalence of hock injuries than the free-stall farms (90% left side, 89% right side). The differences between prevalence of hock injuries on the farm with loosely housed cows and cows housed in free-stalls might be explained by the free-stall design on farms 1, 2 and 3. Previous studies have indicated that poorly bedded mattresses increases risk of hock injuries (*Fulwider et al., 2007*). Stalls that do not allow for proper range of rising and lying motion have been also been shown to increase risk of leg injuries, which lead to an increased likelihood of lameness (*Klaas et al., 2003*).

Conclusions

In conclusion, the prevalence of hock injuries, lameness, and severe lameness indicate that high-producing, lactating dairy cows in Croatia were not housed in environment that fit their needs. This demonstrates the potential for welfare issues related to physical structures within housing systems and the management of those systems. These data also indicate a systematic assessment program focused on identifying the causes of these injuries could lead to improvements in the welfare and productivity of dairy cows in Croatia. Furthermore, routine assessments are needed to evaluate the success of changes made to alleviate hock injuries and lameness.

Procena stanja dobrobiti na farmama za proizvodnju mleka u istočnoj Hrvatskoj

Nicole L. Eberhart, Peter D. Krawczel, Pero Mijić, Vesna Gantner, Maja Gregić, Tina Bobić

Rezime

Cilj rada bio je proceniti dobrobit visoko proizvodnih krava za proizvodnju mleka rase holštajn na komercijalnim farmama sa područja istočne Hrvatske. Podaci o ponašanju kod krava koji se odnose na ležanje u trajanju od najmanje 3 dana (d) prikupljeni su za 278 krava. Istraživanje je sprovedeno na četiri farme na području istočne Hrvatske s različitim izmuzištima i sistemima držanja. Uređaji za kontinuirano merenje (Data logger) u intervalima od 1 minuta (min) su snimali podatke o ponašanju krava koji se odnose na ležanje (vreme ležanja (min /d), interval ležanja (min/ležanju), interval ležanja (n/d) i preferirana strana tela za ležanje. Navedena svojstva kumulativno su prikazana za svaku pojedinu kravu. Za izračunavanje ukupnih prosečnih podataka o ponašanju za ležanje i zdravstvenih ocena po farmi sa 95% sigurnosti korišćena je PROC UNIVARIATE (SAS/STAT). Prosečno vreme ležanja po farmi kretalo se od 11,7 ± 2,7 do 10,4 ± 2,7 h/d.

Prevalenca šepajućih krava kretala se od 28% do 50%. Izrazito prljava vimena kretala su se od 2% do 12%. Prevalenca od manjih do većih otoka na skočnim zglobovima levih nogu bila je u rasponu od 50% do 100%, dok je kod desnih nogu ta vrednost iznosila od 45% do 100%. Može se zaključiti da sve ispitivane farme imaju prostora za poboljšanje ukupne dobrobiti, povećanjem čistoće vimena i smanjenjem povreda na zglobovima.

Ključne reči: mlečne krave, procena, dobrobit, higijena, šepavost

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EVALUATION OF SOME EFFECTIVENESS ELEMENTS OF THE PIG BREEDING INDUSTRY IN BULGARIA, THROUGH CLUSTER ANALYSIS

Ivelina Zapryanova

Agricultural University, 12 Mendeleev Blvd., 4000 Plovdiv, Bulgaria
Corresponding author: ivelina_z@abv.bg
Original scientific paper

Abstract: An evaluation was made of the effectiveness elements of the pig breeding industry in Bulgaria in the period 2001-2016, through cluster analysis. The studied period was divided in 3 subperiods, each one with three similar groups (clusters). Through application of cluster analysis, the proximity of the different administration regions in the country was defined in accordance with certain indicators of the pig breeding effectiveness. It was found that in the first cluster for the period 2001-2006 fall North-Western and South-Western region. The North-Eastern and North-Central region form the second cluster. The South-Eastern and South-Central region fall mainly into the third cluster. In the first cluster for the period 2007-2011, the North-Western, South-Western and South-Central regions have a priority with the lowest number of sold animals. The North-Eastern and the North-Central region, forming a third cluster, remain with the highest effectiveness of the pig farming. After the end of 2013 an aggregation of the sector began. For the period 2012-2016, the second cluster is formed from three regions – North-Western, South-Western, and South-Central in 2013.

Key words: clusters, cluster analysis, effectiveness, pig breeding, regions

Introduction

Pig breeding in Bulgaria, as part of livestock breeding, has its own deep traditions, despite the dynamics in its development through the years. The instable economic environment in the period 1990-2010 led to a decrease in the number of pigs, which inevitably led to a drop in the industry. As a result of this negative statistic, the production of pork is below the level it was in its beginning in the previous century *(Dermendzhieva et al.,2013).* According to *Ivanova et al. (2011)* after the slight stabilization of the livestock breeding industry in 1997-2000, the production has decreased, as a decrease of nearly 2% of the gross added value in it

has been reported. What is worrying is that the biggest decrease in the gross production is reported exactly in pig breeding.

For more than 20 years the economic environment in the country has been too dynamic and insecure, which has put domestic pig breeding in difficult situations. After our country joined the European Union, the qualification of pig carcases under the (S)EUROP system became obligatory for Bulgarian pig breeding, too. According to *(Otouzbirov and Zhelyazkov, 2008)* this will lead to improvement of the selection process, increase of the productive and slaughterhouse qualities of the pigs, but in parallel with this, the consumers shall pay a higher price at the purchase of pork.

The analysis of the state of pig breeding by regions and in different stages of development, allows the making of an evaluation of the effectiveness of the industry, especially when keeping in mind its dynamic state through the years.

All this gave us a reason to make an evaluation of some effectiveness elements of the pig breeding industry in Bulgaria by means of cluster analysis.

Material and Methods

The analyzed material consisted of data from the bulletin of the Agrostatistics Department of Ministry of Agriculture and Food (MAF) about the farm animals in Bulgaria, the activity of slaughterhouses for red meat and meat production in the country, Annual Report on the Status and Development of Agriculture in the period from 2001 to 2017, as well as National Strategy for stable development in Agriculture in Bulgaria in 2014-2020 (MAF, 2013).

The following indicators were analysed: 1. Number of slaughterhouses; 2. Total amount of slaughterhouse meat (t), 2.1. Including meat from pigs (t); 3. Sold pigs (transmitted to slaughterhouses, slaughtered in farms and sold to intermediaries) (in thousands); 4. Number of farms, where sows are bred, 4.1. Number of bred sows; 5. Number of farms, where total pigs bred, 5.1. Number of total pigs bred.

In order to form groups based on the specified indicators, we used the K-Means Cluster method. The Euclidean distance between the groups was used as a measurement of similarity.

The studied period was divided in 3 subperiods (2001-2006, 2007-2011 and 2012-2016), each one with three similar groups. Figures, which represent the grouping of the studied indicators in clusters graphically, were built.

The statistic processing was performed with IBM software product SPSS version 24.

Results and Discussion

After Bulgaria joined the European Union, the geographical zoning of the country is performed in accordance with Regulation (EO) No. 176/2008. According to this document, our country is divided in 6 regions – North-Western, North-Central, North-Eastern, South-Eastern, South-Western, and South-Central one (Fig.1).

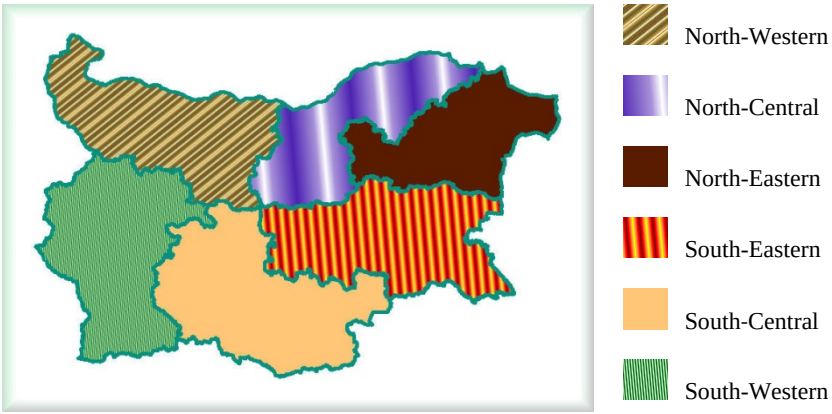


Fig. 1. A common classification of territorial units for statistics (NUTS) by reason of the accession of Bulgaria to the European Union

When forming the clusters for the first studied period, the indicators with the biggest significance are the ones for the amount of the obtained meat, sold pigs in slaughterhouses and farms, as well as their number in different categories. (Table 1). The total number of slaughterhouses does not have an effect on grouping.

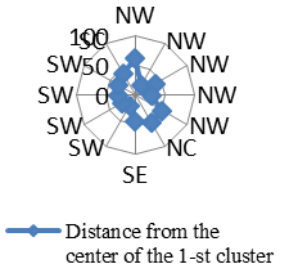
Table 1. F-criterion and degree of reliability (ANOVA table)

	F-criterion and degree of reliability		
	2001-2006	2007-2011	2012-2016
1.NS	2.315	6.065**	0.515
2. TASM	12.386***	8.678**	2.79*
2.1. TASM incl. pork	5.953**	4.344*	3.149*
3. SP	97.623***	134.704***	2.636*
4. NFSB			13.666***
4.1. NBS	16.362***	35.799***	3.534*
5. NFTPB			98.012***
5.1. NTPB	51.884***	75.652***	2.129
***P<0.001; **P<0.01; *P<0.05			

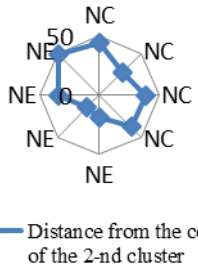
The F-criterion was used for the purposes of the description only, without having to consider hypotheses *(Harizanova-Metodieva et al., 2016)*
1. NS-Number of slaughterhouses; 2. TASM-Total amount of slaughterhouse meat (t), 2.1. TASM incl. pork-Including meat from pigs (t); 3. SP-Sold pigs (transmitted to slaughterhouses, slaughtered in farms and sold to intermediaries) (in thousands); 4. NFSB Number of farms, where sows are bred, 4.1. NBS-Number of bred sows (in thousands); 5. NFTPB-Number of farms, where total pigs bred (in thousands), 5.1. NTPB-Number of total pigs bred (in thousands)

Table 2 shows the final cluster centres, by periods. The first cluster for 2001-2006 displays relative similarity in the studied indicators in the North-Western and South-Eastern regions (Fig 2a). This is the group with the lowest number of bred and sold pigs. The North-Eastern and the North-Central regions form the second cluster (Fig 2b). These are regions, rich in cereal crops, and, where regularly the highest number of pigs from different categories, is bred and sold. The central point of the second cluster includes 32.38 slaughterhouses, with 10.23 thousand tonnes of pork, nearly 269 thousand animals sold in slaughterhouses and farms (Table 2). For the period 2001-2006, the third formed cluster takes a middle ground in all studied indicators, with the exception of the amount of carcass, which is the lowest, compared to the two other similar groups. The South-Eastern and South-Central regions fall into this cluster, the South-Western, with its indicators from 2003, and North-Eastern in 2001 (Fig. 2c).

2001-2006 (Fig.2a)



2001-2006 (Fig.2b)



2001-2006 (Fig.2c)

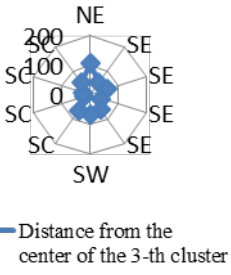


Table 2. Final cluster centres

Period	2001-2006			2007-2011			2012-2016		
Cluster	1	2	3	1	2	3	1	2	3
Traits									
1. NS	27.75	32.38	20.80	9.82	12.67	14.70	12.21	11.00	11.50
2. TASM	7.33	15.60	5.52	6.78	5.57	14.83	12.37	10.11	7.60
2.1. TASM incl. pork	5.31	10.23	4.62	5.92	4.88	10.63	10.88	8.77	ll
3. SP	59.25	269.4	138.4	60.5	137.9	297.3	206.1	39.00	128.9
4. NFSB							274.53	726.00	669.50
4.1. NBS	7.25	21.44	15.17	4.61	10.53	15.09	9.18	3.07	5.76
5. NFTPB							2375.5	13422.3	6631.5
5.1. NTPB	94.33	245.4	173.1	53.6	132.1	189.6	114.1	41.3	74.5

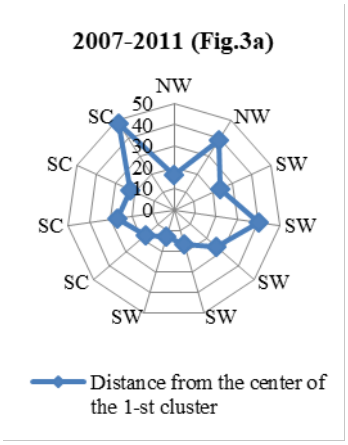
1. NS-Number of slaughterhouses; 2. TASM-Total amount of slaughterhouse meat (t), 2.1. TASM incl. pork-Including meat from pigs (t); 3. SP-Sold pigs (transmitted to slaughterhouses, slaughtered in farms and sold to intermediaries) (in thousands); 4. NFSB Number of farms, where sows are bred, 4.1. NBS-Number of bred sows (in thousands); 5. NFTPB-Number of farms, where total pigs bred (in thousands), 5.1. NTPB-Number of total pigs bred (in thousands)

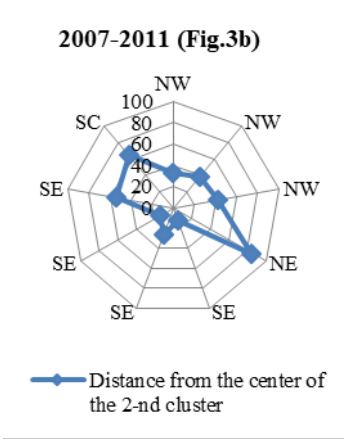
Table 3. Distances between the final cluster centres

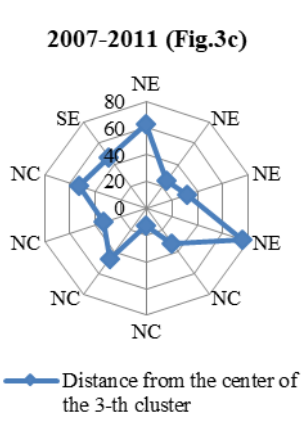
	2-nd cluster	3-th cluster
2001-2006		
1-st cluster	259.364	112.162
2-nd cluster	-	150.608
2006-2011		
1-st cluster	110.483	273.460
2-nd cluster	-	169.815
2012-2016		
1-st cluster	11057.532	4275.148
2-nd cluster	-	6791.745

The proximity between the separate homogeneous groups, reported through the Euclidean distance, shows that the first and third cluster are more similar in the studied indicators (112.162), compared to the second and the third (150.608). During the first analysed period, the first and second cluster share the lowest number of common characteristics (Table 3).

The state of pig breeding in the economic year of 2007 is defined, to a certain extent, by the unfavourable weather conditions and subsequent low grain yields, and the high prices of concentrated fodders. That was the first year of Bulgarian membership in the European Union. The total number of pigs decreased by 12.3% compared to 2006 and by 8.8% compared to 2005. The same trend to a decrease is observed with the sows as well as in the number of pig-breeding farms *(Zapryanova, 2015).*





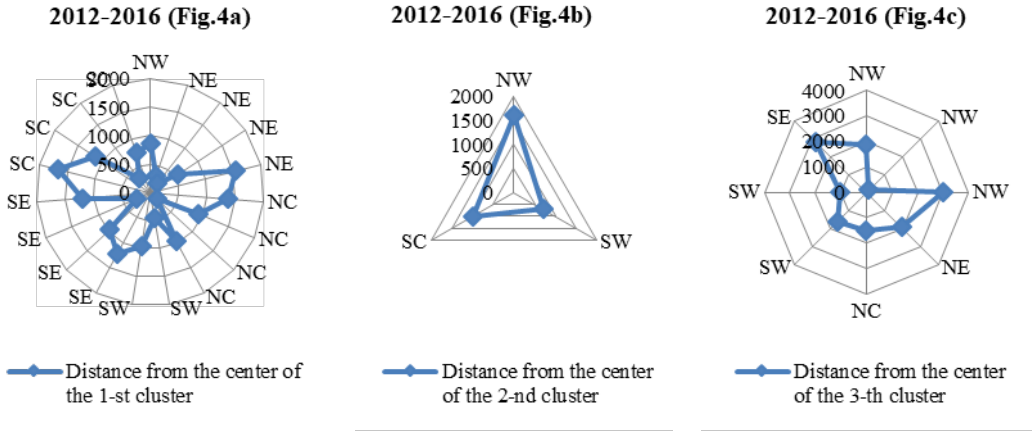


The yield of pork carcass after 2000 is relative parallel through the years in all regions, without sharp amplitudes in the values. The North-Eastern region makes an exception in the period 2006-2008, as from relatively moderate, it turns into a centre of the produced amount of carcass with 22.95 thousand tonnes in 2007 *(Zapryanova, 2018).*

In the first cluster for the period 2007-2011, the North-Western, South-Western and South-Central regions have a priority (Fig. 3a). The lowest number of sold animals (60.4 thousand animals) is characteristic of the group, as well as the number of the animals from all categories, which are bred in these regions (Tabl.2). The number of the slaughterhouses is also the lowest, but unlike the previous period, this indicator has significant effect on formation of the cluster (Tabl.1). Regardless of the serious decrease of the values of the indicators, the North-Eastern and North-Central region, forming the third cluster, remain with the higher effectiveness in pig breeding (Tabl. 2, Fig. 3c). The second homogeneous group, including mainly the North-Western and South-Eastern region has the characteristics of 12.67 number of slaughterhouses, 4.88 thousand tonnes of pork (which is the lowest for the studied period), the number of the bred sows and the total number of pigs take a middle position, respectively with 10.53 and 132.1 thousand animals (Tabl. 2, Fig. 3b).

The most distant in their homogeneity are the first and third cluster (the Euclidean distance is 273.460), and most similar are first and second cluster (the Euclidean distance is 110.483) (Tabl. 3).

Within the last studied period, we have the possibility to add two more indicators – number of farms, where sows are bred, as well as animals from the other categories. These two indicators have reliable effect on the formation of the clusters (Tabl.1). In this time span, however, the number of slaughterhouses as well as the total number of pigs, appears an unreliable source at the formation of homogeneous groups.



Reviewing the three formed clusters (Fig 4a, b, c) the one that makes the most impression in the second one, which is formed from three regions – the North-Western, South-Western and South-Central one – in 2013. Main features of this cluster are the lowest number of sold animals (39 thousand animals), the lowest total number of pigs, but with the highest number of farms, where these animals are bred (Tabl. 2).

The difference between the first and third cluster is the higher number of observations in all regions and years, which form the first group. At the same time, here is the highest number of slaughterhouses (12.21), sold pigs (206.05 thousand animals), lowest number of farms, where the highest number of pigs of different categories are bred.

In our previous research we concluded that consolidation of the sector was reported after the end of 2013, when the number of pigs on farms with more than 200 animals increased by 5.3 thousand, although the number of farms dropped down. In the last five years the largest share of the pigs has been concentrated in three regions of the country – North Central, South-Eastern and North-Eastern *(Zapryanova, 2015).*

Conclusions

The first cluster for the period 2001-2006 shows a relative similarity in the studied indicators in the North-Western and South-Western region. The North-Eastern and North-Central region form the second cluster. The South-Eastern and South-Central region fall mainly into the third cluster.

In the first cluster for the period 2007-2011, the North-Western, South-Western and South-Central regions have a priority with the lowest number of sold

animals. The North-Eastern and the North-Central region, forming a third cluster, remain with the highest effectiveness of the pig farming. The second homogeneous group includes mainly North-Western and South-Eastern region.

After the end of 2013 an aggregation of the sector began. For the period 2012-2016, the second cluster is formed from three regions – North-Western, South-Western, and South-Central in 2013. The difference between the first and the third cluster is the higher number observations in all regions and years. The highest number of slaughterhouses is in the first group (12.21), sold pigs (206.05 thousand animals), the lowest number of farms where the highest number of pigs from different categories is bred.

