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Occurrence and prevention of mastitis in dairy farms situated in marginal regions

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Preface

The relatively large parts of V4 territory with agricultural production are peripheral or the so-called “marginal regions” or “less flavoured areas” where, with the optimum use of all factors of production (labor, land, capital), animal commodities can be effectively produced only occasionally. Geographic, social and economic stability of these regions is strongly influenced by breeding of ruminants with market milk production. Dairy cattle farming accounts for up to 75% of the animal production of these areas and milk production has an irreplaceable role, especially in nutrition of consumers as several dairy specialties can be classified as functional food. The dairy farms in marginal regions have difficulties to comply with all the requirements for dairy production and animal health care.

Preserving the good health of dairy cows is a daily challenge for all involved in primary milk production. Despite the increasing level of technological support and veterinary measures, inflammation of the mammary gland - mastitis, is still one of the main health problems and reasons for economic losses faced by cow farmers. The mammary gland of high-yielding dairy cows requires making the right decisions and enforcing the proper measures aimed at minimizing external and internal factors that increase the risk of intramammary infection. Due to the polyfactorial nature of mastitis related to its reduction, the effectiveness of commonly used antimastitis methods tends to be limited and therefore it is necessary to find the areas of risk in udder health programs and monitoring systems. Only by implementing of complete udder health programs should be accompanied by research efforts to further development these complete udder health control. The scientific book analyses the current knowledge dealing with diagnostic and prevention of mastitis include methods of udder pathogens detection and their virulence factors, control of somatic cell count, proper nutrition and imunnostimulation through peroral supplementation of mineral-vitamin products or organic aditives as well as improving housing and milking practiced in dairy farming conditions. This informations may help to improve the health of the mammary gland and welfare of the dairy cows with the production of safe milk for consumers,

editors.

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Diagnosis and etiology of dairy cows' mastitis

The first part of the scientific publication consists of a set of two studies focused on the aetiology and methods of detection of intrammamary infection - mastitis in cows including identification of forms and causative agents of mastitis, as well as evaluation of the impact of pathogenic bacteria on qualitative changes in the produced milk. In clinical practice, determination of individual oxidative and inflammatory markers is becoming an implementation component of diagnosis intrammamary infection. Due to the complexity of the diagnosis of mastitis, increased especially by their diverse aetiology, pathogenesis, manifestation and duration, the first study focused on the selection of suitable oxidative and inflammatory markers that are detectable already at latent and subclinical forms. Early detection of mastitis and evaluation of the relationships between bacterial pathogens and production of the oxidative inflammatory marker – milk malondyaldehyde provided valuable knowledge that contributes to the improvement of mammary gland health through early initiation of treatment and application of suppressive measures.

The second study deals with the detection of bacterial agents of ruminant mastitis and their occurrence on dairy farms located in the marginal areas of the V4 countries. The knowledge obtained included detection of relationship between occurrence of subclinical and clinical mastitis caused by contagious udder pathogens and non-aureus staphylococci characterized by production of selected virulence factors, such as resistance to selected intramammary antibiotics, haemolysin and enterotoxins production, enterotoxin production and biofilm formation.

1 Chapter

THE OCCURRENCE OF MASTITIS AND ITS EFFECT ON MILK MALONDIALDEHYDE CONCENTRATIONS AND BLOOD ENZYMATIC ANTIOXIDANTS IN DAIRY COWS