Evaluacija nekih elemenata efikasnosti svinjarstva u Bugarskoj, kroz klaster analizu

Ivelina Zapryanova

Rezime

Izvršena je evaluacija elemenata efikasnosti svinjarstva u Bugarskoj u periodu 2001-2016, kroz klaster analizu. Proučavani period je podeljen u 3 podperioda, svaki sa tri slične grupe (klasteri). Primenom klaster analize utvrđena je blizina različitih administrativnih područja u zemlji u skladu s određenim pokazateljima efikasnosti uzgoja svinja. Utvrđeno je da u prvi klasteru za period 2001-2006 pada severozapadni i jugo-zapadni region. Severoistočni i severno-centralni region čine drugi klaster. Jugoistočni i južno-centralni region uglavnom spadaju u treći klaster. U prvom klasteru za period 2007-2011, severozapadni, jugo-zapadni i južno-centralni region imaju prioritet sa najmanjim brojem prodatih životinja. Severoistočni i severno-centralni region, koji formiraju treći klaster, su regioni sa najvećom efikasnošću u svinjarstvu. Nakon kraja 2013. godine počela je agregacija sektora. Za period 2012-2016, drugi klaster je formiran iz tri regiona - severozapadni, jugo-zapadni i južno-centralni u 2013. godini.

Ključne reči: klasteri, klaster analiza, efektivnost, uzgoj svinja, regioni

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THE FATTY ACID COMPOSITION OF SHEEP'S MILK OF AN AUTOCHTHONOUS BREED

Amina Hrkovic-Porobija[1], Aida Hodzic[1], Mensur Vegara[2], Husein Ohran[1], Almira Softic[1], Aida Kavazovic[1], Maja Varatanovic[1]

[1]University of Sarajevo, Faculty of Veterinary Medicine, Zmaja od Bosne 90, 71000 Sarajevo, Bosnia and Hetzegovina
[2]University of Life Sciences, NORAGRIC, Universitetstunet 3, 1430 Ås, Norway
Corresponding author: Amina Hrkovic-Porobija, amina.hrkovic@vfs.unsa.ba
Original scientific paper

Abstract: The study included a total of 127 sheep milk samples from two different areas (Livno and Travnik) in summer feeding period (July, August and September). Fatty acids in milk were determined by gas chromatography (GC). The animals were marked with the appropriate number of ear tags on the basis of which we always took samples from the same animals through different periods. Fatty acids in milk were determined by gas chromatography and the following fatty acids composition: butyric acid, caproic acid, caprylic acid, capric acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, rumenic acid**.** The fatty acid content of sheep's milk in this study showed a tendency of variation, both within and between sampling areas, and characterized by its relatively high content of saturated fatty acid (SFA) during the period of harvest.

Key words: milk, fatty acid composition, sheep

Introduction

Milk and milk products are well balanced nutritious food in human diet. Milk fat contains approximately 400 different fatty acids, which make it the most complex of all natural fats (*Lindmark Mansson, 2008*).The premium nutritional quality of dairy products is highly correlated with milk fat quality and concerns: high concentration of fat soluble vitamins and n-3 fatty acids, as well as high content of conjugated linoleic acid (CLA) (*Markiewicz-Keszycka et al. 2013*). The large number of research studies with the aim of increasing the biological value of animal products - more specifically the milk of ruminants, comes from the FAO recommendations, which in 2003 established the consumption of SFA for humans. However, only two procedures can change the fatty acid profile of products derived from ruminants: modification of fatty acids during the processing, or change in the

fatty acid profile of the diet (*Palma Rennó et al. 2013*). Most studies focus primarily on the effect of feeding the sheep on the fatty acid profile (*Addis et al. 2005*). The influence of physiological factors (breed, lactation) is of less importance (*Tsiplakou et al. 2006*)**.** The aim of this study is to determine the fatty acid composition of sheep's milk as well as to monitor the influence of diet on their composition.

Materials and methodS

A total of 127 sheeps were used to investigate the effects of different sampling period and areas on milk fatty acids (FA) profiles. The research was conducted during July, August, and September at Livno area (village Guber -724 m altitude) and Vlašić mountain (village Mudrike – 1300 m altitude) in Bosnia and Herzegovina (B&H). The animals were marked with numbered ear tags and the sampling was done through different sampling periods (July-I, August-II and September-III). In the area of Livno, two milk samples were taken - July (n = 20) and August (n = 20), while the samples for the third sampling were quantitatively insufficient for performing all the foreseen analyzes, and in the area of Travnik the milk was sampled in three terms - July (n = 25), August (n = 25) and September (n = 25). Fatty acids in sheep milk were determined by gas chromatography in the laboratory Vitas As Oslo Innovation Centre, Norway. Sample preparation was performed according to the procedure described in Luna et al. (2005), which includes the separation of milk fat by centrifugation and fatty acid methylation to produce fatty acid methyl esters (FAME) which are analyzed on a gas chromatograph. The following fatty acid composition was determined: butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), stearic acid (C18:0), oleic acid (C18:1 cis-9), linoleic acid (C18:3 n-3), arachidonic acid (C20:4 n-6, ARA), eicosapentaenoic acid (C20:5 n-3, EPA), docosahexaenoic acid (C22:6 n-3, DHA), rumenic acid (C18:2 cis 9, trans-11, CLA)). Statistical analysis was performed using the software package/SPSS 21.00. Nonparametric statistics were used for processing, Friedman (for the Travnik area) and Wilcoxon test (distribution free tests) (for the area of Livno) were used. The differences were considered statistically significant at $p<0.05$, $p<0.01$ and $p<0.001$.

Results and Discussion

The mean values of fatty acid in milk of sheep breed in the area of Livno and Travnik expressed in grams of each fatty acid per 100 g of total fatty acid (g / 100g FA) are shown in Tables 1 and 2, as well as the statistical significance of differences between sampling periods. Table 3 shows the statistical significance differences of the content of fatty acids in milk depending on the locality and the sampling period. By testing the differences in the fatty acid content between the

Livno and Travnik areas during the sampling periods, statistically significant differences were found in the content of 17 of the total of the given 24 fatty acids.

The results of this research showed, that a significant influence on the profile of fatty acids of sheep milk from both sampling areas (Livno, Travnik) had the botanical composition of pastures and the period of lactation. A total of 24 fatty acids were determined over three sampling periods (July, August and September). During the sampling period, sheep milk from Livno and Travnik areas contained a higher proportion of SFA compared to unsaturated fatty acids (UFA) and polyunsaturated fatty acids (PUFA). Most SFA in sheep milk from the Livno area showed differences between the sampling periods most likely to be due to differences in the composition of pastures (vegetation) at the time they were used for animal feeding.

Saturated fatty acids from the Livno area were individually statistically significantly related to the sampling period, in order to reduce their content to the end of the lactation period. The content of C4:0 acid in sheep milk samples from the Livno area was significantly lower than the value found in Merino sheep milk by *Mierlita et al. (2011)*. In both sampling areas, the trend of decline in sampling periods was noticed for C4:0, especially for the Travnik area (Tables 1 and 2).

Meals containing a higher amount of sugar cause the formation of C4:0, and for these meals a higher content of C4:0 in milk fat is characteristic. In the area of Livno for C6:0, C8:0 and C10:0 acids, a statistically difference was observed between the sampling period, again with the trend decreasing following the end of lactation, and the established values were lower values mentioned by other authors (*Goudjil et al., 2004; Mierlita et al., 2011*). In raw milk, high concentrations of C4:0, C6:0, C8:0 and C10:0 are not preferred, as it may result in distortion of taste /aroma of milk.

Table 1. Mean values of fatty acids in sheep milk from Livno area

SFA			
Fatty acids (g/100gFA**)**	I sampling	II sampling	p
C4:0	3.86	3.69	
C6:0	2.08	1.40	***
C8:0	1.64	0.98	***
C10:0	4.29	2.81	***
C12:0	2.66	2.07	***
C14:0	9.55	8.45	***
C15:0	1.18	1.07	***
C16:0	22.30	21.85	
C17:0	0.81	0.82	
C18:0	8.64	9.72	**
C20:0	0.42	0.43	

MUFA			
C14:1cis-9	0.25	0.27	
C16:1cis-9	0.90	1.00	
C18:1cis-9	17.93	22.27	***
C18:1 cis-11	0.89	0.95	
C18:1trans-9	0.28	0.40	
C18:1trans-10	0.50	0.57	
C18:1trans-11	2.87	2.48	
PUFA			
C20:4	0.16	0.17	
C20:5 n-3 (EPA)	0.15	0.12	
C22:6 n-3 (DHA)	0.10	0.09	
C18:2 n-6	2.46	2.70	
C18:3 n-3	2.26	1.34	***
C18:2cis-9, trans-11(CLA)	1,63	1,49	
∑n-3	2.52	1.62	***
∑n-6	2.61	2.91	*
∑SFA	57.29	53.78	**
∑MUFA	23.97	28.09	***
∑PUFA	6.89	6.01	*
∑UFA	31.30	33.86	**
n-6/n-3	1.05	1.92	***
SFA/MUFA	2.36	1.97	***
SFA/PUFA	8.36	8.98	
MUFA/PUFA	3.48	4.63	***
SFA/UFA	1.82	1.61	**
UFA/MUFA	1.29	1.22	***
UFA/PUFA	4.48	5.63	***

Mean values in the same row with different letter codes differ significantly,
*** p< 0,001, ** p<0,01; I, II – ; I, II, III–represent sampling periods: July, August and September
SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; UFA – unsaturated fatty acid

Table 2. Mean values of fatty acids in sheep milk for Travnik area

SFA				
Fatty acids (g/100gFA)	I sampling	II sampling	III sampling	p
C4:0	3.43[a]	3.30[a]	2.86[b]	***
C6:0	1.86[a]	1.77[a]	1.49[b]	*
C8:0	1.47	1.32	1.22	
C10:0	3.87	3.60	3.68	
C12:0	2.51	2.24	2.83	
C14:0	9.01[a]	9.05[a]	10.19[b]	*
C15:0	1.21	1.16	1.13	
C16:0	21.62[a]	22.58[a]	23.74[b]	**
C17:0	0.70	0.73	0.66	
C18:0	9.22[a]	9.37[a]	7.70[b]	***
C20:0	0.37	0.41	0.38	
MUFA				
C14:1cis-9	0.55	0.37	0.35	
C16:1cis-9	1.01	1.04	1.16	
C18:1cis9	20.90	20.77	20.83	
C18:1 cis-11	0.74[a]	0.71[a]	0.59[b]	***
C18:1trans-9	0.26	0.26	0.23	
C18:1trans-10	0.35	0.31	0.26	
C18:1trans-11	3.20[a]	2.61[b]	2.55[b]	**
PUFA				
C20:4	0.23	0.24	0.24	
C20:5 n-3 (EPA)	0.14	0.14	0.15	
C22:6 n-3 (DHA)	0.11[a]	0.15[b]	0.18[b]	**
C18:2 n-6	2.44	2.57	2.19	
C18:3 n-3	1.91[b]	1.98[b]	1.64[a]	**
C18:2cis-9, trans- 11 (CLA)	2.21[a]	1.69[b]	2.04[a]	***
∑n-3	2.08	2.29	2.02	
∑n-6	2.64	2.74	2.55	
∑SFA	55.93	56.85	56.73	
∑MUFA	27.39	26.11	27.38	
∑PUFA	6.71	6.98	6.66	
∑UFA	33.84	33.25	34.16	
n-6/n-3	1.26[ab]	1.21[b]	1.31[a]	*
SFA/MUFA	2.02	2.11	2.01	
SFA/PUFA	8.23	8.30	8.66	
MUFA/PUFA	3.97	3.79	4.27	
SFA/UFA	1.64	1.73	1.64	
UFA/MUFA	1.25	1.26	1.23	
UFA/PUFA	4.97	4.79	5.27	

Mean values in the same row with different letter codes differ significantly, *** p<0,001, ** p<0,01, * p<.0,05; I, II, III–represent sampling periods: July, August and September

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; UFA – unsaturated fatty acid

In addition to the absolute content of n-3 fatty acids in the meal nothing less is a significant relationship between n-3 and other UFA, which is n-6 fatty acids. Acid EPA, in addition to C18: 3 n-3 and DHA, is the most significant n-3 fatty acid. The acids C18: 2 n-6 and C18: 3 n-3 are the most dominant PUFA in Livno's milk and C18: 2 n-6 and rumenic acid from the Travnik area. The content of C18: 3 n-3 in milk samples from Livno and Travnik ranged from sampling periods, and very high statistically significant differences were found between I and II sampling periods.

The content of C6:0 and C8:0 in sheep milk fat from the Travnik area also had a trend of decreasing values going to the end of the lactation period, with the differences in C6:0 being statistically significant. Saturated fatty acid from the Livno area had a slight decreasing trend in August-II except for C18:0, where a slight increase in the second sampling period was found, while in the examined samples of Travnik milk in the III sampling period there was a decrease in value. Statistically significant differences in the content of fatty acid milk were determined within and between the areas by sampling periods. Depending on the locality and the sampling period statistically significant differences at the level ($p<0.05$) were observed in MUFA, PUFA, UFA and fatty acid sums ratio (Table 3).

The content of the fatty acids investigated in our research showed a tendency of variation over the months and is characterized by its relatively high content of SFA through the period, so it is possible that the sheep breed may have a greater effect on the SFA concentration than the sampling period. Differences in the content of fatty acids are a possible consequence of the aforementioned differences in nutrition, and probably other factors that affect the fatty acid composition of milk fat. The floral composition of hill pastures is better than the mountainous in terms of botanical composition (*Žan et al., 2006*). During the spring, a more intense growth of biomass is made, compared to the summer when it is influenced by high temperatures and lack of precipitation, the pastures are considerably less expensive (*Ljubicic et al., 2012*).

Table 3. Statistical significance differences in the content of fatty acid sheep milk from Livno and Travnik areas between the sampling period

Fatty acids	LI/TI	LI/TII	LI/TIII	LII/TI	LII/TII	LII/TIII
C4:0	*	*	*	*	*	*
C6:0					*	*
C8:0					*	
C10:0						
C12:0				*		
C14:0		*	*			
C15:0		*				
C16:0			*		*	*
C17:0						
C18:0						
C20:0		*				*
C14:1 cis-9		*				
C16:1 cis-9						
C18:1cis-9		*				
C18:1 cis -11						
C18:1 trans-9			*		*	
C18:1 trans-10		*			*	
C18:1 trans-11						
C20:4 n-6		*			*	
C20:5 n-3 (EPA)		*				
C22:6 n-3 (DHA)						
C18:2 n-6		*		*	*	*
C18:3 n-3		*			*	
C18:2 cis-9, trans- 11(CLA)						*
∑n-3	*		*	*	*	*
∑n-6						*
∑SFA						*
∑MUFA	*	*	*		*	
∑PUFA				*	*	*
∑UFA	*	*	*			
n-6/n-3	*	*	*	*	*	*
SFA/MUFA	*	*	*		*	
SFA/PUFA						
MUFA/PUFA	*		*	*	*	*
SFA/UFA	*		*			
UFA/MUFA	*		*	*	*	*
UFA/PUFA	*		*	*	*	*

*p<0,05. L –sampling area LIVNO; T – sampling area TRAVNIK. I, II, III - –represent sampling periods: July, August and September; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; UFA – unsaturated fatty acid

There are particularly pronounced differences when comparing the values of fatty acids content between different sampling periods of different areas (LI / TI, LI / TII, LI / TIII, LII / TI, LII / TIII). Attention should be paid to the flora of Vlasic Mountain and its age. Vlasic Mountain with its geographic position, terrain configuration and mountain climate significantly influences the composition, layout and dynamics of the appearance of certain plant species in this ecosystem. If the quality of the pasture is observed from this site it can be concluded that the best quality food is given by plant species from the legume group.

The Livno area with its geographical position, terrain configuration and characteristic climate represents the area of a unique flora with a large number of interesting plant species. The botanical composition of fodder plants and their percentage distribution in these localities of the Livno Canton, which can be classified as mountainous lawns in terms of altitude and other climatic edafic conditions, is characterized by the content of hornbeam (*Scabiosa columbaria*, *Knautia arvensis*) and grass (*Nardus stricta, Festuca sp*.) indicating a certain acidity of the soil because these plants inhabit the soil of acid reactions (*Hrkovic, 2009*).

The quantity, composition and characteristics of milk produced, especially sheep held on pasture, in given environmental conditions, depend on the combined effects of seasonal changes in the climate and available food, as well as variations in the metabolic status of sheep resulting from lactation, can explain the established changes in fatty acid composition of milk during this study. In the class of SFA, C4:0, C6:0 and C18:0 in sheep milk from the Travnik area, they were statistically significant for the sampling period, in terms of reducing their content in the month of September-III.

The content of C16:0 in analyzed samples of milk from the Livno area was opposite to the samples from the Travnik area. The content of C16:0 in milk samples from the Livno area was higher in the I sampling period but without statistically significant difference compared to the II period (August), which is consistent with the results of the research (*Mihaylova et al., 2005*) who point out that the content of C16:0 and C18:0 was the highest in sheep's milk in July. The content of C16:0 in analyzed milk samples from the Travnik area grew by sampling periods and a statistically significant value in comparison to the previous two sampling was recorded in September Table 2. There was no extremely dry period during this research (data from the Federation Hydrometeorological Institute). However, in August-II, fewer precipitation and high temperatures were reported, resulting in less vegetation, and the sheep's meals were solely pond. The annual season as a whole does not act equally to the animal organism, and therefore individual factors (temperature, humidity, air flow and light) need to be observed and their potential impact on production performance. High air temperatures can adversely affect milk fat and milk fat content, which may also affect the fatty acid composition of milk. Leading SFA in most nutrients is C16:0

which, together with C12:0 and C14:0, is considered hypercholesterolemic. In the examined samples of milk from both sampling areas, the dominant fatty acids were C14: 0, C16: 0, C18: 0 and C18: 1 cis-9.

Analysis of the fatty acid composition of sheep milk from the Livno area showed very high statistically significant differences between sampling periods mainly for SFA (C6: 0-C15: 0). The content of SFA in dairy fat from both sampling areas was higher in July-I compared to August-II and September-III, which is likely to be related to nutrition and climatic factors during that period. The content of C12:0 in Livno milk samples was very statistically significantly different between sampling periods (Table 1). *Valvo et al. (2007)* found that the content of C12:0, C14:0 and C16:0 was higher in the milk of sheep which were kept alive than sheep in the pasture, which was the result of a higher proportion of C14: 0 and C16: 0 in the hay and barley compared to pastures of pastures. The most abundant MUFA milk fat in the Livno and Travnik areas was C18:1 cis-9 whose value varied depending on the locality and sampling period, which may be due to the seasonal effect associated with the feeding mode in summer period. *Popović-Vranješ et al. (2010)* found that at the beginning of the past season C18: 1 cis-9 in organic milk gradually increased in August to reach a value higher than the average value found in conventional milk.

In the majority of other MUFA analyzed samples of sheep milk from the Travnik area there was a decrease in the value by sampling periods, ie at the end of lactation. The content of VA in milk samples from the Livno and Travnik areas showed a fall in values according to sampling periods with high statistical significance for the Travnik area. Changes in VA content in analyzed milk fat samples may be the result of changes in the content of C18: 3 n-3 in plants depending on the vegetation phase and differences in the length of grain. Determining the concentration of ARA, EPA and DHA in the field of Livno is not significantly different between the endpoints, while the emphasis on the concentration of DHA highlights the significance of the difference between the two groups. The acid EPA is incapable of partially blocking the conversion of n-6 fatty acids into harmful eicosanoids, thereby decreasing the risk of the cardiovascular lesions *(Popović-Vranješ et al., 2010).*

Both areas contain C18 : 3 n-3 had a trend of falling to the end of the lactation period, and this would be the result of nutrition, that is, the stage of vegetation, because the younger plants are richer in C18: 3 n-3**,** and its content decreases by decaying the vegetation. Some authors suggest that the increased intake of C18:2 n-6 and feeding on pastures increases the CLA content of milk (*Popović-Vranješ et al., 2010*). The CLA content in both sampling areas has a variation trend over the months of sampling, which may be due to feeding on pastures, especially where vegetation is present in grasslands, as our CLA research shows a downward trend of values going to the end of lactation, and at the end of the drooping period when the nutritional value of the herbicide is reduced.

It is possible to manually manipulate fatty acidic milk by the summer feeding on the beans (*Purchas et al., 2005*). Feeding on pasture increases CLA in milk, especially the presence of grass in the early stage of growth. It should be noted that in the Travnik CLA area was the second most representative of PUFA (immediately after C18: 2 n-6 acids) in sheep's milk. Lower CLA values in Livno's milk dairy can be due to an increased inflow of C18: 1 cis-9 degradation intermediate from buraga, in particular isomer C18: 1 trans-10. The content of these isomers was higher in milk of Livno's sheep, and it was found that they, and without diminishing desaturase activity in milk, could lead to lower CLA values in milk (*Marenjak et al., 2005*). Once milk is richer with n-3 FA and CLA than cow's milk, and one of the reasons may be that sheep are more often eaten by crap, while cows are more prone to pasture and are more fed with concentrates (*Marenjak et al., 2005*). Tables 1 and 2 also show the total quantities of SFA, MUFA, PUFA and UFA milk from Livno and Travnik. By examining the samples of sheep milk from the Livno and Travnik areas, statistically significant differences were found in most of the SFA and PUFA acids in the area and between the areas per sampling period. The fatty acids of MUFA in milk fat from the Livno and Travnik areas had statistically significant changes in the value during a different sampling period only in several cases. Despite the differences in the content of certain fatty acids between the sampling periods, the trend remained the same in both areas. The total share of SFA in milk from both areas was greater than the total share of MUFA and PUFA, but without statistical significance for the Travnik area as well as between the areas. Examination of the relationship between SFA / PUFA and statistically significant differences between SFA / MUFA, MUFA / PUFA, UFA / MUFA and UFA / PUFA was found in the samples of milk from the Livno area significant difference. Such SFA values from the Livno area were expected due to the predominantly found values of C14: 0, C16: 0 and C18: 0. Acid PUFAs fulfill many structural and functional roles that are incomparable among fatty acids due to the wide spectrum of biological processes (*Andrišić, 2013*). The majority of MUFA in the samples from both areas is C18: 1 cis-9 with its value was higher in samples from the Travnik area. UFA / MUFA in milk samples from Livno area was significantly statistically significantly different between sampling periods. No statistically significant difference was found in the samples of milk from the Travnik area of SFA, MUFA, PUFA and UFA, nor between fatty acid sums.

The fatty acid content of sheep's milk in this study showed a tendency of variation, both within and between sampling areas, and characterized by its relatively high content of saturated fatty acid (SFA) during the period of harvest. It is possible that the lactation period had a greater effect on SFA concentration than the breed type because the differences are particularly pronounced when comparing the values of fatty acids content between the different sampling periods within both examined areas.

Conclusion

The fatty acid content of sheep's milk in this study showed a tendency of variation, both within and between the sampling area, and characterized by relatively high content of SFA during the grazing period.It is possible that the lactation period had a greater effect on SFA concentration than the type of strain because the differences are particularly pronounced when comparing the values of fatty acids content between the different sampling periods within both examined areas. Sheep milk samples from both areas of dominant fatty acids were expected to be myristic, palmitic, stearic and oleic. Optimizing the diet and the source of fatty acids in animal foods can improve the fatty acid profile in milk.

The presence of VA in the rumen is a result of incomplete biohydrogenization, and changes in its content in milk fat can be the result of a change in the content of LNA in plants depending on the stage of vegetation and the difference in the length of the grazing period. In the milk samples from both areas, the LNA content had a downward trend towards the end of the lactation period. This can be a consequence of feeding or vegetation, as the content of LNA in plants decreases with the release of vegetation, and / or its more intense metabolism to DHA and EPA, whose share in milk for the Travnik area is growing towards the end of the lactation period. The content of DHA acid in milk from the area of Livno was lower in relation to milk from the area of Travnik, but without statistically significant differences.

Our research shows a trend in the decline in CLA content in milk going towards the end of lactation, and at the end of the gaseous period when the nutritional value of the plant cover decreases.

The content of total n-3 fatty acids in milk from the Livno region had a tendency to decline by the end of the lactation period, and n-6 fatty acids reversed the trend, and these differences between I and II sampling were statistically significant. The highest values of the total n-3 and n-6 fatty acid content for the Travnik area were determined, however, in the II period of sampling, but without statistically significant differences between the sampling period.

By examining the ratio of the sum of different classes of fatty acids in the milk samples from the Livno area, statistically significant differences were found between the sampling period for SFA / MUFA, MUFA / PUFA, UFA / MUFA and UFA / PUFA, with the exception of the SFA / PUFA ratio. In the milk from the Travnik region, the same relationships did not statistically significantly differ from the sampling period, possibly due to the more stable composition of the plant cover. Milk samples from the Travnik area contained more PUFA than milk from the area of Livno and a more favorable SFA / PUFA ratio.

Masno-kiselinski sastav ovčjeg mleka autohtone rase

Amina Hrkovic-Porobija, Aida Hodzic, Mensur Vegara, Husein Ohran, Almira Softic, Aida Kavazovic, Maja Varatanovic

Rezime

Istraživanjem je obuhvaćeno ukupno 127 uzoraka mleka ovaca iz dva različita područja (Livno i Travnik), u letnjem periodu hranjenja (juli, avgust i septembar). Životinje su obeležene odgovarajućim brojem ušnih markica, na temelju čega su uvek prikupljani uzorci od istih životinja kroz različita razdoblja. Masne kiseline u mleku određene su gasnom hromatografijom (GC), te je utvrđena sledeća masno-kiselinska kompozicija: buterna kiselina, kapronska kiselina, kaprilna kiselina, kaprinska kiselina, stearinska kiselina, oleinska kiselina, linoleinska kiselina, arahidonska kiselina, eikozapentaenska kiselina, dokozaheksaenska kiselina, rumenska kiselina. Sadržaj masnih kiselina ovčjeg mleka u ovoj studiji pokazao je tendenciju varijacije, kako unutar tako i između područja uzorkovanja, te je karakterističan po svom relativno visokom sadržaju zasićenih masnih kiselina (SFA) tokom razdoblja žetve.

Ključne riječi: mleko, masno-kiselinski sastav, ovce

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DETECTION OF ENROFLOXACINE RESIDUES BY MICROBIOLOGICAL SCREENING METHOD

Jelena Petrović[1], Brankica Kartalović[1], Radomir Ratajac[1], Jasna Prodanov Radulović[1], Igor Stojanov[1], Marina Žekić[1], Srdjan Stefanović[2]

[1]Scientific Veterinary Institute Novi Sad, Rumenacki put 20, Novi Sad, Serbia
[2]Institute of Meet Hygiene and Technology, Kacanskog 13, Belgrade, Serbia
Corresponding author: Jelena Petrović, jelena@niv.ns.ac.rs
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Abstract: The usage of microbiological screening tests is widespread in control of presence of antimicrobial drug residues in meat samples. Screening tests must be capable to detect antimicrobial drug residue of interest and detection limits must comply with MRL (Maximum Residue Limit). The aim of this study was to examine the performance of a microbiological screening test with *E. coli* as test microorganism: capability of detecting enrofloxacina and it's main metabolite ciprofloxacine at MRL levels in both fortified and incurred chicken tissue samples. Detection limits of microbiological screening test with *E. coli* was 50 ng/g for enrofloxacin and 25 ng/g for ciprofloxacin. Screening test had positive results in all samples of fortified and incurred meat with residue concentrations above MRL level. The results of this examinations shows that microbiological screening test with *E. coli*, as simple and cost effective test, is capable to detect enrofloxacine and it's metabolite ciprofloxacine in treated poultry at MRL level ie test is capable to detect unsafe poultry meat.

Key words: enrofloxacine, residue, microbiological screening test

Introduction

Antimicrobial drug treatment is widespread in food producing animals breeding. Meat for human consumption should be safe i.e. residues of antimicrobial drugs in meat should be below MRL (Maximum Residue Limit). Safety of poultry meat is very important in Serbia becasue this type of meat and poultry products are often consumed in Serbia (*Tolimir et al., 2016*)

Fluoroqunolone antimicrobial drugs are *semi*-synthetic antimicrobial agents. Fluoroquinolonesa are used both in human and veterinary medicine.

Enrofloxacin is fluoroquinolone antimicrobial developed exclusively for the use in veterinary medicine. Intensive poultry production, housing systems, management practices have influence on poultry health (*Lolly et al., 2013*). Common poultry infections, such as mycoplasmal infections, colibacilosis and pasteurelosis, frequently are treated with enrofloxacin (*Martinez et al., 2006*). Ciprofloxacin is the main methabolite of enrofloxacin and also has bactericidal activity, as parent compound is approved in human medicine.

The widespread use of fluoroquinolone compounds as therapeutic agents, particularly in intensive poultry production, has become a matter of great concern in recent years due to the identification of resistant *Campylobacter* and *Salmonella* strains in meat and possible transfer to humans via food chain (*Petrovic et al., 2008*). MRL values as sum of enrofloxacin and ciprofloxacin are 100 ng/g for chicken meat and 200 ng/g for liver are regulated by the Commission Regulation (EU) No 2377/2009 and ammending annexes. In Serbian legislation MRL values are defined as "quantities that can be proven by known or recognized methods", only for sulfonamide resudes MRL value is defined as 100 ng/g for meat (*Anonymous, 1992*).

Presence of antimicrobial drug in meat usualy is controled by screening methods and confirmation of residue (identification and quantification) in suspected samples is performed by physico-chemical methods (*EC, 2002*). There is broad range of methods for detection of fluoroquinolone drug residues: microbiological tests, ELISA, immunoasay and Biosensor tests as screening methods (*Song et al., 2015, Chen et al., 2017*). Microbiological screening methods are capable to detect broad spectrum of antimicrobial drug presence but these methods are not capable to identify and quantify the exact antimicrobial drug residue present in the sample. For confirmation more precise and reliable methods are used such as HPLC with ultraviolet and fluorescence detection (*Marschiello et al., 2001*), liquid chromatography tandem mass spectrometry (*Zhang et al., 2019*).

Screening methods must satisfy the following requirements: they must detect antibiotics of interest, detection limits must comply with the requirements (MRLs), they must be easy to perform and cost effective, test results are to be obtained rapidly, and the tests must be standardized (low variability within and between batches/laboratories) (*Chafer-Perices et al., 2010*). Microbiological inhibitory tests are widely used as a standard for screening purposes. The test principle is based on measurement of the inhibition zone, which presents the inhibition of multiplication of test microorganism in presence of antibiotics. These tests can serve as rapid tests as the result can be obtained within 24 hours (*Petrovic et al., 2008*). Sistematic control of residues is one of important steps which helps broiler production in Serbia to reach European standards. According to *Petrovic et al. (2012)* it is necessary to build efficient livestock production that can compete in the European market contributing to the growth of farmers and national income.

The aim of this study was to examine the performance of screening test microbiological method with *E. coli* as test microorganism: capability to detect enrofloxacin and ciprofloxacin at MRL levels in both fortified and incurred chicken tissue samples. LOD of diffusion method were determined in tissue samples fortified wih enrofloxacin and ciprofloxacin. Incurred samples were obtained in experimental design where chickens were treated with therapeutical doses of enrofloxacin. The presence of fluoroquinolones in breast muscle and liver was detected by microbiological inhibition test and HPLC method.

Material and methods

Chemicals and reagents

Enrofloxacin and ciprofloxacin analytical standards was purchased from Sigma Company, USA. In experiment was used preparation Enrocin® 10% ad us. vet. (Hemovet - Serbia and Montenegro), 1 ml of solution contains 100 mg enrofloxacin.

Microbiological method: test agar pH 8.0 was prepared in our laboratory (Caseine hydrolisate 2%, dextrose 0.4%, NaCl 1%, agar agar 1.6%). *Escherichia coli* NCIMB 11595 was used as test microorganism. Paper disks containing 0.003 ciprofloxacin µg/disk (Mast Diagnostic, Mereyseyside, UK) were used as positive control on each plate.

Liquid chromatography method with fluorescence detection: methanol, acetonitrile, n-hexane and phosphoric acid were purchased from J. T. Baker, Holland. All the solvents were of HPLC purity. Waters "Sunfire" column, C18, 150x4.6mm, 3.5µm particle size was used for separation at flow rate of 0.8 mL/min. Mobile phase was 0.01M phosphoric acid (pH 3)/acetonitrile; 80:20 v/v1-10. min and 60:40 - 10-20 min.

Determination of LOD – fortified samples

Detection limit of qualitative screening techniques must have a percentage of false negative results below 5% (ß error) at MRL value (Decision 2002/657/EC). The limit of detection (LOD) of the microbiological method was determined by the method recommended by *Reichmuth et al.*, (1997). Series of 7 concentrations of each antibiotic were analyzed in 12 replicates. Meat without antibiotics and meat fortified with 2-3 times higher concentration of antibiotics then expected limit of detection were used as negative and positive controls, respectively. Expected LOD was determined in preliminary examinations. Three different concentrations between the negative control sample and expected positive sample were analyzed. The following concentrations were examined (ng/g) 0.00, 0.78, 1.56, 3.12, 6.25,

12.50, 25.00, 50.00, 100.00, 200.00 and 400.00. The results are shown in the form of dose-response curve. For this examination LOD is defined as that concentration, where 95% of the results were evaluated positive. LOD was determined by plotting the line for 95% positive responses. The place where the line cuts the dose-response curve presents LOD.

Animals, drug and protocol of study – incurred samples

The study was performed on 65 healthy chickens (*Arbor Acres*); 1-day old chickens were included in the experiment. At the age of two weeks the chickens were randomly divided into two groups. Group A (30 animals) was the control group, which was not treated with antimicrobials. At the age of 28 days the chickens in group B (35 animals) were given daily doses of enrofloxacin (10 mg/kg bw/day), via drinking water, for five consecutive days.

The chickens were euthanized day before starting the therapy and during the withdrawal period. At each sampling three chickens were euthanized. The samples of breast muscle and liver were obtained. The samples were stored at -20^0C until assayed for the presence and concentrations of enrofloxacin and ciproflokacin.

Qualitative analysis: microbiological method

Test agar pH 8.0 was seeded with *Escherichia coli* NCIMB 11595. Working solution of *E. coli* was made of freshly prepared culture. The culture was diluted in peptone-salt solution to give optical density of 0.452 at 620 nm in a 10 mm cell, with the use of peptone-salt solution as a reference. Sterile Petri dishes were filled with inoculated test agar. All plates were subjected to a quality control. Paper discs containing 0.003 ciprofloxacin μg/disk were placed in the center of the Petri dish. Meat and liver were sampled while still frozen. An 8 mm diameter cork borer was used to remove a cylinder of frozen meat. The meat cylinders were cut into 2 mm thick discs. Four discs of meat were placed on opposite ends of the plate. Each sample was examined in 12 replicates. The plates were kept in refrigerator for 2 hours and than incubated on 37^0C for 24 h. After incubation the plates were inspected for inhibition zones around the meat discs and inhibition zones (IZ) for all 12 replicates were recorded (2 mm width was considered positive result).

Quantitative analysis – HPLC with fluorescence detection

Liquid chromatography method with fluorescence detection at excitation wavelength of 280 nm and emission wavelength of 455 nm was used for determination of enrofloxacin and ciprofloxacin residues in meat and liver (*Ramos*

et al., 2003). Detection limit was 10 ng/g and quantification limit was 20 ng/g. Enrofloxacin and ciprofloxacin were detected isocratically in 7-10 minutes. Quantification was performed using external standard method and the results were obtained from the calibration curve of blanks fortified at four levels.

Statistical analysis

Statistical analysis was performed using the Microsoft Office Excel 2000 and statistical software SPSS for Windows 8.0.0. Screening method data were analyzed by the use of descriptive statistic methods. Differences in IZ diameters were analyzed for statistical significance by the use of Student's t – test. The differences of $p<0.05$ were considered significant.

Results and discussion

Limit of detection (LOD) is the basic parameter in determining the test sensitivity. Test sensitivity is the probability of obtaining positive test result in truly positive samples. In a view of antimicrobial residue detection in food, a positive sample is the sample that contains residues at level above the MRL. This value is the basic parameter for sample assessment, since samples containing residues below MRL level are considered negative, i.e. safe. An ideal screening test would yield a LOD exactly at MRL level for each particular antimicrobial. However, performing of such tests is not always feasible in daily practice. Thus, the test is considered enough sensitive if the detection limit is at or below the MRL level, an never above the MRL. The LOD of a microbiological test depends of the innate sensitivity of the test bacterium, pH and thickness of growth medium (*Gaudin et al.*, 2010).

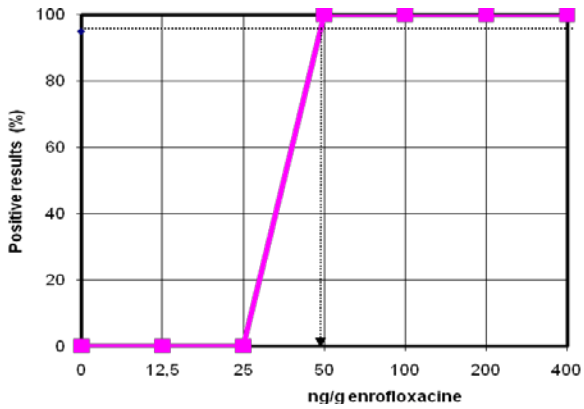

Figure 1. LOD of microbiological method for enrofloxacin

Figures 1 and 2 demonstrate the results of the examination of the microbiological method sensitivity to enrofloxacin and ciprofloxacin in the form of dose-response curve. Concentrations 0.78 - 25.00 ng/g of enrofloxacin did not have any positive response, while the concetrations 50 ng/g and above gave 100% positive responses. For this examination, LODs were defined as concentrations, where 95% of the results were evaluated as positive. LODs can be derived from figures 1 and 2 as 50 ng/g for enrofloxacin and 25ng/g for ciprofloxacin.

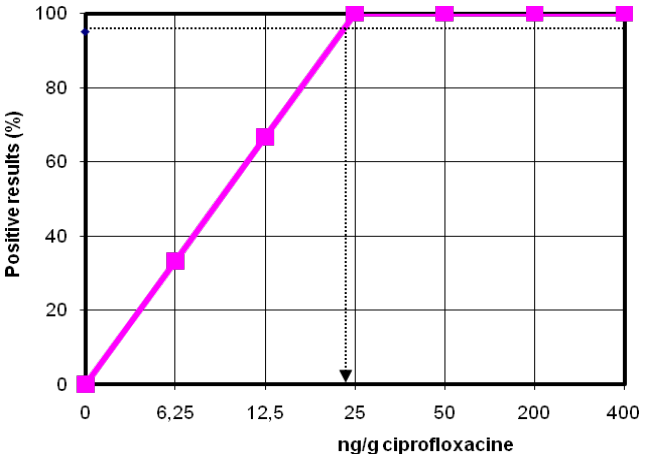

Figure 2. LOD of microbiological method for ciprofloxacin

The results obtained in this research corresponds to the reports of *Choi et al., (1999)* on detection limits for *E. coli* strain 128 ranging from 35 to 50 ng/g for enrofloxacin and 30 ng/g for ciprofloxacin. According to *Okerman et al., (1998)* detection limits of the pH6 plate *E. coli* ATCC 11303 were 50 ng/g for enrofloxacin and 30 ng/g for ciprofloxacin. In 2001, the same autors investigated sensitivity of another strain of *E. coli*- Bayer 14 and established detection limits of 150 ng/g and 30 ng/g for enrofloxacin and ciprofloxacin, respectively. Lower LOD was determined in milk compared to meat: in milk enrofloxacine and ciprofloxacine had LOD of 20ng/g and 10 ng/g respectively and in meat LOD was 200 ng/g. These results were obtained by STAR test, microbiological screening method with *E.coli* as test microorganism in pH8 plate *(Gaudin et al., 2010).* Sensitivity differences that occur in various authors are mainly related to diverse strains of *E. coli* as well as to differences with respect to test-design (nutritive medium, incubation temperature).

Examination of negative control samples did not revealed any false positive response. The established detection limit corresponds with MRL values for enrofloxacin and, ciprofloxacin in poultry meat and liver. Within MRL for examined fluoroquinolones microbiological inhibition method revealed 100% postive results. The demands of Serbian national regulative are also fulfilled, because residues could be detected at unsafe level.

After oral application, fluoroquinolones are well absorbed, distributed into tissues and excreted in urine and feces at high concentrations *(Prescott et al., 2000*). Enrofloxacin is metabolised in liver to main metabolite ciprofloxacin and some minor metabolites (*EMEA, 1998*). Breast muscle and liver samples from day 1 before dosing and day 1 and 4 of withdrawal period and day 1 post withdrawal were analyzed by the microbiological and HPLC method (Table 1).