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Abstract: Early identification of mastitis is a serious challenge for dairy farmers and veterinarians in ensuring the health of an animal and the hygienic quality of the produced milk. The purpose of this study was to detect the occurrence and aetiology of mastitis in a dairy herd of 153 milked cows localised in a farm in west Slovakia. During the complex investigation, 606 quarter milk samples were examined (6 quarters were discarded) and classified based on the clinical status, the presence of abnormal udder secretions, the result of the California mastitis test (CMT), the somatic cell count (SCC) and the bacteriological identification of the pathogens causing the intramammary infection (IMI). The study was augmented by the detection of malondialdehyde (MDA) in the milk and the measurements of the blood enzymatic activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) as potential biomarkers for the udder health screening. A positive CMT score was recorded in 19.5% (118) of the examined quarters and 12.5% (76) of the quarters were infected with bacterial pathogens causing latent mastitis (LM; 1.3%), subclinical mastitis (SM; 8.3%), and clinical mastitis (CM; 2.9%). The most commonly isolated bacteria from the infected quarters were coagulase-negative staphylococci (55.2%), *Staphylococcus aureus* (11.8%) and streptococci (10.5%). The concentration of MDA and SCC were significantly higher from both the SM and CM cases than in the milk samples from the healthy cows, while the blood activities of SOD and GPx were lower in the cows with CM compared to the healthy cows. The higher MDA concentrations in the SM and CM milk observed in this study showed the presence of an oxidative stress in the infected milk, accompanied by a decrease in the antioxidative enzymatic activity in the blood of the cows. Therefore, the measurement of the milk MDA concentration and the activity of the blood SOD and GPx may prove insightful for the better screening of the udder health in the early diagnosis of mastitis.

1.1 Introduction

The economic value of dairy cows is determined mainly by their milk yield and longevity. The most important factor affecting the quantity and quality of milk produced is the occurrence of production diseases, especially mastitis (Zajac et al., 2012).

Mastitis or intramammary infection (IMI) is characterized by physicochemical changes of the milk, accompanied by an increase in SCC and bacterial pathogens, as well as by changes in the mammary tissue depending on the type of the disease (Malinowski et al., 2006; Zigo et al., 2019).

Worldwide, mastitis is known as a multifactorial disease, and it is closely related to the production system and the environment. The incidence of mastitis increases when the immunological and antioxidant defense mechanisms of the mammary gland are impaired. Dairy cows are exposed to numerous genetic, physiological, and environmental factors associated with both the host and pathogens that can compromise host immunity and increase the incidence of mastitis (Turk et al., 2012; Andrei et al., 2016).

According to Vasiľ et al. (2009), more than 140 different microorganisms are considered to cause mastitis. Bacteria are the most common causative factor, recognized in more than 95% of mastitis cases. Globally, most common mastitis-causing bacteria in dairy ruminants are *S. aureus* and coagulase-negative staphylococci (CNS), as well as streptococci and *E. coli*, which may have a similar or higher prevalence than that of staphylococci (Taponen et al., 2007).

Based on the intensity and severity of clinical signs, mastitis is usually divided into subclinical and clinical disease. In clinical mastitis (CM), signs range from mild to severe and can be systemic, local, or milk related, whereas in subclinical mastitis (SM) no signs are observed. The most prominent symptoms of CM are swelling, heat, hardness, redness or pain of the udder. The milk of a cow with CM has a watery appearance, and flakes, clots or pus is often present. During SM the udder and milk appear normal, but the infection is still present. The increase in SCC is associated with reduced milk production to the tune of 60 to 140 liters per cow per year in animals with SM (Sztachańska et al., 2016).

Authors Ashfaq and Muhammad (2008) report that subclinical cases of mastitis are more common than CM. It is estimated that in the herd of dairy cows there are approximately 15 - 40 undetected cases of SM for each case of CM.

Often due to the lack of symptoms, early identification of mastitis is an essential problem for dairy farmers and veterinarians to ensure not only the animal health but also the hygienic quality of produced milk. Economic aspects interfere with the routine assessment of California mastitis test (CMT) because the score evaluation according to color reaction is often insufficient. In addition to the assessment of the CMT, bacteriological examination of raw milk samples complemented by alternative parameters may aid the better screening of udder health. Oxidative stress parameters such

as malondialdehyde (MDA) concentration and activity of antioxidant enzymes may belong to these complementary indicators (Suriyasathaporn et al., 2006, 2010; Turk et al., 2017).