Table 1. Determination of residues before and after enrofloxacine administration

Treatment day		Microbiological method (IZ in mm)							HPLC (ng/g)	
		x	SD	SE	Cv	Iv	t	% posit	Enro	Cipro
-1	M	0	-	-	-	-	-	0	0	0
	L	0	-	-	-	-		0	0	0
1 W	M	15.0	1.5	0.4	9.7	4.0	2.3*	100	580	50
	L	16.2	1.3	0.3	7.9	3.0		100	1200	820
4 W	M	1.4	-	-	-	9.0	-	16.7	30	<10
	L	8.0	0.9	0.2	2.4	3.0		100	50	70
1 PW	M	0.0	-	-	-	-	-	0	20	<10
	L	6.7	1.0	0.2	14.6	4.0		100	50	<10

M- meat; L- liver; -1- before therapy; W- withdrawal; PW -day after the end of withdrawal period; * – significant difference ($p < 0.05$), enro- enrofloxacin; cipro-ciprofloxacin; /- not examined; SD- standard deviation; SE- standard error; CV-coefficient of variation; Iv-interval of variation; t-t test value

During the withdrawal period, enrofloxacin and ciprofloxacin concentrations in breast muscle and liver exceeded the MRL values on day 1 of withdrawal period (Figure 3). Ciprofloxacin was not detected in muscle on day 4 of withdrawal period, but it was detected in liver in concentrations below MRL. During the withdrawal period, all muscle samples gave positive results in 100% microbiological method examinations on day 1, while on day 4 only 16.7% muscle samples were positive. One day after the end of withdrawal period, enrofloxacin was detected by HPLC method, i.e. in meat 20 ng/g and 50 ng/g in liver, while there was no ciprofloxacin. Similar results were obtained by *Schneider (2001)*, on day 3 of withdrawal period there was 38.2 ng/g of enrofloxacin and 0.9 ng/g

ciprofloxacin in meat, but in liver there were 142.0 ng/g of enrofloxacin and 51.0 ng/g of ciprofloxacin. More recent data from the same author, using lower doses of enrofloxacin (50 ng/g) for the same period, revealed the following: in meat there were 28.8 ng/g enrofloxacin and 0.0 ng/g of ciprofloxacin, and in liver 70.8 ng/g of enrofloxacin and 25.1 ng/g of ciprofloxacin *(Schneider and Donoghue, 2002)*. In the *EMEA (1998 a,b)* reports, three days after withdrawal period 42 ng/g of enrofloxacin were found in chicken liver.

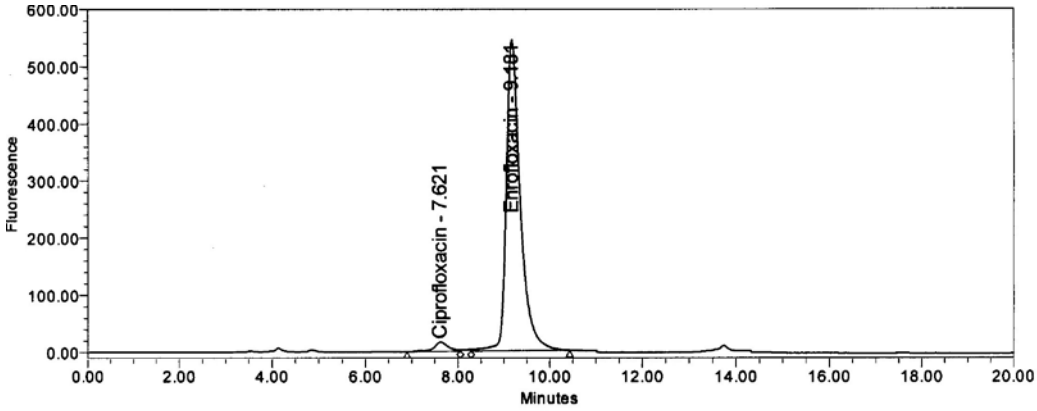

Figure 3. Chromatographic determination of enrofloxacin residues in chicken muscle on the first day of withdrawal period

A four-day withdrawal period for enrofloxacin allowed enough time to decrease drug concentration in meat and liver to an acceptable level prior to slaughter (below EU MRL).

Conclusion

The results of examining the residues in tissues of treated animals using screening microbiological method fulfill the demands for a qualitative method. Examining of treated animals using screening method gave positive results in all samples where the residues content was above MRL level.

Detekcija rezidua enrofloksacina primenom mikrobiološke skrining metode

Jelena Petrovic, Brankica Kartalović, Radomir Ratajac, Jasna Prodanov Radulović, Igor Stojanov, Marina Žekić, Srdjan Stefanovic

Rezime

Mikrobiološki skrining testovi se često koriste u kontroli prisustva rezidua antimikrobnih lekova u mesu. Skrining testovi moraju detektovati rezidue antimikrobnih lekova od interesa a granica detekcije mora biti u saglasnosti sa MDK rezidua (maksimalno dozvoljena koncentracija). Cilj rada je ispitivanje performansi mikrobiološkog skrining tesa sa *E. coli* kao test mikroorganizmom: mogućnost testa da detektuje enrofloksacin i njegov glavni metabolit u koncentracijama bliskim MDK vrednostima, u uzorcima u kojima su veštački dodati antimikrobni lekovi i u uzorcima dobijenim od tretiranih životinja. Granica detekcije mikrobiološkog skrining testa sa *E. coli* je 50 ng/g za enrofloksacin a 25 ng/g za ciprofloksacin. Skrining test je dao pozitivne rezultate u svim uzorcima u koje su veštački dodati enrofloksacin i ciprofloksacin, takođe i u uzorcima lečenih pilića u kojima je koncentracija rezidua bila znad MDK nivoa. Rezultati ovih ispitivanja pokazuju da je mikrobološki skrining test sa *E. coli* , kao jednostavan i finansijski isplativ test, može detektovati enrofloksacin i njegov glavni metabolit ciprofloksacin iznad MDK u jestivim tkivima lečenih pilića odnosno mogu detektovati meso koje nije bezbedno za ishranu.

Ključne reči: enrofloxacin, rezidue, mikrobiološki skrining test

Acknowledgement

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MYCOBIOTA AND AFLATOXIN B_1 IN POULTRY FEEDS

Vesna Krnjaja[1], Tanja Petrović[2], Slavica Stanković[3], Miloš Lukić[1], Zdenka Škrbić[1], Violeta Mandić[1], Zorica Bijelić[1]

[1]Institute for Animal Husbandry, Autoput 16, 11080, Belgrade-Zemun, Serbia
[2]Institute of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia
[3]Maize Research Institute "Zemun Polje", Slobodana Bajića 1, 11185, Belgrade-Zemun, Serbia
Corresponding author: vesnakrnjaja.izs@gmail.com
Original scientific paper

Abstract: In this study, a total of 30 poultry (chicken and laying hens) feed samples collected from different poultry farms in Serbia in 2016 were tested for fungal and aflatoxin contamination. Using the plate count and standard mycological methods, total fungal counts and potentially toxigenic fungal genera were determined. Natural occurrence of aflatoxin B_1 (AFB_1) was detected by ELISA (enzyme-linked immune sorbent assay) method.

The total fungal count was in the range from 1×10^2 (2 log CFU g^{-1}) to 1.83×10^5 CFU g^{-1} (5.26 log CFU g^{-1}). The majority of the chicken feeds (78.57%) had the total fungal count in the ranged from 1×10^2 to 4.8×10^4 CFU g^{-1}, whereas in 68.75% of the laying hens feeds it was ranged from 5.3×10^4 to 1.83×10^5 CFU g^{-1}. In 21.43% of the chicken feeds fungal contamination reached the level above the regulation limits. Three potentially toxigenic fungal genera, *Aspergillus*, *Fusarium,* and *Penicillium*, have been identified. In the tested poultry feed samples, more samples contaminated with *Aspergillus* were determined compared to samples contaminated by *Fusarium* and *Penicillium* species. The AFB_1 was detected in concentrations from 1.34 to 18.29 μg kg^{-1}, with an average of 4.47 and 4.56 μg kg^{-1} in the chicken and laying hens feed samples, respectively. In 14.29% of the chicken feeds, the level of AFB_1 was above the regulation limits.

The obtained results confirmed the importance of continuous mycological and mycotoxicological control of poultry feed, as well as need to improve risk assessments of such contaminants along the food chain.

Key words: poultry feed, total fungal count, aflatoxin B_1

Introduction

The majority of cereals (maize, wheat, barley, ray and oats) commonly used as poultry feed may be contaminated with toxigenic fungi, mainly from

genera *Aspergillus*, *Fusarium* and *Penicillium* which may produce poisonous secondary metabolites called mycotoxins. Thus mycotoxins can easily enter food chain via meat and meat products produced of animals fed with mycotoxin contaminated feed. Primarily the cereals contamination may appear in the field, where fungal spores are spread by the wind, rain, mechanical injuries or insects to the crops (*Aliyu et al., 2016*). The infection process can be further continued during the grain storage, due to the effect of abiotic and biotic factors (*Krnjaja et al., 2015*).

Aflatoxins (AFs) and ochratoxins have been the most common contaminants of poultry feed. Cereal kernels are a very suitable substrate for the development of *Aspergillus* species (*Fareed et al., 2014*). *Aspergillus flavus* and *A. parasiticus* are the main producers of aflatoxins. Among the different types of aflatoxins (B_1, B_2, G_1, G_2 and M_1), aflatoxin B_1 (AFB_1) has been the most toxic (*Babu et al., 2014*). Consumption of poultry feed contaminated with AFs causes aflatoxicosis in animals and a severe economic losses in the poultry production. Aflatoxins have negative impact on important poultry production parameters such as feed intake, feed conversion, weight gain, etc. An immune response in poultry can also be reduced, which raises the risk to diseases (*Fareed et al., 2014*).

In order to avoid harmful effects of AFs on animal health, the European community set maximum permissible levels for AFB_1 to 20 μg kg^{-1} for complete and complementary poultry feed (except for young animals). The regulation limits for feeds of young animals have been set to 10 μg AFB_1 kg^{-1} (for complete feed) and 5 μg AFB_1 kg^{-1} (for complementary feed) (EC, 2003). In Serbia, according to the Regulation on the quality of feedstuffs (*Službeni Glasnik RS, 4/2010, 113/2012, 27/2014, 25/2015, 39/2016, 54/2017*), the maximum permissible levels in complete and complementary feeding stuffs have been set to 20 μg AFB_1 kg^{-1} for adult poultry, and 5 μg AFB_1 kg^{-1} for young poultry.

Since mycotoxins are inevitable contaminants of cereals as a main constituent of poultry feeds, the aim of this study was to determine the fungal contamination and aflatoxin presence in the samples of poultry feed collected from different farms in Serbia. These investigations are important in order to highlight the importance of quality control along the food and feed chains.

Materials and Methods

In this study, the mycological and mycotoxicological evaluation of 30 poultry feed samples (14 of chicken and 16 of laying hens feed) was performed. The group of the tested chicken feed samples was used for the feeding of the broilers and pullets. The samples were complete or complementary feed mixtures, collected from different poultry farms in Serbia in 2016. The samples of about 1 kg were stored for 2-3 days at 4°C, prior to analysis. The moisture content was

determined using a laboratory moisture analyzer (OHAUS MB35, Parsippany, NJ, USA). The presence of fungal species was determined using the ISO 21527-2 method (2008).

Fungal species were identified according to fungal morphology and identification key of *Watanabe* (*2002*). The isolation frequency of potentially toxigenic fungi from genera *Aspergillus*, *Fusarium*, and *Penicillium* in the tested samples was calculated as the percentage of poultry feed samples contaminated with fungal species in relation to the total number of poultry feed samples.

The presence of aflatoxin B_1 (AFB_1) was detected by ELISA (enzyme-linked immune sorbent assay) method according to the manufacturer's instructions Celer Tecna® ELISA kits. The absorbance was determined at a wavelength of 450 nm on an ELISA plate reader spectrophotometer (Biotek EL x 800TM, Winooski, VT, USA). The lower and upper detection limits of AFB_1 were 1 μg kg^{-1} and 40 μg kg^{-1}, respectively.

The SPSS software (IBM, Statistic 20) was used for data comparison of the tested parameters. The significance levels were determined by t-test and Pearson correlation coefficient.

Results

Total fungal counts in the tested poultry samples are shown in Table 1. In Serbia, according to the Regulation on the quality of feedstuffs (*Službeni Glasnik RS, 4/2010, 113/2012, 27/2014, 25/2015, 39/2016, 54/2017*), the acceptable limits for fungal contamination in plant origin feed mixtures has been set to 200,000 CFU g^{-1} (for adult animals) and 50,000 CFU g^{-1} (for young animals). Following this regulation, in 21.43% (3/14) of the chicken feeds the values were exceeded the permitted limits (Table 1). The mean moisture contents were 10.57% and 10.62% in the chicken and laying hens feed samples, respectively.

Table 1. Level of fungal contamination of tested poultry feed samples

Values above and under regulations limits (CFU g^{-1})	Fungal counts		Fungal frequency (%)/Number of positive samples	
	Colony forming units per g of sample (CFU g^{-1})	log_{10}CFU g^{-1}	Chicken feed	Laying hens feed
> 200,000	0	-	0/0	0/0
> 50,000	$5.3 \times 10^4 – 1.83 \times 10^5$	4.72 – 5.26	21.43/3	68.75/11
< 50,000	$1 \times 10^2 – 4.8 \times 10^4$	2 – 4.68	78.57/11	31.25/5
Number of total samples			14	16

A significantly higher fungal count was found in the laying hens feeds than in the chiken feeds (Table 2).

Table 2. Statistical analyses of total fungal counts (log_{10}CFU g^{-1}) in tested poultry feed samples

Types of feed	Mean (log_{10}CFU g^{-1} ± S.D.)	Minimum (log_{10}CFU g^{-1})	Maximum (log_{10}CFU g^{-1})
Chicken feed	4.18b ± 0.83	2	4.96
Laying hens feed	4.90a ± 0.26	4.51	5.26
Level of significance	**	-	-

CFU g^{-1}, colony forming units per g of sample; **significant at P<0.01

The occurrence of potentially toxigenic fungal species from the *Aspergillus* genus was more common in the laying hens feeds (93.75% positive samples) than in the chicken feeds (85.71% positive samples). On average, the most number of *Aspergillus* spp. contaminated samples (89.73%) were established, followed by *Fusarium* spp. (79.47%) and *Penicillium* spp. (34.38%) contaminated samples (Table 3).

Table 3. The frequency of contaminated poultry feed samples with potentially toxigenic fungi from genera *Aspergillus*, *Fusarium* and *Penicillium*

Fungal genus	The frequency of fungal contaminated samples (%)		
	Chicken feed	Laying hens feed	Average
Aspergillus	85.71	93.75	89.73
Fusarium	71.43	87.50	79.47
Penicillium	50.00	18.75	34.38

Table 4 shows the frequency, ranges and average concentrations of AFB_1 occurence in the tested poultry feed samples. A higher percentage of positive AFB_1 samples was detected in the laying hens feeds (100%) then in the chicken feeds (85.71%). Average concentrations of AFB_1 investigated in the chicken and laying hens feeds were 4.47 and 4.56 μg kg^{-1}, respectively (Table 4). The level of AFB_1 which was above the regulation limit (5μg kg^{-1}) was recorded in two chicken feed samples (14.29%), whereas in all the laying hens feed samples, the levels of AFB_1 were under the permissible limit (20 μg kg^{-1}).

Table 4. Level of AFB_1 in tested poultry feed samples

Item	Aflatoxin B_1 (AFB_1)	
	Chicken feed	Laying hens feed
Number of positive samples/Number of total samples	12/14	16/16
Frequency %	85.71	100
Range (μg kg^{-1})	1.79 – 16.01	1.34 – 18.29
Average concentration in positive samples (μg kg^{-1})	4.47	4.56

According to data analyses, there was no significant positive correlations between the total fungal counts and the moisture contents (r = 0.39) and between the total fungal counts and the levels of AFB_1 (r = 0.41), while a statistically significant (P < 0.01) positive correlation was registered between the levels of AFB_1 and the moisture contents (r = 0.76) in the laying hens feeds. Further, there was no significant negative correlations between the total fungal counts and the moisture contents (r = – 0.15) and the levels of AFB_1 (r = – 0.24), while there was positive but not significant correlation between the levels of AFB_1 and the moisture contents (r = 0.31) in the chicken feeds.

Discussion

Fungi are ubiquitous plant pathogens that are common agents of foods and feedstuffs deterioration. Fungal and mycotoxin contamination of animal feed are the major threats to animal and human health worldwide.

In the tested poultry samples, the lower level of the total fungal counts was 1×10^2 whereas the highest level was 1.83×10^5 CFU g^{-1}. According to the Serbian Regulation the 21.43% of the chicken feeds exceeded the maximum permitted level set to provide food safety and quality assurance. The most of the samples were contaminated with *Aspergillus* spp. with average AFB_1 concentrations from 4.47 μg kg^{-1} in the chicken feeds to 4.56 μg kg^{-1} in the laying hens feeds. These results are similar to those of previous mycological investigations of poultry feed samples in Serbia (*Krnjaja et al., 2010*). However, according to the results of *Cegielska-Radziejewska et al. (2013)*, the fungal count was below 1×10^4 CFU g^{-1} in feeds for broilers collected in Poland in 2010, with *Aspergillus* and *Rhizopus* as the most common genera. The same authors observed that fungal contamination in poultry feeds from western Poland in 2010 was much lower than in the period of 2006-2008 which accounted 10^4–10^5 CFU g^{-1}. Additionally, in Argentina, *Monge et al. (2013)* established low values of total fungal counts (1×10^2) in pelleted poultry feed samples with relative high percentages (>40%) of *Aspergillus flavus* and *A. parasiticus* isolates. Similarily, total fungal count ranging from 10–10^6 CFU g^{-1}, and 43.5% of *Aspergillus* spp. isolates have been determined in poultry feed samples by *Greco et al.* (*2014*).

Feed ingredients such as cereals, sunflower, soybean, etc. are suitable for fungal development and mycotoxin contamination. The extreme high aflatoxin levels in maize crops has been recorded in Serbia during the summer of 2012 due to extreme high temperatures and low rainfalls which provoke the high incidence of *Aspergillus* species (*Kos et al., 2012, 2014; Lević et al., 2013; Krnjaja et al., 2013*). In the present study, 85.71% of the chicken feeds and 100% of the laying hens feeds were contaminated with AFB_1. There were 14.29% of the chicken feeds with unacceptable concentrations of AFB_1. Similarly, *Parvathi et al. (2017)*

reported that aflatoxins have been the most common contaminants in different poultry feeds and feed ingredients collected in India. Furthermore, in Pakistan, *Fareed et al. (2014)* reported a higher incidence and contamination levels of aflatoxins then ochratoxin A (OTA) in local poultry feeds and feed ingredients.

The growth of *Aspergillus* species and aflatoxin biosynthesis in cereals and other feed crops are conditioned with suitable environmental factors such as temperature and relative humidity (*Patel et al., 2015*). In addition, water activity (a_w) and temperature of cereal grains are the main factors that influence the fungal growth and mycotoxin synthesis (*Medina et al., 2017*). In warm and humid areas, *A. flavus* and *A. parasiticus* as the main producers of aflatoxins have been dominant species on maize ears. Optimal conditions for their growth are defined with temperature of 35°C and $a_w = 0.95$, whereas the higher value of water activity, $a_w = 0.99$ and temperature of 33°C are necessary for aflatoxin production (*Milani, 2013*). It has also been reported that physical factors, such as moisture, relative humidity, temperature, and mechanical damage are critical for mycotoxin production (*Bryden, 2012).* Higher values of moisture content (20-25%) provide convenient conditions for fungal infections of crops prior to harvest (*Magan 2006*).

Proper field management practice and use of resistant cereals cultivars are particularly important in mycotoxin control. In addition, during harvest, as a first stage in the cereals production chain, regular and accurate moisture and temperature determination becomes the dominant control measure in the prevention of mycotoxin synthesis. The excessive moisture along the cereal production chain has been the most critical factor affecting the growth and proliferation of fungi, which further increases the risk of feedstuffs mycotoxin contamination (*Kana et al., 2013*). In this study, even the mean moisture content of the tested poultry feeds was relatively low (<11%), toxigenic fungi and AFB_1 were recorded in the most of the samples, confirming that contamination may occur not only during harvest but also during pre-harvest, incorrect storage and transportation conditions or during poultry feed processing (*Binder et al., 2007*). The positive correlations between the moisture content and the total fungal count and the levels of AFB_1 were detected which was in accordance with the observations of *Greco et al. (2014).*

Conclusion

In conclusion, it can be emphasized that the tested poultry feed samples collected in 2016 were mainly contaminated with toxigenic species from the genus *Aspergillus*, followed by *Fusarium* and *Penicillium* genera. In 21.43% of the chicken poultry feeds, the fungal contamination was above the maximum permitted values. The high percentage of positive AFB_1 samples has been registered, whereas in 14.29% of the chicken poultry feeds, the AFB_1 level was also above the

regulation limit. These results confirm the necessity of continuous mycological and mycotoxicological control of feeds as the most important measure of control in feed and food safety strategy.

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Mikobiota i aflatoksin B_1 u hrani za živinu

Vesna Krnjaja, Tanja Petrović, Slavica Stanković, Miloš Lukić, Zdenka Škrbić, Violeta Mandić, Zorica Bijelić

Rezime

U ovom radu je 30 uzoraka hrane za živinu sakupljenih tokom 2016. godine iz različitih živinarskih farmi u Srbiji, ispitivano na prisustvo gljiva i aflatoksina u uzorku. Primenom metode razređenja i standardnih mikoloških metoda utvrđeni su ukupan broj gljiva i identifikovani su potencijalno toksigeni rodovi gljiva. Prirodna pojava aflatoksina B_1 (AFB_1) utvrđena je primenom biohemijske imunoadsorpcione metode (ELISA).

Ukupan broj gljiva bio je od 1×10^2 (2 log CFU g^{-1}) do $1{,}83 \times 10^5$ CFU g^{-1} (5.26 log CFU g^{-1}). Najveći broj uzoraka hrane za piliće (78,57%) imao je ukupan broj gljiva u rangu od 1×10^2 do $4{,}8 \times 10^4$ CFU g^{-1}, dok je 68,75% uzoraka hrane za nosilje imalo ukupan broj gljiva u rangu od $5{,}3 \times 10^4$ do $1{,}83 \times 10^5$ CFU g^{-1}. U 21,43% hrane za piliće ustanovljen je nedozvoljen ukupan broj gljiva. Identifikovana su tri potencijalno toksigena roda gljiva *Aspergillus*, *Fusarium* i *Penicillium*. Najveći broj ispitivanih uzoraka hrane za živinu bio je kontaminiran *Aspergillus* vrstama, u odnosu na *Fusarium* i *Penicillium* vrste koje su kontaminirale manji broj uzoraka. Rang sadržaja AFB_1 bio je od 1,34 do 18,29 μg kg^{-1}, sa prosečnim sadržajem od 4,47 μg kg^{-1} u uzorcima hrane za piliće, i 4,56 μg kg^{-1} u uzorcima hrane za nosilje. U 14,29% uzoraka hrane za piliće ustanovljen je nedozvoljen sadržaj AFB_1.

Dobijeni rezultati potvrđuju značaj stalne mikološke i mikotoksikološke kontrole hrane za živinu, kao i potrebu za usavršavanjem procene rizika od štetnih (gljivičnih) kontaminenata u lancu ishrane.

Ključne reči: hrana za živinu, ukupan broj gljiva, aflatoksin B_1

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CHEMICAL COMPOSITION *AND IN-VITRO DIGESTIBILITY* OF SUGARCANE BAGASSE AND RICE HUSK TREATED WITH THREE STRAINS OF WHITE ROT FUNGI AND EFFECTIVE MICROORGANISM

Regasa Begna[1], Mengistu Urge[2], Tegene Negesse[3], Getechewu Animut[4]

[1]Mizan Tepi University, Department of Animal Science, P.O.Box 260, MizanTeferi, Ethiopia
[2]Haramaya University, School of Animal and Range Science, P.O.Box 138, Dire Dawa, Ethiopia
[3]Hawassa University, School of Animal and Range Science, P.O.Box 5, Hwassa, Ethiopia
[4]Agriculture Transformation Agency, P.O.Box 708, Addis Ababa, Ethiopia
Corresponding author: Regasa Begna, begnaregasa@gmail.com
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Abstract: A study was conducted to evaluate the effect of biological treatments of sugarcane bagasse (SCB) and rice husk (RH) with three strains of white-rot fungi (WRF) (*Pleurotusostreatus (Po), Pleurotusflorida (Pf) and Trichodermaviride (Tv)* and effective microorganism (EM) on the chemical composition and in-vitro digestibility. The experiment consisted of 2x5 factorial arrangements, two levels of feed (SCB and RH) and five levels of biological treatments (Control, Po, Pf, Tv, and EM).Treatment of RH with EM, Tv, Po and Pf, significantly increased crude protein content from 7.90% in untreated to 7.92, 10.46, 10.61 and 11.35%, respectively. The corresponding increase in CP% of sugarcane from 2.61% was 3.41, 5.96, 5.89 and 5.95%.Treatments significantly ($P<0.001$) decreased neutral detergent fiber, acid detergent fiber, acid detergent lignin cellulose and hemicelluloses contents with the lowest value recorded for Tv. The IVOMD, IVDMD and metabolizable energy (ME) were significantly ($P<0.001$) increased. In conclusion, the study indicates that treatment of RH with *Trichodermaviride* and SCB with EM is more effective than others in improving the nutritive value of the roughages. We suggest evaluation of the treated roughages on animal performance.

Key words: *In-vitro* digestibility, white-rot fungi, effective microorganism, sugar cane bagasse, rice husk.

Introduction

Biological treatment employs microorganisms and their enzymatic machineries to break down lignin and alter lignocelulose structures. The use of white rot fungi (WRF) under solid-state fermentation (SSF) is the most promising biological treatment to mineralize lignin component to CO_2 and water in pure culture *(Isroi et al., 2011)*. Several studies during the last decades indicated that colonization of lignocelluloses agro-industrial by-product with white-rot fungi had positive impact on *in vitro* degradability *(Jalc, 2002; Tripathiet al., 2008)*. During SSF, the fiber fraction of feed such as NDF and ADF could be reduced, while the crude protein content increased. Studies also showed that dry matter is lost during SSF, but digestibility of ADF and NDF is improved. However, the improvement in nutritive value of lignocelluloses by WRF varies with feed type, fungal strains, temperature and fungal growing techniques *(Tripathi and Yadav, 1992)*. Among the white-rot fungi, *Trichoderma species* such as *Trichodermaviride* and *Trichodermareesie (Abdel-Azim et al., 2011),* and *Pleurotus species* such as *Pleurotusdjamor, Pleurotussajor-caju, Pleurotusostreatus and Pleurotus florid (Singh et al. 1990; Nasehi et al., 2013)* have efficiently reduced indigestible cell wall component and increased dry matter digestibility (DMD) of lignocelluloses. Effective microbes (EM) is another biological treatment method that has been utilized to improve the nutritive value of lignocelluloses by-products.

Despite the promising potential of biological treatment with WRF and availability of large volume of lignocellulose by-products that can be utilized as animal feed in Ethiopia, research has not been conducted to evaluate the effects of biological treatment on the nutritive value of these potential feed resources under the production and environmental scenario of the country. Hence, there is no information to advice producers and users concerning appropriate and efficient use of these resources as animal feed. Therefore, the present study was conducted to evaluate the effects of three strains of WRF and effective microbes on chemical compositions, *in-vitro,* and *in-sacco* rumen degradability of sugarcane bagasse and rice husk.

Materials and Methods

Chemical analysis was conducted at Haramaya University Animal Nutrition Laboratory and In-vitro digestibility was done at Holeta Research Center. Haramaya University is located at 9^{o} 26'N latitude, 42^{o}3' E longitudeand at about 1980 meters above sea level (m.a.s.l.). The mean annual rainfall of the study area is about 870 mm with a range of 560-1260 mm, and the mean maximum and minimum temperatures are 23.4^{o}C and 8.25^{o}C, respectively (Haramaya University

Meteorological Station). Whereas, Holeta Research Center is located at 9° 3' N latitude, 38° 30' E longitude and at about 2400 m.a.s.l. with mean minimum and maximum temperatures of 6.1°C and 22.2°C, respectively.

The sugarcane bagasses (SCB) was obtained from Wonji Sugar Factory and rice husk (RH) from Bench Maji Zone. Three strains of WRF, namely *Trichodermaviride, Pleurotusflorida and Pleurotusostreatus*–were obtained from plant protection section of the school of plant sciences of Haramaya University. The samples were kept under 4°Cuntil used. Each fungi were grown on a Petri dish containing potato dextrose agar (PDA) as nutrient for three days and the activated microorganisms were sub cultured on Petri dish (9 cm) containing 25ml potato dextrose agar (PDA) for another seven days. The slant culture samples were used to inoculate the spawn flasks. At this stage, the fungal strains were cultured in a media containing: 4% molasses, 0.4% urea, 0.2% KH_2PO4 and 0.03% MgSO4 ($7H_2O$) per one liter of water. Two hundred ml of this media was incubated in 500 ml sterilized conical flask contains 100g ground waste. The flasks were sterilized (121°C for 15 minutes), cooled and inoculated with the prepared inoculums and incubated at 25°C for seven days to prepare spawn. The spawn was used to inoculate polyethylene bag containing 500 g of sugar cane bagasse and rice husk moisten with 500 ml of fungal medium (w/v) and distributed in polyethylene bag in four replications. Each polyethylene bag was inoculated with 10 % (w/w) spawns of *Trichodermaviride, Pleurotusflorida and Pleurotusostreatus (Jahromi et al., 2010).* A control sample was treated with the above mentioned media without any fungal strain. The control and treated materials were adjusted with media to approximately 60% humidity and was incubated at room temperature for 21 days. Then after, the control and fungal treated feeds were dried in an oven to a constant weight in order to stop fungi growth.

Adequate quantities of an inert form of EM (EM-1) packed in plastic bottles was purchased from Weljijie PLC (Debrezeit). Molasses was added and mixed with EM at equal proportion in order to initiate the microbial (EM) activity such as multiplication and metabolism activities and diluted with a mixture of chlorine free water and molasses (18:1 ratio per liter) *(Higa and Wididana, 2007).* After stirring, the mixture was sprayed over the SCB and RH until they achieve moisture content of 60%. After preparation of the cultures, five hundred gram of treated sugarcane bagasse and rice husk were packed in airtight polyethylene bag each in four replications and incubated at room temperature for21 days. Then, the samples were removed from the bag, dried in an oven at 60°C for 48 hours and used for determination of chemical compositions, *in-sacco* and *in-vitro* degradability of the treated feeds.

A 2*5 factorial treatment arrangements with 4 replications were used to study the effect of feed type and treatment method. Samples of SCB and RH treated with three white rot fungi (*Trichodermaviride Pleurotusflorida,*

Pleurotusostreatus) and essential microbes. Hence, the treatment combination consisted of eight treated and two untreated feed samples (Table 1).

Table 1.Treatment combination and layout for chemical composition and *in vitro* digestibility of treated sugar cane bagasse and rice husk

Treatment	Fungal type					Feed type		Replic
	UT	**EM**	**Tv**	**Pf**	**Po**	**SCB**	**RH**	
T1	+					+		4
T2		+				+		4
T3			+			+		4
T4				+		+		4
T5					+	+		4
T6	+						+	4
T7		+					+	4
T8			+				+	4
T9				+			+	4
T10					+		+	4

UT=Untreated;EM=Effective microbes; Tv= Trichodermaviride;Pf=Pleurotusflorida;Po=Pleurotusostreatus; SCB = Sugarcane bagasse; RH = Rice husk.

One half of the sample was ground to pass 1mm sieve size Wiley mill and used for chemical analysis. The DM and ash contents of the feed samples were determined following *AOAC (2002).* The NDF, ADF and ADL were determined based on the method described by *VanSoest and Robertson (1985).* Hemicelluloses and cellulose were calculated as NDF-ADF and ADF-ADL, respectively. The N content of the samples was determined by the micro-Kjeldahl method and CP was calculated as N X 6.25. Metabolizable energy per kilogram was determined indirectly by conversion factors from its *in-vitro* organic matter digestibility *(MAFF, 1984)* as ME (Mcal/kg DM) = 0.16*IVOMD.

The samples used for *in vitro* digestibility test was ground to pass through 1mm sieve size Wiley mill, labeled and transported to animal nutrition laboratory of Holeta Agricultural Research Center. The *In vitro* digestibility was determined according to *Tilley and Terry (1963)* two stage techniques for *in vitro* digestion of forage crops, as modified by *Van Soest and Robertson (1985)*, where a second stage (HCL-pepsindigestion) was substituted by neutral detergent extraction to simulate true digestibility.

The results of feed sample chemical assay and *in vitro* digestibility of feeds were analyzed by using SAS software version 9.1 *(SAS, 2008).* When there was significant difference between means, the mean separation was made by

adjusting with Tukey honestly significant difference test. The model employed for the analysis is described below: Model: yijk=μ +ai + bj + a*bij+ εijk Where: Yijk= the dependent variables, μ=overall mean; ai= theith feed type; bj= thejth treatment method, a*b= The ijth interaction (between feed type and treatment method) εijk=random error.

Results

The chemical composition was significantly affected ($p<0.01$) by the interaction of feed and treatment types (Table 2). Biological treatments significantly($p<0.01$) decreased organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) of the RH and SCB as compared to untreated samples. However, the treatments were significantly ($p<0.01$) improved crude protein (CP) content as compared to untreated samples. The crude protein content of sugarcane bagasse and rice husk was significantly ($p<0.001$) improved in all treatments as compared to the untreated sample, except for rice husk treated with EM. The treatments raised CP content from 7.90% in untreated to 10.5%, 10.5% and 11.35% for RH treated with T*v*, Po and Pf, respectively while the CP content of sugar cane bagasse increased from 2.61% of untreated to 3.5%, 5.96%, 5.89% and 5.95% for sugarcane treated with EM, T*v*, Po and Pf, respectively. The interaction show that increase in CP content of RH was higher when treated with Pf than the EM and the other WRF, while the improvement in CP content of SCB treated with all WRF is similar and higher than for EM treated SCB.

Table 2. Chemical composition (%DM) of sugarcane bagasse and rice husk as affected by treatment with white-rot fungi and effective microorganisms

Factors	Levels	DM	OM	ASH	CP	NDF	ADF	ADL
Feed	RH	91.94^{b}	80.24^{b}	19.76^{a}	9.47^{a}	58.34^{b}	38.44^{b}	13.59^{b}
	SCB	94.22^{a}	91.76^{a}	8.24^{b}	4.76^{b}	81.74^{a}	59.89^{a}	14.35^{a}
	SEM	0.12	0.03	0.028	0.044	0.07	0.10	0.05
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Treatments	U	93.97^{a}	87.12^{a}	12.88^{c}	5.26^{c}	76.35^{a}	53.65^{a}	14.60^{a}
	EM	93.92^{a}	86.94^{a}	13.06^{c}	5.21^{c}	71.54^{b}	48.47^{b}	14.31^{a}
	Pf	92.38^{b}	85.10^{c}	14.90^{a}	8.65^{a}	67.21^{c}	48.65^{b}	14.14^{b}
	Po	92.54^{b}	85.34^{b}	14.66^{b}	8.25^{b}	67.38^{c}	48.25^{b}	13.68^{c}
	Tv	92.54^{b}	85.50^{b}	14.50^{b}	8.20^{b}	67.72^{c}	46.79^{c}	13.13^{d}
	SEM	0.22	0.048	0.047	0.077	0.12	0.17	0.09
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Feed * Treatments	RH,U	93.16^{b}	80.89^{e}	19.11^{c}	7.90^{c}	65.82^{d}	44.39^{d}	14.08^{bc}
	RH,EM	92.53^{c}	80.98^{e}	19.02^{c}	7.92^{c}	59.62^{e}	38.61^{e}	14.37^{b}
	RH,Pf	90.88^{d}	79.50^{g}	20.50^{a}	11.34^{a}	55.30^{f}	37.11^{f}	13.61^{cd}
	RH,Po	90.98^{d}	79.90^{f}	20.10^{b}	10.61^{b}	55.30^{f}	36.99^{f}	13.24^{de}
	RH,Tv	91.12^{d}	79.93^{f}	20.07^{b}	10.45^{b}	55.68^{f}	35.12^{g}	12.66^{e}
	SCB,U	94.79^{a}	93.35^{a}	6.65^{g}	2.61^{g}	86.88^{a}	62.92^{a}	15.12^{a}
	SCB,EM	92.31^{c}	92.90^{b}	7.10^{f}	3.41^{f}	83.46^{b}	58.33^{c}	14.25^{b}
	SCB,Pf	93.88^{b}	90.69^{d}	9.31^{d}	5.95^{e}	79.12^{c}	60.20^{b}	14.67^{ab}
	SCB,Po	94.11^{b}	90.78^{cd}	9.22^{de}	5.89^{e}	79.46^{c}	59.52^{bc}	14.13^{bc}
	SCB,Tv	94.03^{b}	91.07^{c}	8.93^{e}	5.96^{e}	79.76^{c}	58.46^{c}	13.59^{cd}
	SEM	0.30	0.068	0.068	0.11	0.17	0.23	0.13
	P.value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a-g}$LSMeans with different superscripts within the same column and factor are significantly different at P<0.01; SEM= Standard Error Mean;RH=Rice husk; SCB= Sugarcane bagasse; U=Untreated; EM= effective microorganism; Po=Pleurotusostreatus; Pf.=Pleurotusflorida; Tv.=Trichodermaviride; RHU= untreated Rice husk; RHPo.= Rice husk treated with Pleurotusostreatus; RHPf.= Rice husk treated withPleurotusflorida; RHTv.=Rice husk treated with Trichodermaviride; RHEM =Rice husk treated witheffective microorganism; SCBU= untreated Sugarcane bagasse; SCBPo.= Sugarcane bagasse treated with Pleurotusostreatus; SCBPf.= Sugarcane bagasse treated with Pleurotusflorida; SCBTv.= Sugarcane bagasse treated with Trichodermaviride; SCBEM = Sugarcane bagasse treated with effective microorganism; DM=Dry matter; OM=Organic matter; CP=Crude protein; NDF=Neutral detergent fiber; ADF= Acid detergent fiber; ADL=Acid detergent lignin.

In-vitro digestibility, cellulose, hemicelluloses, and Metabolizable energy content of treatments are significantly ($p<0.01$) affected by interaction of feed and biological treatment types (Table 3). Biological treatments significantly ($p<0.01$) reduced cellulose and hemicelluloses content of RH and SCB, while *in-vitro* organic matter digestibility, dry matter digestibility and Metabolizable energy content were significantly ($p<0.01$) increased as compared to untreated RH and SCB. Large decrease in cellulose and hemicelluloses content of RH and SCB occurred when treated with Tv and EM, respectively. Similarly, large improvement in IVOMD of RH and SCB were recorded when treated with Tv and EM, respectively. Improved IVOMD resulted in leads increased Metabolizable energy from 4.5MJ/kg in untreated to 7.5MJ/kg in Tv. treated RH and from 4MJ/kg in untreated to 6MJ/kg in EM treated SCB.

Table 3. Cellulose, hemicelluloses, *invitro* digestibility and Metabolizable Energy content of sugar cane bagasse and rice husk treated with white rot fungi and essential microbes

Factors	Levels	Cellulose	Hemi-Cellulose	IVDMD	IVOMD	ME MJ/Kg DM
Feed	RH	24.85^{b}	19.90^{b}	36.01^{a}	37.59^{a}	6.01^{a}
	SCB	45.53^{a}	21.85^{a}	29.27^{b}	30.59^{b}	4.89^{b}
	SEM	0.12	0.08	0.12	0.11	0.02
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001
Treatments	U	39.05^{a}	22.69^{a}	27.53^{d}	28.73^{d}	4.59^{a}
	EM	34.16^{cb}	23.07^{a}	35.35^{b}	36.88^{b}	5.9^{b}
	Pf	34.51^{cb}	18.56^{c}	31.65^{c}	33.17^{c}	5.3^{c}
	Po	34.57^{b}	19.12^{c}	31.8^{c}	33.11^{c}	5.29^{c}
	Tv	33.66c	20.93^{b}	36.88^{a}	38.57^{a}	6.17^{a}
	SEM	0.2	0.13	0.2	0.19	0.03
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001
Feed*Treatments	RH,U	30.31^{c}	21.43^{c}	26.89^{ef}	28.09^{ef}	4.49^{ef}
	RH, EM	24.24^{d}	21.01^{c}	35.74^{c}	37.14^{c}	5.94^{c}
	RH ,Pf	23.5^{de}	18.19^{e}	37.42^{b}	39.35^{b}	6.3^{b}
	RH, Po	23.75^{de}	18.32^{cd}	35.09^{c}	36.39^{c}	5.82^{c}
	RH ,Tv	22.46^{e}	20.56^{c}	44.94^{a}	47.02^{a}	7.52^{a}
	SCB,U	47.80^{a}	25.13^{a}	28.17^{de}	29.36^{de}	4.7^{de}
	SCB,EM	44.08^{b}	24.96^{a}	34.97^{c}	36.64^{c}	5.86^{c}
	SCB, Pf	45.52^{b}	18.93^{e}	25.88^{f}	26.99^{f}	4.32^{f}
	SCB, Po	45.39^{b}	19.94^{e}	28.53^{d}	29.84^{d}	4.77^{d}
	SCB,Tv	44.87^{b}	21.30^{c}	28.83^{d}	30.13^{d}	4.82^{d}
	SEM	0.28	0.19	0.28	0.27	0.04
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a-g}$LSMeans with different superscripts within in the same column and factor are significantly different at $P<0.05$. SEM= Standard Error Mean, RH=Rice husk, SCB= Sugarcane bagasse, U=Untreated EM= effective microorganism, Po=Pleurotusostreatus, Pf.=Pleurotusflorida, Tv.=Trichodermaviride, RHU= untreated Rice husk; RHPo.= Rice husktreated withPleurotusostreatus; RHPf.= Rice husk treated withPleurotusflorida; RHTv.=Rice husk treated withTrichodermaviride; RHEM =Rice husk treated

witheffective microorganism; SCBU= untreated sugarcane bagasse; SCBPo.= Sugarcane bagassetreated withPleurotusostreatus; SCBPf.= Sugarcane bagassetreated withPleurotusflorida; SCBTv.= Sugarcane bagassetreated withTrichodermaviride; SCBEM = Sugarcane bagassetreated witheffective microorganism; IVOMD=In-vitro organic matter digestibility, IVDMD= In-vitro dry matter digestibility, ME= Metabolizable energy.