MDA is one of the relatively stable peroxidation product and is the most widely used indicator to assess oxidative stress (Castillo et al., 2006). Oxidative stress is related to some disorders in cattle as mastitis, retained placenta, udder edema or muscle and metabolic diseases (Sharma et al., 2011). It was proved that bacterial infection and other inflammation processes affecting the mammary tissue causing an increase SCC in milk, especially in the polymorphornuclear leukocytes (PMN) and macrophages number, are associated with the generation of reactive oxygen species (ROS; Castillo et al., 2006). The accumulation of ROS correlating with a reduction of antioxidative activity due to catalysis of various hydrogen and lipid peroxides can lead to oxidative stress (Suriyasathaporn et al., 2009).

1.2 Objectivites of this chapter

Oxidative stress detection using biomarkers is a relatively young field of research in ruminant health. Therefore, the aim of this study was to determine the incidence and etiology of mastitis according to standard methods complemented by the detection of milk MDA and blood activity of glutathione peroxidase (GPx) and superoxide dismutase (SOD) as potential biomarkers for udder health screening.

1.3 Materials and methods

1.3.1 Animals and milking

The study was conducted in the farm of west Slovakia of 180 dairy cows of Slovak spotted cattle with average annual milk production 6 971 kg. Cows were kept in free housing system with deep litter based on the repeated spreading of straw every two days. The basis of cows feeding was total mixed ration consisted of grass silage, maize silage, hay, and concentrate according to international standards (NRC, 2001) to meet the nutritional requirements of a 600 kg cow yielding 15 - 25 kg of milk/d. Cows were milked twice a day in fishbone milking parlor (DeLaval, UK) 2x10 stalls with standard exit and single return lane. The first milking starting at 4.30 a.m. and the second at 4:30 p.m. The milking routine consisted from udder washing with water from hose to remove impurities. Subsequently, the udder was thoroughly wiped with disposable paper wipes. The forestripping from each quarter was hand-drawn into a dark-bottomed cup for visual assessment of milk consistency. Milking and pulsation vacuum was set at 42 kPa. Pulsation ratio was 60:40 at a rate of 52 c/min and termination was automatically signaled when the milk flow dropped to 0.2 l/min.

After the milking process, the teats were disinfected by teat-dipping. The milk was stored in refrigerated milk tanks at + 5 °C and removed daily around 11.30 a.m.

1.3.2 Herd examination and milk samples collection

During the practical part of the study, 27 cows from herd were separated in calving boxes or were milked into separate vessels for the first 12 days after calving. A thorough evaluation of udder health in 153 lactating cows included veterinary history, clinical examination, sensory analysis of milk from forestripping of each udder quarter followed by assessment of CMT with subsequent collecting of milk samples. Veterinary history included important information as changes in behaviour or physical state, date of last calving, date of insemination, hygiene of holdings, milking performance, feeding, milking procedure and recent changes in daily procedures. After considering the history was examined the udder by adspection and palpation for clinical signs of oedema, spontaneous triggering of milk, signs of inflammation or atrophy.

CMT (Indirect Diagnostic Test, Krause, Denmark) from 606 quarters (6 quarters were discarded) of 153 milked cows was performed (Table 1). According to procedure Jackson and Cockcroft (2002) milk from every quarter was mixed with the reagent and the result was read as trace, score 1, 2, 3, 4 or negative depending on the gel formation in the milk sample.

Afterward from all 153 milked cows were collected aseptically two samples (10 ml) of quarter milk for bacteriological cultivation and SCC measurement in accordance with the guidelines of the National Mastitis Council (2001). The cooled samples were immediately transported to the laboratory of the University of Veterinary Medicine and Pharmacy in Kosice.