Discussion

The biological treatment on average reduced ADF, NDF and ADL from 55.5 to 47%, 79 to 67 % and 14.5 to 13%, respectively, which was significant among treatments with the lowest cell wall components recorded for feed treated with *Trichodermaviride.* Similar to the result of the present study, *Hassan et al. (2015)* noted that treatment of rice husk with *P. ostreatus* decreased the cell wall components as compared to untreated. *Salman et al. (2011)* reported that treatment of sugarcane bagasse with fungi, yeast and bacteria decreased the cell wall components. *Abdel-Azim et al. (2011)* stated that treatment of rice straw and corn stalks by *Trichodermaviride* decreased NDF and ADF. Similarly, *Baraghit et al. (2009)* reported that biological treatments with different fungal and bacteria strains decreased cell wall constituents of different crop residues. The decrease in cell wall components might be due to the breakdown of lignocelluloses bonds resulting into hydrolysis of cellulose by fungi and bacteria *(El-Ashryet al., 2002; El-Shafie et al., 2007; Fayed et al., 2009 and Mahrous et al., 2010). Fazaeli et al. (2004)* noted that fungi treatment solubilize and utilize the cell wall components as carbon source and thus change the ratio of insoluble to soluble carbohydrates in the by-products. The result of treatment inoculated with EM agree with the finding of *Yonatan (2010)* who reported that treatment of coffee husk with EM decreased the cell wall components as compared to the untreated husk. Similarly, *Mullgeta (2015)*noted inoculating crop residues with EM reduced cell wall components as compared to untreated crop residues.

The CP content of feed samples on average increased from 4.61 to 8.42%with highest value recorded in feed treated with white rot fungi species. This result is in agreement with *Shoukry et al. (1985)* who noted that treatment of sugarcane bagasse with different microorganisms leads to increased CP and ash content as compared to the untreated treatment. *Nasehi et al.(2013)* noted that fermentation of barley and wheat straw with *Pleurotusflorida* decreased the cell wall components and increased the CP content. *Yonatan (2010) and Mulgeta (2015)* reported that EM treatment improve the CP content of roughage as compared to untreated. The increased crude protein content when feed is treated with biological media is attributed to the growth and production of mycelium *(Ragunathan et al., 1996).* Mycelium relatively contain high protein, hence it is expected that the treated by-products containing fungal mycelium to have a higher

concentration of CP. There is secretion of certain proteineous extra cellular enzymes into the waste during breakdown and their subsequent metabolism *(Kadiri, 1999; Akinfemiet al., 2009),* which also adds to the protein content of the treated feed. Moreover, the increased CP could be due to the capture of excess nitrogen by fermentation *(Sallam et al., 2007).* Increased CP content in general suggests that the treated feed could be a good source of protein for livestock.

Significantly higher improvement in IVOMD, IVDMD and ME were recorded for feed treated with *Trichodermaviride* followed by EM, *Pleurotusostreatus* and Pf. The higher digestibility of lignocelluloses by-products treated with WRF and EM could mainly be attributed to lower cell wall components and higher CP content of biological treated by-products due to the action of microorganisms during fermentation. *Surinder and Suman(1986)* reported that *Pleurotusostreatus* and *S. pubverulentum* used as biological treatment of paddy straw increased IVDMD. *Shah and Rehman (1988)* noticed increased IVDMD when cotton seed hulls were fermented by *Bacillus polymexa* and *Trichodermaviride* as compared to unfermented. *Bassuny et al. (2003)* found that IVDMD and IVOMD ofrice and bean straw treated with biological treatment were significantly improved compared to the control. *Yonatan (2010)* noted that treatment of coffee husk with EM improve the IVOMD. Similarly, *Mulgeta (2015)* reported that treatments of different crop residues resulted in to improvement of IVOMD as compared to untreated roughages.

Conclusion

The results of the experiment indicate that biological treatments can improve the nutritive value of rice husk and sugarcane bagasse through decreasing cell wall content, improving percent crude protein and *in-vitro* dry matter digestibility. The results of fermentation characteristics of treatments suggested that the best biological treatment is obtained from feed treated with EM. The best result of IVOMD, IVDMD and ME were achieved for rice husk treated with Tv. and sugarcane bagasse treated with EM. The overall result implies that biologically treated sugarcane bagasse and rice husk can be incorporated into other ruminants' diet for better productivity. We also suggest *in-vivo* metabolism and feeding trial study on Tv. and EM treated rice husk and sugarcane bagasse for more complete information.

Hemijski sastav i *in-vitro* svarljivost šećerne trske i ljuske pirinča tretirane sa tri soja gljivica bele truleži i delotvornim mikroorganizmima

Regasa Begna, Mengistu Urge, Tegene Negesse, Getechewu Animut

Rezime

Sprovedena je studija za procenu uticaja biološkog tretmana šećerne trske (SCB) i pirinčane ljuske (RH) sa tri soja gljivice bele truleži (VRF) (*Pleurotusostreatus* (Po), *Pleurotusflorida* (Pf) i *Trichodermaviride* (Tv)) i efikasnim mikroorganizmima (EM) na hemijski sastav i in-vitro svarljivost. Eksperiment se sastojao od 2x5 faktorijalnog ogleda, dva nivoa hraniva (SCB i RH) i pet nivoa bioloških tretmana (kontrola, Po, Pf, Tv i EM). Tretman RH sa EM, Tv, Po i Pf, značajno povećava sadržaj sirovog proteina od 7,90% u netretiranom do 7,92; 10,46; 10,61 i 11,35%, respektivno. Odgovarajući porast sadržaja sirovog proteina šećerne trske od 2,61% bio je 3,41; 5,96; 5,89 i 5,95%. Tretmani su značajno (P <0,001) smanjili NDF, ADF, ADLC i hemicelulozu sa najnižom vrednošću evidentiranom za tretman Tv. IVOMD, IVDMD i metabolička energija (ME) su bile značajno (P <0,001) povećane. U zaključku, studija ukazuje da je tretman RH sa *Trichodermaviride* i SCB sa EM efikasniji od drugih u poboljšanju nutritivne vrednosti krmiva. Predlažemo procenu tretiranih krmiva prema proizvodnim performansama realizovanim u stočarskoj proizvodnji.

Ključne reči: *In-vitro* svarljivost, gljivice bele truleži, efikasan mikroorganizam, šećerna trska, pirinač

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BACTERIAL INOCULANT EFFECT ON QUALITY OF ALFALFA SILAGE AND HAYLAGE

Snežana Đorđević[1], Violeta Mandić[2*], Nikola Đorđević[3], Biljana Pavlović[3]

[1]Agrounik d.o.o., Research and Development Center, Belgrade, Republic of Serbia
[2]Institute for Animal Husbandry Belgrade-Zemun, Belgrade, Republic of Serbia
[3]Biounik d.o.o., Research and Development Center, Šimanovci, Republic of Serbia
[*]Corresponding author: violeta_randjelovic@yahoo.com
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Abstract: Using of silage and haylage of forage legumes in ruminant nutrition and promotion of promoting proper forage conservation techniques should be an important strategy in livestock production in our country. Forage legumes are difficult to ensile, so it is necessary to apply the starter culture of selected strains of lactic acid bacteria that support the ensiling process and prevent bacterial butyric fermentation and thus contribute to the preservation and improvement of silage and haylage quality. In this paper, the influence of bacterial inoculant 'Silko for alfalfa' on the quality of silage and haylage of alfalfa in two separate trials is presented. The inoculant is a combination of homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. The first-cut alfalfa in the second year was used for silage and haylage. The silage was examined in mini-silos in the laboratory, and the haylage at the cattle farm where the plant material was cuts were collected in experimental silo bags. The treatments were control (untreated silage, i.e. haylage) and silage, i.e. haylage treated with inoculant 'Silko for alfalfa' (rate of 5 ml t^{-1} fresh material). The silages were analyzed after 90 days, and haylage after 40. The inoculant 'Silko for alfalfa' has been found to maintain the nutritive value of silage and haylage and to improve their chemical, energy and fermentation parameters relative to the control. Since 'Silko for alfalfa' positively affects the correct lactic acid fermentation of silage and haylage and contributes to a lesser loss of nutritional value and energy it is expected that it can promote a high level of productivity of ruminants, and thus contribute to the growth of profit in livestock production.

Key words: alfalfa, bacterial inoculant, haylage, silage, quality

Introduction

Profitability of dairy and fattening cattle farms depends significantly on the preparation and use of high quality silage and haylage in combination with a concentrate. In Serbia, legume hay is a widespread form of feed, while silage and haylage are less represented. In our country silage and haylage are used in season when there is no plant production, that is, in the winter and in the early spring when there is no production of feed in the fields, meadows and pastures. Alfalfa is a useful source of protein essential for the nutrition of domestic animals, especially ruminants with high levels of lysine and tryptophan, which are deficient in maize. It is rich in vitamins, primarily carotene and has a high content of mineral substances, especially calcium. Silage and haylage of alfalfa are a very important for combining with maize. In addition to high nutritional value, alfalfa is a very economical plant species due to high yields of green mass and hay, which in the second year of life with 3-4 cuts can be up to 100 t ha^{-1} of green mass and 20 t ha^{-1} hay.

However, alfalfa is difficult to ensile due to the low content of water-soluble carbohydrates and moisture and high buffering capacity of the fresh mass. Because it does not contain the required sugar minimum for successful lactic acid fermentation, it is necessary to apply chemical or bacterial inoculants in the conservation of alfalfa *(Repetto et al., 2011)*. Otherwise, untreated silage accelerates the activity of *Clostridium butyricum* that uses existing sugars for its activity which leads to produce small amounts of lactic acid and increase in the content of butyric acid and intense degradation of proteins and amino acids *(Pys et al., 2002)*. Epiphytic lactic acid bacteria (<1% microflora) are not sufficient for rapid stimulation of the ensiling process, so it is necessary to apply the starter culture of selected strains of lactic acid bacteria that support the ensiling process and prevent the loss of dry matter and butyric fermentation *(Schmidt et al., 2009)*. Various bacterial inoculants that contain homofermentative microorganisms in a monoculture or a combination of several species, strains of homofermentative and heterofermentative bacteria and bacterial-enzymatic additives are available on the world market *(Đorđević et al., 2009)*. Lactic acid bacteria comprise a heterogeneous group of Gram-positive, non-spore forming, catalase negative microorganisms that synthesize lactic acid as the main product of fermentative metabolism. They belong to the genera *Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc (Pahlow et al., 2003)*. *Jatkauskas et al. (2013)* point out that the use of bacterial and bacterial-enzymatic inoculants is necessary in the ensiling of alfalfa, grass-clover mixtures, grasses and maize as they contribute to faster decrease in pH, inhibit growth of harmful microorganisms, prevent loss of dry matter and increase the aerobic stability of silage. *Kizilsimsek et al. (2007)* find that alfalfa silage treated with a combination of three

homofermentative bacteria *Lactobacillus lactis*, *Lactobacillus plantarum* L-54 and *Lactobacillus plantarum* Aber F1 show higher lactic acid content and lower acetic acid, ethanol and ammonium nitrogen content compared to untreated silage. *Zielińska et al. (2015)* find in the alfalfa silages tretated with *Lactobacillus plantarum* K KKP 593p, *Lactobacillus plantarum* C KKP 788p, *Lactobacillus buchneri* KKP 907p and a mixture of all three strains, a smaller total number of molds, *Clostridium perfringens* and *Listeria* spp. and improved fermentation characteristics, quality and aerobic stability in relation to control silage. *Silva et al. (2016)* recommend *Pediococcus pentosaceus* strain 6.16 to inoculate the alfalfa silage as it rapidly reduces pH and increases the concentration of lactic acid. Selected strains of lactic acid bacteria in inoculants, in addition to improving the quality and aerobic stability of silage, also have probiotic activity in the digestive tract of animals *(Holzer et al., 2003)*. Thus, *Han et al. (2014)* conclude that inoculated silage may be a potential agent for the transmission of probiotics because in faeces of cow several species of lactic acid bacteria have been found which have been applied with inoculants in silage. Similarly, silage inoculants increase appetite and daily consumption in animals, thereby contributing to the increase in milk or meat production, higher total gain, lower feed conversion ratio, and low production costs *(Merry et al., 1993)*.

Forage legumes are difficult to ensile because they have low content of soluble carbohydrates and high buffering capacity. Different types of chemical and bacterial additives for ensiling of forage legumes have been developed in the world. 'Silko for alfalfa' is a homemade product intended for silage and haylage of forage legumes. The aim of this research was to examine the influence of inoculant 'Silko for alfalfa' on the quality of silage and haylage of alfalfa in two separate experiments.

Materials and Methods

Bacterial inoculant

Bacterial inoculant 'Silko for alfalfa' is a combination of homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. which are isolated from maize rizosphere and then identified according to the instructions given in the Bergey's Manual of determinative bacteriology (*Bergey, 2009*). The genotypic identification of bacteria was performed by sequencing the 16S rRNA by PCR method, and the determination of the strain by computer analysis in the Basic Local Aligment Search Tool where the obtained nucleotide sequence with the sequences available in the GenBank database, or NCBI (National Center for Biotechnology Information, Nacional Institutes - http://vvv.ncbi.nlm.nih.gov). Species that are contained in the inoculant are defined. The number of colony forming units in inoculant is $1x10^{10}$ CFU / ml.

Experiment 1
Fresh forage and silages

The influence of 'Silko for alfalfa' on the chemical composition and fermentation characteristics of alfalfa silage was tested in laboratory conditions. The cultivar Mirna (Bc Institute Zagreb) was used as the material. The cultivar was grown at the experimental plot of the Bc Institute at Zagreb, Croatia. For the silage, the first-cut in the second year of exploitation was used. The harvesting was done at the beginning of the flowering stage in May 2017. Masses were chopped with chopper harvester at about 20 mm chop length. Samples were taken to a laboratory where two treatments were formed: 'Silko for alfalfa', where the mass was treated with inoculant per rate of 5 ml t^{-1} green mass, and control where the mass was treated with distilled water in the same amount as the inoculant so that the moisture content of the silage was the same. A manual sprayer was used to spray liquid on the green mass. Glass jars of 1.5 l volume were mini-silos, with lids with water-valve. The jars were filled with about 850-1150 grams of compacted chopped mass and left in a dark place at room temperature for 90 days after which the silage was analyzed. Each treatment had three replicates. The chemical composition of the starting material before the ensiling was as follows: dry matter (DM) 355.0 g kg^{-1}, crude protein 225 g kg^{-1} DM, ADF 414.4 g kg^{-1} DM and NDF 527.5 g kg^{-1} DM.

Experiment 2
Fresh forage and haylage

In order to determine how inoculant the 'Silko for alfalfa' influences the alfalfa haylage quality, an experiment was set up on a commercial cattle farm in the Serbia during 2017. The cultivar Banat ZMS II (Institute of field and vegetable crops, Novi Sad) was used as the material. The first-cut in the second year of exploitation was used for the haylage. Plants were harvested at the beginning of the flowering stage (May) and wilted for 24h. Then the wilted mass was chopped with chopper harvester at about 15-40 mm chop length and packed in experimental silo bags. A special applicator was placed on the chopper harvester by which the chopped mass was treated with a new inoculant which represented the treatment of 'Silko for alfalfa' and distilled water for the control. The bacterial inoculant was administered in a rate of 5 ml of t^{-1} of green mass. Each treatment had three replications. The chemical composition of the starting material was as follows: dry matter content (DM) 467.0 g kg^{-1}, crude protein 208.8 g kg^{-1} DM, NDF 494.71 g kg^{-1} DM and ADF 408.6 g kg^{-1} DM. Haylage was analyzed after 40 days by taking three composite samples from each treatment. The composite sample included ten samples taken from different locations in silo bags, and then mixed in a clean plastic bin to form a common sample of about 2.0 kg. On the same day the samples were delivered to the laboratory for chemical analysis.

Determination of chemical, energy and fermetation parameters

The dry matter content was determined as a difference in weight before and after drying at 105 ° C in a oven to a constant mass. Kjeldahl method is used to determine crude protein content *(AOAC, 1990)*. Van Soest method is used to determine neutral (NDF) and acid detergent fiber (ADF) *(Van Soest et al., 1991)*. The silage pH value was determined from silage extract using a pH meter (Hanna Instruments HI 83141 pH meter). The distillation method is used to determine NH_3-N / total nitrogen (TN) using a Kjeltec 1026 analyzer *(Bijelić et al., 2015)*. Gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) is used to determine the amount of milk, acetic and butyric acid (*Faithfull, 2002)*. The energy parameters are calculated according to the following formulas:

Total digestible nutrients (TDN) (%) = (-1,291 × %ADF) + 101.35 according to *Horrocks and Vallentine (1999)*; Relative feed value (RFV) (%) = Digestible Dry Matter (DDM) × Dry Matter Intake (DMI) × 0.775; DDM (%) = 88.9 - (0.779 x % ADF) and DMI (%) = 120 / (% NDF) according to *Horrocks and Vallentine (1999);*Metabolisable energy (ME) (MJ kg^{-1}) = 14.07 + 0.0206 × ether extract (g kg^{-1}) - 0.0147 × crude fibre (g kg^{-1}) - 0.0114 × crude protein (g kg^{-1}) ± 4.5 % according to *Nauman i Bassler (1993)*;Net energy for lactation (NEL) (MJ kg^{-1}) = 9.10 + 0.0098 × ether extract (g kg^{-1}) - 0.0109 × crude fibre (g kg^{-1}) - 0.0073 × crude protein (g kg^{-1}) according to *Baležentienė i Mikulionien (2006)*.

Statistical analysis

The experiments were set by a randomized block system in 3 replications. Experimental data were statistically analyzed by the ANOVA using software SPSS 18 (IBM Corporation). Tukey test was used for the comparison of mean values at the level of $p \leq 0.05$.

Results and Discussion

Experiment 1

The results of the study show that the values of dry matter, crude protein, lactic acid, TDN and RFV were higher, and ADF, NDF, ammonium nitrogen in total nitrogen, acetic and butyric acid and pH were lower in the silage treated with inoculant than in control (Table 1).

Table 1. Chemical, energy and fermentation parameters untreated silage and silage treated with inoculant

Item	Control	'Silko for alfalfa'	F test
Chemical composition			
Dry matter (DM) (g kg^{-1})	325,1[b]	337,1[a]	*
Crude protein (g kg^{-1} DM)	202,4[b]	212,2[a]	*
Acid detergent fibre (ADF) (g kg^{-1} DM)	355,8[a]	313,1[b]	**
Neutral detergent fibre (NDF) (g kg^{-1} DM)	424,7[a]	394,2[b]	**
Energy parameters			
Total digestible nutrients (TDN) (%)	55.4[b]	60.9[a]	**
Relative feed value (RFV) (%)	134.0[b]	152.2[a]	**
Fermentation parameters			
pH	4,98[a]	4,43[b]	*
NH_3-N/TN (g kg^{-1} TN)	65,6[a]	43,9[b]	**
Lactic acid (g kg^{-1} DM)	34,1[b]	73,5[a]	**
Acetic acid (g kg^{-1} DM)	19,7[a]	6,5[b]	**
Butyric acid (g kg^{-1} DM)	0.39[a]	0.08[b]	**

TN – Total nitrogen; Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

Higher dry matter and protein content and lower content of ADF and NDF indicate that silage treated with inoculant has a better chemical composition compared to untreated silage. The greater loss of dry matter in control is the result of slowed lactic acid fermentation that is regulated only by epiphytic bacteria found on plants, and whose number according to *Schmidt et al. (2009)* <1% microflora. On the other hand, by adding bacterial inoculants, lactic acid fermentation was more intense and less dry matter was lost, while lactic acid synthesis was increased. The content of crude proteins in the investigated silages was over 200 g kg^{-1} DM (control - 202.4 g kg^{-1} DM, treated silage - 212.2 g kg^{-1} DM) which from the aspect of protein content is considered as high quality silage. High protein content in the control silage can be the result of cutting of plants at early flowering stage when the share of leaves in the fodder is greater than the share of the stem. The leaves contain the ¾ of proteins. The content of NDF and ADF was higher in untreated silage which reflects a reduced digestibility. Generally, lower ADF in silage results in higher TDN, while lower ADF and NDF result in higher RFV. Therefore, treated silage has a better energy value compared with untreated silage. According to *Horrocks and Vallentine (1999)*, high quality alfalfa should have a RFV 151% (Standard Prime). Alfalfa with a RFV value of 125% to 140% can be used only in the nutrition of cows in the final lactation phase *(Dunham, 1998)*. *Redfearn et al. (2008)* conclude that alfalfa with a RFV value of 160% can only be used as feed for cows in lactation. In our case, inoculant-treated silage belongs to high quality and can be used for feeding cows at an early stage of lactation since its RFV (152.2%) is more than 151% (Standard Prime). On the other hand, untreated

silage has a RFV value of 134% and can be used for feeding cow in the final lactation phase. *Kung and Muck (1997)* have already proved in 47% of their studies that silage treated with inoculants leads to an average increase in milk yield of 1.4 kg per cow per day. According to *Kung et al. (2003),* silage treated with *Lactobacillus plantarum* MTD/1 gives better results in milk production. Therefore, we can assume that silage treated with inoculant 'Silko for alfalfa' due to its good qualitative and energy properties can improve the performance of animals.

Examined inoculant contributed to the intensification of lactic acid fermentation as it affected the decrease in the pH, the increase in lactic acid content and the reduction in the content of acetic and butyric acid in silage. It preserved the nutritive value of silage compared with control. In the treated silage, the lower NH_3-N/TN content (43.9 g kg^{-1} TN) compared to the control (65.6 g kg^{-1} TN) indicated a lower degradation of the protein. On the other hand, in untreated silage, proteolytic enzymatic activity was enhanced. The pH in the treated silage was lower by 0.55 than in control, which promoted better activity of homofermentative lactic acid bacteria and higher lactic acid content. A higher content of acetic acid was observed in control compared to the treated silage. In both silages the butyric acid content was low (<0.05% of dry matter). Therefore, the treated silage was well preserved due to lower pH and production of a higher amount of lactic acid compared to the control. Our study has shown that used inoculant can improve silage quality and reduce protein degradation in silage. It is precisely the role of inoculants to intensify the production of lactic acid, quickly reduce pH and prevent the development of pathogenic microorganisms *(Wang et al., 2006)*. *Ce et al. (2016)* find that alfalfa silages treated with various lactic acid bacteria inoculants (*Lactobacillus casei*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*) have a higher lactic acid content, lower butyric acid content, and NH_3-N / TN compared to control. *Si et al. (2018)* find that the silage treated with *Lactobacillus plantarum* and *Lactobacillus buchneri* has significantly lower pH, butyric and propionic acid content and NH_3-N / TN and higher content of dry matter and lactic acid in comparison with control.

Experiment 2

Bacterial inoculants should have the same effect in silage and haylage of forage legumes, to quickly reduce pH and support aerobic stability with improved animal performance *(Aragón, 2012)*. Our research has proved that the bacterial inoculant 'Silko for alfalfa' affects the faster fermentation and the creation of larger amounts of lactic acid and in the haylage of alfalfa (Table 2).

Table 2. Chemical, energy and fermentation parameters untreated haylage and haylage treated with inoculant

Item	Control	'Silko for alfalfa'	F test
Chemical composition			
Dry matter (DM) (g kg^{-1})	421,4[b]	441,7[a]	**
Ash (g kg^{-1} DM)	86,9[b]	98,2[a]	*
Ether extract (g kg^{-1} DM)	384,1	388,0	ns
Crude protein (g kg^{-1} DM)	177,9[b]	197,3[a]	*
Crude fibre (g kg^{-1} DM)	296,6	274,4	ns
Acid detergent fibre (ADF) (g kg^{-1} DM)	354,1[a]	316,1[b]	*
Neutral detergent fibre (NDF) (g kg^{-1} DM)	419,1[a]	387,9[b]	*
Energy parameters			
Total digestible nutrients (TDN) (%)	55.6[b]	60.5[a]	**
ME (MJ kg^{-1})	15.6	15.8	ns
NEL (MJ kg^{-1})	8.3	8.5	ns
Relative feed value (RFV) (%)	136.1[b]	154.1[a]	**
Fermentation parameters			
pH	4,27[a]	4,16[b]	*
NH_3-N/TN (g kg^{-1} TN)	25,27[a]	20,31[b]	**
Lactic acid (g kg^{-1} DM)	65,1[b]	71,57[a]	*
Acetic acid (g kg^{-1} DM)	34,9[a]	28,43[b]	**
Butyric acid (g kg^{-1} DM)	0	0	ns

TN – Total nitrogen; Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability, * - significant at 5% level of probability and ns – non significant.

The results of the study showed that the values of dry matter, ash, crude protein, lactic acid, TDN and RFV were higher in haylage treated with inoculant than in the control. In contrast, ADF, NDF, NH_3-N/TN, acetic acid and pH were lower in haylage treated with inoculant than in the control. The pH value in examined haylages was below 4.5 which prevented botulism *(Kenney, 2001)* and listeriosis *(Ryser and Marth, 2007)*. The examined haylages did not differ in the content of ether extract, crude fiber, butyric acid, metabolisable energy and net energy for lactation. In general, the inoculant contributed to achieving optimum pH and better lactic acid fermentation, resulting in a lower loss of organic matter and increased digestibility. It is to be expected that the feeding of ruminants with high quality haylage can improve the production result of animals, as research by *Shirley (1996)* shows that haylage treated with inoculant can increase the dry matter intake, production of milk and weight gain of cows.

Conclusions

The results show that the inoculant 'Silko for alfalfa' preserves the nutritive value of alfalfa silage and haylage and improves chemical, energy and

fermentation parameters. In the treated silage and haylage, the content of dry matter, crude protein and lactic acid was higher than in control. Contrary to this, in the treated silage and haylage the values of ADF, NDF, pH, NH_3-N/TN and acetic acid were lower than in the control. The butyric acid content was lower in the treated silage than in the untreated, while the butyric acid was not detected in the haylage. The inoculant 'Silko for alfalfa' applied in silage and haylage positively influenced on lactic acid fermentation by preventing butyric fermentation and contributing to a reduced loss of nutritional value and energy. Also, it is to be expected that silage and haylage treated with the inoculant 'Silko for alfalfa' can promote a high level of productivity of ruminants, and thus contribute to the profitable livestock production.

Uticaj bakterijskog inokulanta na kvalitet silaže i senaže lucerke

Snežana Đorđević, Violeta Mandić, Nikola Đorđević, Biljana Pavlović

Rezime

Važna strategija u stočarstvu treba da bude uvođenje silaža i senaža krmnih leguminoza u ishranu preživara i promovisanje pravilnih tehnika siliranja i senažiranja. Krmne leguminoze se teško siliraju pa je neophodno primeniti starter kulture odabranih sojeva bakterija mlečne kiseline koje podržavaju proces siliranja i sprečavaju buternu fermentaciju i time doprinose očuvanju i unapređenju kvaliteta silaža. U radu je prikazan uticaj primene bakterijskog inokulnata 'Silko za lucerku' na kvalitet silaže i senaže u dva odvojena eksperimenta. Inokulant predstavlja kombinaciju homofermentativnih mlečnih bakterija *Lactobacillus plantarum* i *Pediococcus* spp. Za siliranje su korišćeni prvi otkosi lucerke u drugoj godini eksploatacije. Silaža je ispitivana u mini-silosima u laboratoriji, a senaža u silo vrećama na govedarskoj farmi. Tretmani su bili kontrola (netretirana silaža, odnosno senaža) i silaža, odnosno senaža tretirana sa inokulantom 'Silko za lucerku' (doza 5 ml t^{-1} krme). Silaže pripremljene u eksperimentalnim uslovima su analizirane nakon 90 dana, a senaže dobijene u proizvodnim uslovima nakon 40 dana. Ustanovljeno je da korišćeni inokulant čuva nutritivnu vrednost silaže i senaže i da poboljšava njihove hemijske, energetske i fermentacione parametre u odnosu na kontrolu. S obzirom da ispitivani inokulant pozitivno utiče na pravilnu mlečno-kiselinsku fermentacju silaže i senaže lucerke i doprinosi manjem gubitku hranljive vrednosti i energije za očekivati je da može promovisati visok nivo produktivnosti preživara, a samim tim i doprineti rastu profita u stočarstvu.

Ključne reči: lucerka, bakterijski inokulant, senaža, silaža, kvalitet

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FARM RECORDS IN INVESTIGATION OF EPIDEMIOLOGY, SYMPTOMATOLOGY AND CAUSES OF CLINICAL MASTITIS IN A DAIRY FARM

Dino Haračić[1], Sabina Šerić-Haračić[2]*, Ermin Šaljić[3], Nihad Fejzić[2]

[1] Zoetis portfolio for Western Balkans, Unitic business centre, tower B/20, Fra Anđela Zvizdovića 1, 71000 Sarajevo, Bosna and Herzegovina
[2] University of Sarajevo, Veterinary faculty, Animal health economics department
[3] University of Sarajevo, Veterinary faculty, Internal medicine clinics Zmaja od Bosne 90, 71000 Sarajevo, Bosna and Herzegovina
Corresponding author: Sabina Šerić-Haračić, sabina.seric-haracic@vfs.unsa.ba
Original scientific paper

Abstract: Mastitis is one of the most important diseases in dairy cow farms and one of the most common cause for antibiotic treatment. Aims of this study were: to investigate frequency and trends of clinical mastitis in cows on a large dairy farm, describe clinical characteristics of mastitis and investigate causative infectious agents in selected cases alongside antimicrobial resistance.
In our study we used farm records for clinical mastitis recorded for period 2016 and 2017. We also used results of the regular on farm testing of the somatic cell count for 2017. Samples of milk from all clinical mastitis cases were taken during November and December 2017 in order to investigate causative agents and their antimicrobial resistance.

Occurrence of clinical mastitis was 205 cases (47.7%) in 2017 compared to 93 cases (29.7%) recorded in 2016. In 2017 reoccurrence of clinical mastitis in same animal was recorded for 93 cows (45.4%). In 2016 reoccurrence of clinical mastitis in same animal was recoded for 49 cows (29.7%). Average course of clinical mastitis in 2016 was 3 days, while in 2017 4.5 days (continuous days of recording a case in farm records). Somatic cell count in more than half of tested animals was higher than 200.000 SC/ml according to the measurements from February and July 2017 (number of cows tested 236 and 169, respectively). Out of 23 milk samples, 20 had bacteriological growth. In 9 samples we identified *S.aureus*, in 6 streptococcus spp., in 4 coagulase negative staphylococci (CNS) and one sample contained *E.coli*. Most common resistance was found for lincomycin-spectinomycin (100%) gentamicin (92%), followed by cefquinome (65%), linkomycin (53%) and erythromycin (47%). Isolates of *S.aureus* were resistant on the largest number of investigated antibiotics.

Key words*:* clinical mastitis, trends, etiology, antimicrobial resistance

Introduction

Improvement of production technologies and hygiene on farms accompanied with programs for control of important infectious diseases causing productions losses and public health threats (i.e. brucellosis, tuberculosis) had led to eradication of many of these diseases particularly in developed countries. Simultaneously, other diseases became important especially in intensive production systems, commonly called breeding diseases such as lameness, reproductive and metabolic disorders and mastitis (*Zwart, 1997*).

Mastitis in dairy animals is recognized worldwide as one of the most expensive diseases in modern farm production (*Seegers, 2003*). In addition to burden of infectious mastitis which causative agents have public health importance, mastitis related production losses are one of the most important limiting factors of dairy production in Bosnia and Herzegovina (BiH) (*Alagić, 2006*). Prevalence of either clinical or subclinical mastitis on farms in BiH can reach more than 50% (*Varatanović, 2010*). Most commonly about half of these cases are clinical mastitis, where most commonly isolated causative agents are *Staphylococcus aureus*, *Streptococcus agalactiae*, coagulase positive staphylococci, *Trueperellapyogenes*and coliforms (*Matarugić, 2009; Varatanović, 2009)*.

Some bacteria causing mastitis in dairy cows can also cause different diseases in humans, however important public health aspect of mastitis in animals is linked with occurrence of residues in milk due to non-selective use of antimicrobials in treatment and control of this disease. Residues of antimicrobials in food can harm human health directly, but also lead to increase of the antimicrobial resistance in bacteria and potential for spread of theses resistant agents throughout the food chain, in addition to reducing effectiveness of mastitis treatment in animals (*Tenhagen, 2006*).

Aims of our study were: first to establish annual prevalence of clinical mastitis on large commercial farm, second to describe trends of mastitis occurrence and severity of clinical findings, third to investigate causes of clinical mastitis in some cases and fourth to investigate antimicrobial resistance of isolated agents.

Materials and methods

With consent and guarantee of private and business information protection, this study used following data from farm records of a commercial dairy farm:

- Records of the farm veterinary service for clinical mastitis cases in 2016 and 2017, which contained date of symptoms onset/recognition, ear tag number for

diseased animal and number of affected quarters. Individual animals were kept in records for consecutive days until termination of symptoms and/or therapy,
- Results of the somatic cell count in milk of individual animals measured alternately in stables A and B throughout 2017 (quarterly).

Sampling of the milk from cows with clinical mastitis was done on same farms in order to establish causative agents and their antimicrobial resistance using disk-diffusion method (antibiogram). Laboratory investigations were done at Laboratory for bacteriology and mycology of the Institute of Veterinary faculty University of Sarajevo.

Data management, analysis and graphical representation of study results were done using Excel (Microsoft Office (r)).

Using farm records for clinical mastitis in 2016 and 2017 we created Excel data base containing 556 entries. By eliminating repeated entries of animals with same ear tag number in time frame shorter than 15 days since the same animal was firstly recorded as mastitis case, data base of prevalent cases of clinical mastitis was created containing 370 cases in two year period. Repeated record of the same animal (same ear tag number) longer than 15 days from last entry was considered to be repeated case of mastitis in same animal. Also based on prevalent cases data base we created data base containing new (incident) cases of mastitis on monthly basis, which contained 338 records (animals recorded as cases in previous month were not counted as new cases in following moth).Case definition was observation of clinical symptoms of mastitis and/or changes in milk found in one or more quarters. In addition to data on the frequency of clinical mastitis, we analyzed severity of clinical findings (number of affected quarters, duration of symptoms) on monthly and annual basis.

Results of the somatic cell count in milk of the individual animals were analyzed separately, since sampling was conducted only in clinically healthy animals. Since farm has two stables (A and B), SCC was conducted for animals in stable A in February and September 2017, and in July and November for animals in stable B. Results of the SCC were stratified in 4 categories (<200.000 SC/ml, 200.000 – 500.000 SC/ml, 500.000 – 1.000.000 SC/ml, >1.000.000 SC/ml), and expresses as proportion for each category with respect to overall animal tested in each occasion. By comparing SCC results with data bases of clinical mastitis we identified numbers of occurring cases in period one month before and after SCC testing.

Milk samples were taken from all animals with clinical mastitis registered during November and December 2017, before any treatment was administrated. Microbiological isolation of agents was done using standardized laboratory protocols (*Quinn, 2011*).

For establishing average of affected quarters in clinical mastitis cases on monthly and annual basis we calculated Mode (the most frequent observation), while for average length of mastitis we used the mean (arithmetic average). For

comparison of proportions (prevalence) we used chi-square test for homogeneity of proportion interpreted for level of statistical significance of 5% (α=0.05).

Results and Discussion

Comparing established occurrence of clinical mastitis in 2016 and in 2017 (Figure 1) we established higher frequency of cases in 2017 on monthly and annual basis. Annual prevalence for 2016 was 38.4%, while for 2017 47.7%. Difference between annual prevalence was found to be statistically significant ($\chi 2$=7,6, p value 0,00587).

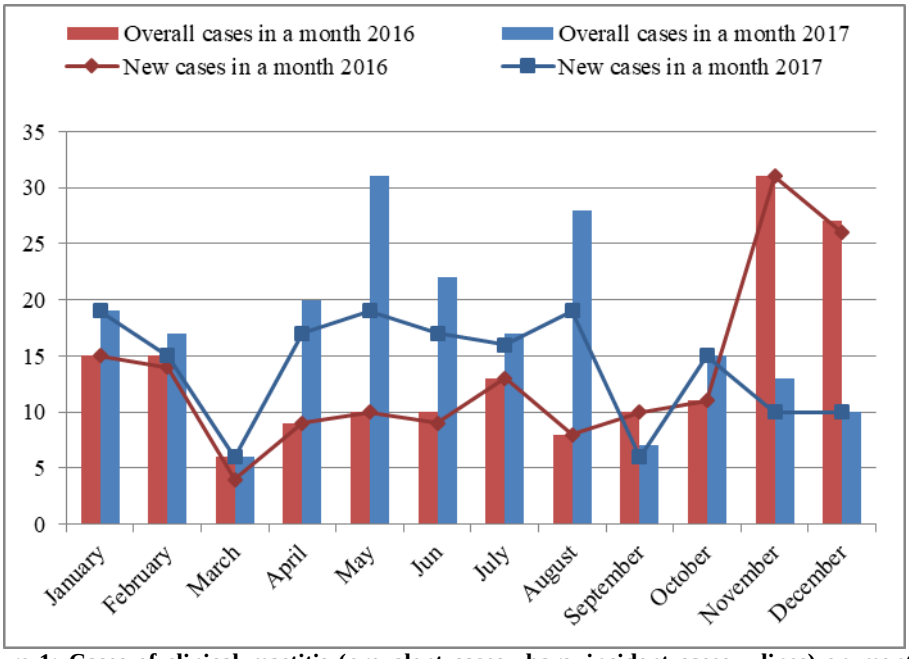

Figure 1: Cases of clinical mastitis (prevalent cases –bars, incident cases – lines) on monthly basis for 2016 (in red) and 2017 (in blue)

Our results also showed that in many animals mastitis is reoccurring within one or two year period (Figure 2). From 205 cases of clinical mastitis in 2017, 45.4% (93/205) were recurrent twice or more times in a same animal. In 2016 from overall 165cases, recurrence was 29.7% (49/165). For two year period (2016 and 2017), proportion of reoccurring cases was 49.5% (183/370).

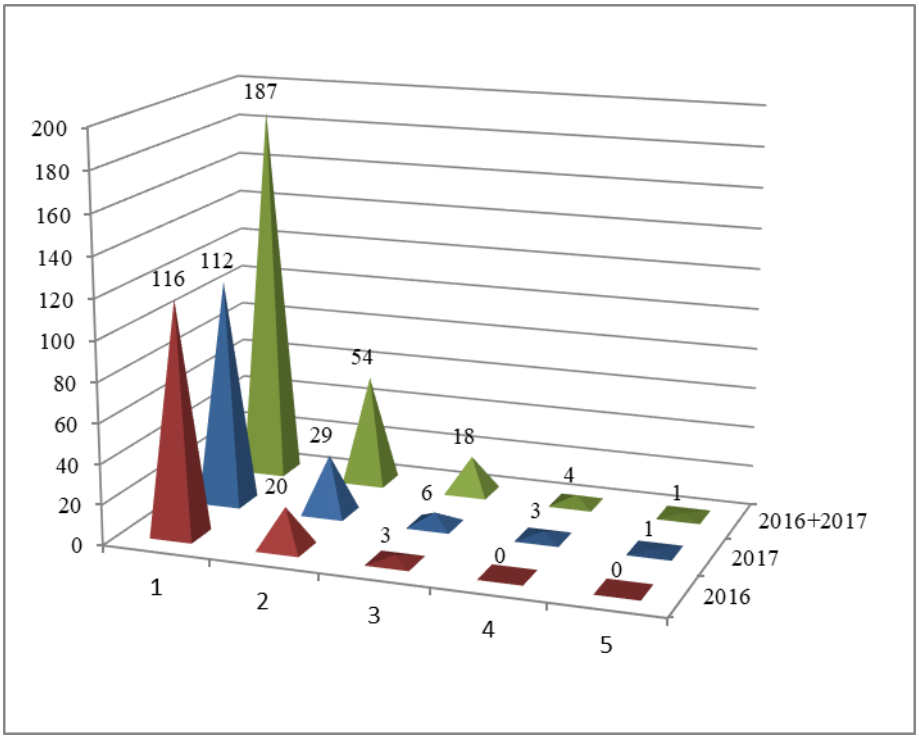

Figure 2: Repeating occurrence (x axes) of clinical mastitis in same animals within a year (2016 – in red, 2017 – in blue), and within two years (2016+2017 in green)

Higher frequency and reoccurrence of clinical mastitis in 2017 was accompanied with higher average number of affected quarters (Figure 3).