1.3.3 Experimental groups selection for oxidative stress determination

Based on veterinary history, CMT assessment and clinical examination for measurement of milk MDA concentration and blood enzymatic activity of GPx and SOD were assigned three groups: control (healthy cows), cows with subclinical (SM) and clinical (CM) mastitis. To a SM group were included 43 cows without clinical signs of mastitis or other illnesses but with positive CMT at least one quarter. Out of them were selected 77 quarters for MDA detection that showed a positive CMT (score 1 - 4). To a CM group were included seven cows with clinical signs (Figure 1) and out of them were selected 15 quarters for MDA detection with high score of CMT ≥ 3. From all health cows with a negative score of CMT and without clinical sign was randomly selected seven animals (28 quarters) to the control group.

Selected quarter milk samples from each group were then collected into the 12 ml plastic tubes for MDA detection. Blood for SOD and GPx measurement from selected cows was withdrawn by a puncture of *vena jugularis* and collected into the heparinized tubes. Samples were placed on ice, and

immediately transported to the laboratory where they were processed according to the instructions of the enzyme assay kits manufacturer and stored at -56 °C until analysis. MDA was measured in milk samples on the day of collection.

Table 1. Evaluation of California mastitis test (CMT) and Somatic cell count (SCC) in monitored herd

All exanimated quarters[1] from 153 cows		Healthy quarters	Quarters with positive CMT score[2]	Infected quarters[3]	Evaluation of CMT score and SCC from exanimated quarters			
					CMT score	**n**	**%**	**SCC x 10^3**
n	606	488	118	76	**0 (negative)**	488	80.5	94.53±9.85
%	100	80.5	19.5	12.5	**T (trace)**	26	4.3	282.00±15.39
					1	38	6.3	505.10±27.25
					2	29	4.8	861.56±33.40
					3	18	3.0	1695.50±31.34
					4	7	1.2	5724±57.96

Note: n - number of quarters, All examined quarters[1] - quarters with milk secretion (6 quarters were rejected), Quarters with positive CMT score[2] - quarters with positive evaluation of California Mastitis Test with score: trace, 1, 2, 3 and 4. Trace score - is seen slight thickening seen but disappear after prolonged mixing, Score 1 – easy thickening of the liquid, which appears after 20 seconds of mixing but no tendency toward gel, Score 2 - mixture thickens after 20 second of mixing and gel formation is suggested, Score 3 - immediate concentration with gel formation, Score 4 – Strong gel formation and the surface of the mixture becomes convex. Infected quarters[3] – positive quarters after bacteriological investigation (see Table 2).

1.3.4 SCC and MDA detection in milk samples and blood antioxidant enzymes

The SCC in quarter milk samples was measured using Somacount 150 apparatus (Bentley Instruments Inc., Minnesota, USA). Lipid peroxidation products in the selected milk samples were determined by spectrophotometric measurement (Shimadzu, UV 160) of thiobarbituric reactive substances at 532 nm, according to the method described by Andrei et al. (2016). The results were expressed in nmol of MDA per ml of milk ($nmol.ml^{-1}$).

After thawing and warming of blood samples with erythrocyte lysate, the detection of SOD activity was performed using the Ransod kit (Randox Laboratories, Crumlin, UK) according to the method of Woolliams et al. (1983). Erythrocyte glutathione peroxidase activity was assessed using the Ransel kit (Randox Laboratories, Crumlin, UK) by the kinetic method (Paglia and Valentine 1967). Chemistry Analyser Olympus AU 400 (Olympus, Mishima, Japan) was used as the spectrophotometer in this study. The activity of SOD and GPx enzymes was normalized to

haemoglobin concentration measured by Drabkin method, expressed as Units per g of haemoglobin (U/g Hb).

Figure 1. Clinical mastitis cases with positive bacteriological cultivation

Note: (A1 – A2) clinical IMI caused of *Str. uberis*, (B1 – B2) clinical IMI caused of *S. aureus*.