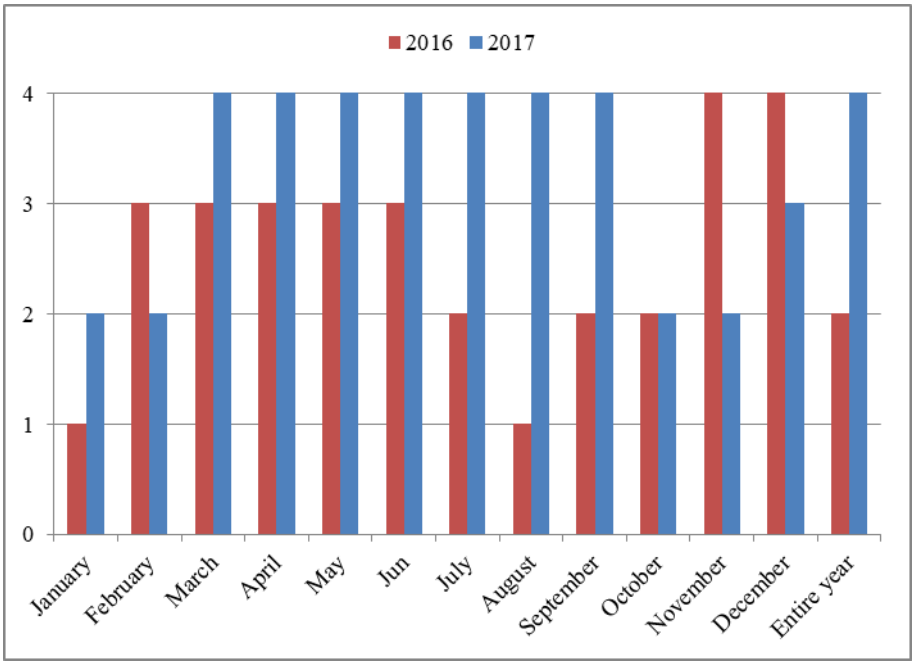

Figure 3: Average number of affected quarters in clinical mastitis cases (mode) on monthly and annual basis (2016 - in red, 2017 – in blue)

In most animals symptoms had disappeared after at most 3 days. Number of animals in which symptoms lasted between 8 and 15 days and over 15 days in 2016 was 12 and 7 respectively, and in 2017 17 and 15, respectively. Average duration on clinical mastitis symptoms in 2016 was 3 days (mean), while in 2017 4.5 days.

Results of the SCC in milk (Figure 4), shows that more than half of tested animals had >200.000 SC/ml according to measurement from February and July 2017, coinciding that in same months the largest number of clinical mastitis cases was observed (in comparison with other two months of SCC testing). In September and November 2017, proportion of animals with >200.00 SC/ml of milk was 34.4%and 32.1%, respectively whereas in same months 7 and 13 cases of mastitis was recorded respectively.

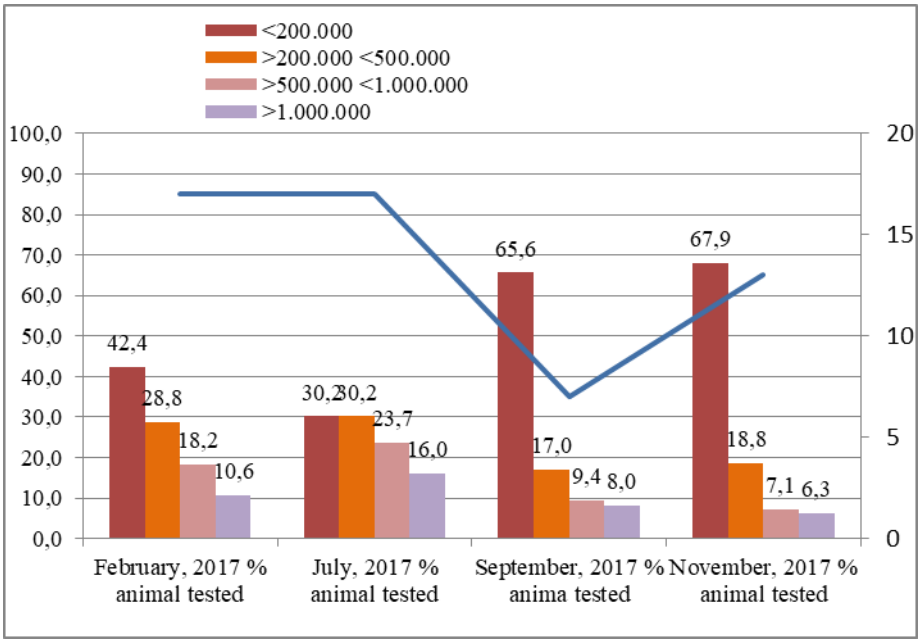

Figure 4: Results of the SCC in milk measured quarterly during 2017, stratified by categories (% (bars) - left axes, February – in red, July – in orange, September – in pink, November- in violet) shown with number of clinical mastitis cases in same months (blue line – right axes)

Because cases of subclinical mastitis recognized by increase of SC/ml commonly progress to clinical mastitis, as well as cases of chronic mastitis, table 1 shows number of clinical mastitis cases recorder one month before and 1 month after SCC testing stratified by given SCC categories. Progression of subclinical cases in clinical is particularly indicative for SCC testing in February (one month after SCC testing). Also for many cases of clinical mastitis recorded in January number of SCC measured in February remained high.

Table 1: Number of recorded clinical mastitis cases one month before and one month after measurement of SCC, stratified by SCC categories

	Month of SCC testing							
SCC	II 2017.		VII 2017.		IX 2017.		XI 2017.	
	-1mo.	+1mo.	-1mo.	+1mo.	-1mo.	+1mo.	-1mo.	+1mo.
<200.000	2	4	-	-	2	2	1	2
200.000-500.000	1	1	-	-	-	-	2	-
500.000-1.000.000	4	1	-	-	-	-	-	-
>1.000.000	4	3	-	12	-	-	-	1

Twenty milk samples, of total 23, had bacteriological growth. *S.aureus* was identified in 9 samples, *Streptococcus* spp. in 6, CNS in 4 samples and one sample contained *E.coli*. Table 2 contains results of testing for antimicrobial resistance in bacterial isolates.

Table 2: Results of the antimicrobial resistance testing or identified causative agent of clinical mastitis shown as proportion (%) of sensitive (S), moderately sensitive (I) and resistant isolates

	Overall isolates N=20			*S.aureus Isolates* N=9			CNS Isolates N=4			*Streptococcus* spp. Isolates N=6			*E.coly* isolate
Antibiotic	S	I	R	S	I	R	S	I	R	S	I	R	S/I/R
Amoxicillin/ clavulanic acid	75	5	20	78	-	22	50	-	50	83	-	17	S
Ampicillin/ sulbactam	75	25	-	78	22	-	50	50	-	100	-	-	I
Cefquinome	25	10	65	22	-	78	50	-	50	-	33	67	S
Ceftiofur	30	40	30	22	67	11	50	50	-	17	-	83	S
Cephalothin	100	-	-	100	-	-	100	-	-	100	-	-	ni*
Ciprofloxacin	50	15	35	67	-	33	75	-	25	-	50	50	S
Linkomycin	5	42	53	-	55	45	25	25	50	-	17	83	ni
Erythromycin	21	32	47	-	22	78	25	25	50	50	50	-	ni
Gentamicin	8	-	92	-	-	100	25	-	75	ni			ni
Marbofloxacin	100	-	-	100	-	-	100	-	-	ni			ni
Penicillin	65	-	35	67	-	33	50		50	67	-	33	S
Cefprozil	100	-	-	100	-	-	100	-	-	100	-	-	ni
Mastijet (neomicin, bacitracin, tetracicline)	95	-	5	100	-	-	100	-	-	100	-	-	- R
linkomicin-spectinomicin	-	-	100	-	-	100	-	-	100	100			ni

* not investigated

Mastitis in cows is inflammation of mammary gland, commonly caused by infection with microorganisms. Large number of microorganisms is recognized as causes of mastitis and many of them have direct or indirect public health importance (*Watts, 1988; Hameed, 2006*). Mastitis is also leading disease according to economic losses in dairy cow farms and most common cause for antibiotic treatment (*Seegers, 2003; Pol, 2007; Saini, 2012).*

Annual clinical mastitis prevalence established in this study (for 2016. 38.4%, and for 2017. 47.7%) coincides with results of similar earlier studies conducted in BiH (*Varatanović, 2010*). Comparative studies from other countries report range of established prevalence; from 21.5% in Ethiopia (*Workineh, 2002*), 26.4% in Germany (*Terhagen, 2006*), 31% in Finland (*Pitkälä, 2004*), up to 52.4% in Uruguay (*Gianneechini, 2002*). Clinical mastitis prevalence at dairy farm in BiH that we established falls into this range; however there is space for improvement

and reaching values of herd/farm prevalence established in developed countries. Further on, we recorded statistically significant increase of prevalence over two year period. During 2017 occurrence of clinical mastitis was higher in period from April to August compared to the rest of the year (118:87 case ratio), while in 2016 this ratio was 50 cases in period April-August compared to115 cases in rest of the year. Increased occurrence of clinical mastitis in warmer season of the year is related to better conditions for growth and spread of environmental mastitis pathogens (*Riekerink, 2007*). Therefore difference in clinical mastitis occurrence and seasonality between 2016 and 2017 can be explained by different etiology. This is also supported by established difference in clinical manifestation of mastitis in each year (average number of affected quarters and length of disease). Clinical mastitis caused by environmental bacteria is most commonly has shorter course and usually affects only one quarter *(Erskine, 2016)*. In samples of milk we investigated in two final months of 2017 leading cause of clinical mastitis was *S.aureus*, which is considered infectious mastitis pathogen, manifested with more severe clinical manifestation. Norwegian study conducted in 1997 established that leading causative agent of mastitis found in milk samples collected during late fall and early winter is *S.aureus* followed by *A.pyiogenes* (Waage, 1999). Same study found more *E.coli* in samples collected during summer months.

Early detection of mastitis on farms is accomplished by monitoring of the SCC, especially for subclinical mastitis. One month after SCC measurement on investigated farm conducted in February and July 2017 subclinical mastitis (SCC>200.000 SC/ml) evolved in clinical in 5 and 12 animals respectively. This shows that monitoring and control programs for subclinical mastitis are important preventive measure against clinical mastitis.

By microbiological cultivation of milk samples we established following causative agents; S.*aureus*, CNS, streptococci and *E.coli*. Study conducted in France in 2007 and 2008 found that most common causative agents of mastitis in dairy cows were *S.uberis* (22.1%), *E.coli* (16%) and coagulase positive staphylococci (15.8%) (*Bortel, 2010*). On the other hand German study (2001/2002) indicated that leading causes of mastitis in cattle was CNS, followed by *Corynebacterium bovis* and *S.aureus* (*Terhagen, 2006*). In Finland most commonly isolated bacteria associated with mastitis were CNS and *S.aureus* (*Pitkälä, 2004*). Obviously primary causes of mastitis in developed countries are environmental mastitis pathogens, indicating success of mastitis control programs primarily aimed against infectious pathogens such as *S.aureus* are *S.agalactiae (Hillerton, 2005).* However, infectious mastitis pathogens are still major issue on dairy farms in developing countries, as shown by our study as well (*Gianneechini, 2002; Workinch, 2002*). Together with shift in importance of different mastitis pathogens, increased occurrence of resistance in *S.aureus* and CNS isolates is observed, particularly for beta lactam antibiotics (*Myllys; 1998*).

Primary importance of increased resistance in these bacteria is resulting risk for human health, however simultaneously this impairs efficiency and options for mastitis treatment. Commonly it is very difficult to obtain antimicrobial usage data (type of antibiotic used, dosage, treatment regimen), especially if farmers themselves without consulting veterinarians are able to acquire and apply antibiotic treatment. Studies report proportion of resistant isolates on penicillin is for *S.aureus* from 17% to 52% (compared to overall number of *S.aureus* isolates from milk of mastitis cases), and for CNS from 30,7% to 40% (*Pitkälä, 2004; Terhagen, 2006; Botrel, 2010)*. This concurs with our results, however same research report much less proportion of resistant isolates of these bacteria to erythromycin, gentamycin and lincomycin. Moreover established resistance of our *S.aureus* and CNS isolates to antibiotics not used in food animals or in some cases contraindicated for treatment of mastitis indicates former unselective usage. Penicillin resistant S.*aureus* isolates are found in only 4% in Norway where legislation prescribes that only veterinarians make decision and administer antibiotic treatment of animals, while in countries where this is legally enabled to farmers as well, proportion of resistant strains is much higher up to a point when this antibiotic (most commonly used in mastitis treatment) is uttermost ineffective (*Oliver, 2012*).

Mastitis in dairy cattle is therefore complex disease occurring as a result of interaction of many factors related to animal itself, causative pathogen and the environment. Earlier epidemiological studies have led to establishment and widespread of simple mastitis prevention measures such as tit disinfection after milking and dry cow treatment (*Watts, 1988*). In order to reduce occurrence of mastitis in our farms it is recommended to introduce and fully implement these standard preventive measures alongside ensuring early detection of mastitis (using SCC and California mastitis test), isolation of diseased animals, culling of repeated cases, microbiological monitoring and testing for antimicrobial resistance before treatment is administered (*Workinech, 2002*). Keeping farm records and using them to improve effectiveness of decisions regarding treatment options for individual animals represents a basis for sound and responsible dairy production.

Conclusion

Annual clinical mastitis prevalence established in this study corresponds to same figures found in the country earlier, however it could be reduced to the levels recorded in developed countries. Different seasonality, clinical course and severity compared between 2016 and 2017 indicate different etiology of disease. Comparison of SCC measurements and occurrence of clinical mastitis confirms that this is an important tool in recognizing subclinical mastitis but also chronic (reoccurring) mastitis cases. We established following causative agents; S.*aureus*,

CNS, streptococci and *E.coli*. Antibiotic resistance results from our isolates, concurs other research, however there was much less proportion of resistant isolates of isolated bacteria to erythromycin, gentamycin and lincomycin. Established resistance of *S.aureus* and CNS isolates to antibiotics not used/ contraindicated in food animals indicates former unselective usage.

Farmska evidencija za istraživanje epidemiologije, simptomatologije i uzročnika kliničkog mastitisa na farmi mlečnih krava

Dino Haračić, Sabina Šerić-Haračić, Ermin Šaljić, Nihad Fejzić

Rezime

Mastitis je jedna od najvažnijih bolesti na farmama mlečnih krava i predstavlja jedan od najčešćih povoda primene antibiotika. Ciljevi ovog istraživanja bili su: istražiti učestalost i trendove kliničkog mastitisa krava na velikoj komercijalnoj farmi, opisati kliničke karakteristike i odrediti infektivne uzročnike u određenom broju uzoraka mleka, kao i njihovu antimikrobnu rezistenciju.

U našem istraživanju smo koristili evidenciju farme o kliničkom mastitisu za period 2016. i 2017. Godina. U istraživanju su korišćeni rezultati redovnog testiranja broja somatskih ćelija u mleku iz 2017. godine. Uzorci mleka od svih krava kod kojih je ustanovljen klinički mastitis tokom novembra i decembra 2017. godine su mikrobiološki ispitani i određena je antimikrobna rezistencija dobijenih bakterijskih izolata.

Ustanovili smo da je broj krava sa kliničkim mastitisom iznosio 205 (47,7%) u 2017. godini, a broj krava sa kliničkim mastitisom u 2016. godini bio je 165 (38,4%). Pojava kliničkog mastitisa bila je veća u 2017. godini po mesecima i ukupno u odnosu na 2016. godinu. U 2017. godini ponovljeni slučajevi kliničkog mastitisa su iznosili 45,4% (93/205). U 2016 godini ponovljeni slučajevi kliničkog matitisa su iznosili 29,7% (49/165). Prosečno trajanje kliničkog mastitisa u 2016. godini bilo je 3 dana, a u 2017. godini 4,5 dana (broj dana u kontinuitetu u evidenciji). Broj somatskih ćelija u mleku kod više od polovine testiranih životinja u februaru i julu 2017. godine bio je veći od 200.000 SC/ml (testirano 236 odnosno 169 krava). Od 23 uzorka mleka, 20 je imalo bakteriološki rast. U 9 uzoraka ustanovljena je *S.aureus*, u 6 streptococcus spp., u 4 koagulaza negativne stafilokoke i u jednom *E.coli*. Kod svih bakterijskih izolata najraširenija je rezistencija na lincomycin-spectinomycin (100%) gentamicin (92%), koje slede

cefquinome (65%), linkomycin (53%) i erythromycin (47%). Izolati *S.aureus* su bili rezistentni na najveći broj ispitanih antibiotika.

Ključne reči*:* klinički mastitis, trendovi, etiologija, antimikrobna rezistencija

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Milan M. Petrović[1], Stevica Aleksić[1], Milan P. Petrović[1], Milica Petrović[2], Vlada Pantelić[1], Željko Novaković[1], Dragana Ružić-Muslić[1]

[1]Institute for Animal Husbandry, Belgrade – Zemun, 11080 Zemun, Serbia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia
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

Institute for Animal Husbandry, Autoput 16, 11080 Belgrade, Serbia
Corresponding author: Zdenka Škrbić, **e-mail address**
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Text and titles of tables, figures and graphs, Times New Roman, font size 9, **bold**, in the following form:

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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:

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

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Tekući račun br. 205-65958-94
Komercijalna banka

INSTRUCTION FOR EUR PAYMENTS
AIK BANKA AD BEOGRAD

Please pay as per instruction given below:

56A: Intermediary bank:	**SOGEFRPP** **SOCIETE GENERALE** **F-92978 PARIS** **FRANCE**
57A: Account with institution:	**AIKBRS22** **AIK BANKA AD BEOGRAD** **BULEVAR MIHAILA PUPINA 115Đ** **11070 NOVI BEOGRAD** **REPUBLIKA SRBIJA**
59: Beneficiary customer:	**RS35105050120000062319** **INSTITUT ZA STOČARSTVO ZEMUN** **Autoput Beograd-Zagreb 16** **Zemun** **REPUBLIKA SRBIJA**

ACCOMMODATION AND SYMPOSIUM LOCATION

The Symposium will be held in Hotel Park, Belgrade Njegoševa street 2, 11000, Belgrade, Serbia (www.hotelparkbeograd.rs)

Single room at special rate of 50 € daily per room
Double room at special rate of 70 € daily per room
City tax is not included and is approximately 1.2 € per person daily.

Accommodation at SPECIAL RATES is possible for reservations before **August, 31st 2019.**

Hotel reservation telephone: +381114146800
Hotel reservation e-mail: reception@hotelparkbeograd.rs

On behalf of Organizing Committee

Dr. Milan P. Petrović,
Principal Research Fellow
Serbia

HOTEL RESERVATION FORM

Hotel Park Beograd welcomes the guests of
INSTITUTE FOR ANIMAL HUSBANDRY SYMPOSIUM
9-11th October 2019
We are pleased to advise the special rates and conditions available for this event:
(Please check appropriate box)

	Single room at special rate of 50 € daily per room
	Double room at special rate of 70 € daily per room

All the above mentioned rates are INCLUSIVE of full buffet breakfast in our Continental restaurant, VAT, complimentary internet access.
City tax is not included and is approximately. 1.2 € per person daily.

GUEST INFORMATION:

Family name:	
First name:	
e-mail:	
Contact phone number:	
Date of arrival:	Date of departure:

PAYMENT INFORMATION:

BY CREDIT CARD:	Visa	Master	Diners	American Express
Card Holder name:				
Card Number:			Exp. Date:	

Cancellation Policy: If you wish to cancel, please do so at least 72 hours prior to arrival. For all cancellations or no shows after this period, one night charge will be charged to your credit card.

PLEASE COMPLETE THIS FORM AND SEND TO HOTEL PARK BEOGRAD VIA FAX OR E-MAIL reception@hotelparkbeograd.rs by August 31st 2019.