1.3.5 Microbiological examinations

Bacteriological examinations and identification were performed according to generally accepted principles (Malinowski et al., 2006). Quarter milk samples (10 µl) were inoculated on Petri dish with Columbia Blood Agar Base (Oxoid, UK) with 5% of defibrinated ram blood and incubated for 48 h at 37° C; the dish was examined after 24 and 48 h of incubation. Suspected colonies were inoculated and cultured on selective media such as *Staphylococcus* medium N°110, Baird-Parker agar, Brilliance^{TM} UTI Clarity Agar, Edwards Medium, Mac Conkey Agar (Oxoid, OXOID Ltd., Basingstoke, Hants, UK). Parameters such as colony size and appearance, pigment production and

coagulase, catalase activity, hemolysis, Gram staining have also been taken into account in the determination of bacterial species. Colonies of *Staphylococcus* spp. were tested for coagulase activity (Staphylo PK, Imuna Pharm, SR). Growth-confirmed colonies of *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were further identified biochemically using the STAPHYtest 24, STREPTOtest 24, resp. ENTEROtest 24 (Erba-Lachema, CZ) and the software TNW Pro 7.0 (Erba-Lachema, CZ).

1.3.6 Statistical analysis

All data were analyzed statistically using GraphPad Prism Software, Version 4.00 (Graphpad Prism, 2003). The data are presented as the mean (M) ± standard error of the mean (SEM). Differences in selected groups of control, SM and CM cows were determined by analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Statistical analysis by variable probabilities for Post Hoc Test was used to compare milk MDA concentrations from negative and positive milk samples with CMT score 1 – 4. The criteria for statistical significance was set at $P < 0.05$.

1.4 Results

The udder health status assessed by CMT and SCC is shown in Table 1. CMT was negative in 488 of 606 quarter milk samples (80.5%) with mean SCC of 94.5 x 10^3 cells/ml in healthy lactating cows. The positive CMT with a score from trace to 4 were noted in 118 quarter milk samples (19.5%). In 92 quarter milk samples with CMT score 1 - 4 was increased the SCC over the regular limit (400 x 10^3 cells/ml) at mean 505.1, 861.6, 1695.5 and 5724 x 10^3 cells/ml, respectively.

Numbers and percentages of isolates grouped according to the severity of mastitis are shown in Table 2. From a total of 76 infected quarter milk samples, 36 samples were found positive for CNS, 15 for coagulase-positive staphylococci (CPS), 8 for *Streptococcus* spp., and 9 for other bacteria. CNS (*S. chromogenes*, *S. haemolyticus*, *S. warneri*, and *S. xylosus*) were the most abundant etiologic factors (47.4% of mastitis). The SM was noted in 50 milk samples (8.3%). SM caused by staphylococci were represented by 43.4% of all mastitis samples. At quarter levels, 8 (1.3%) and 18 (2.9%) quarters were classified as latent and clinical mastitis, respectively. The most common pathogens in CM were *S. aureus*, *Str. uberis* and *S. chromogenes.*

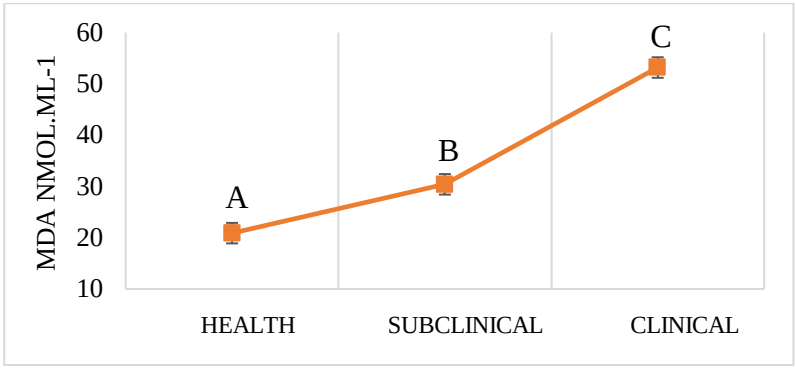
Table 2. Isolated microorganisms from infected quarters in monitored herd

Isolated microorganisms	n	%	Latent[1]		Subclinical[2]		Clinical[3]	
			n	%	n	%	n	%
***Staphylococcus* spp.**								
S. aureus	9	*1.5*			3	*0.5*	6	*1.0*
S. chromogenes	13	*2.1*	2	*0.3*	8	*1.3*	3	*0.5*
S. haemolyticus	8	*1.3*			6	*1.0*	2	*0.3*
S. warneri	8	*1.3*	2	*0.3*	6	*1.0*		
S. xylosus	7	*1.2*	1	*0.2*	6	*1.0*		
S. intermedius	6	*1.0*	2	*0.3*	4	*0.7*		
***Streptococcus* spp.**								
Str. uberis	4	*0.67*					4	*0.6*
Str. faecalis	2	*0.3*			2	*0.3*		
Str. suis	2	*0.3*			2	*0.3*		
Other bacteria								
E. coli	4	*0.7*	1	*0.2*	2	*0.3*	1	*0.2*
Pseudomonas spp.	3	*0.5*			3	*0.5*		
Enterobacter aerogenes	2	*0.3*			2	*0.3*		
Mixed infection*	8	*1.3*			6	*1.0*	2	*0.3*
Total	**76**	***12.5***	**8**	***1.3***	**50**	***8.3***	**18**	***2.9***

Note: n - number of isolated bacteria from 606 examined quarters, Mixed infection* - mixed infection caused two or more bacteria, Latent mastitis[1] – are characteristic with normal milk consistency, but infection is present in samples of raw milk without changing of SCC and negative CMT score, Subclinical mastitis[2] - no signs are observed, the udder and milk appears normal, but infection is still present with positive CMT score and increased SCC. Clinical mastitis[3] – signs range from mild to severe with positive CMT score, high level of SCC, positive bacteriological cultivation, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production with clinical signs.

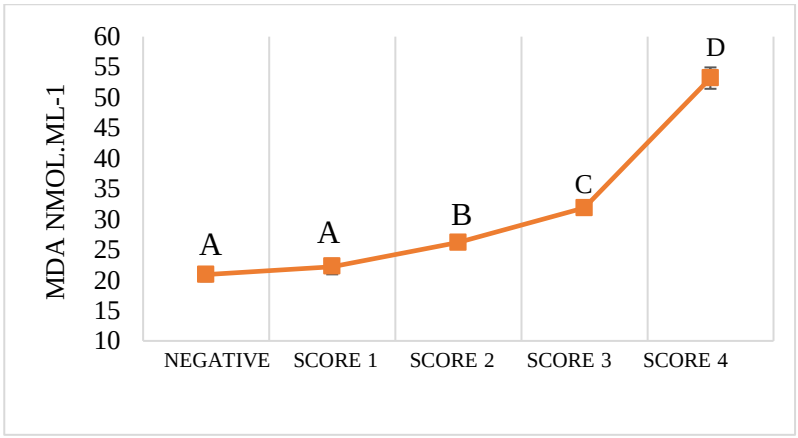
The differences in lipid peroxidation products (MDA) concentration in the milk of dairy cows with SM and CM are shown in Graph 1. Increased ($P < 0.01$) milk MDA concentrations were found in the CM and SM milk samples. Graph 2 shows milk MDA concentrations from CMT-positive quarters with scores 1 - 4. The highest MDA concentrations were observed in milk from positive quarters with CMT score 4.

Samples with CMT scores from 2 to 4 had a significantly higher ($P < 0.05$) concentration of MDA when compared to samples negative for CMT (Graph 2). The differences in erythrocyte antioxidant enzymes activity among selected groups of dairy cows are shown in Graph 3 and 4. There was significant decrease ($P < 0.05$) in erythrocyte GPx and SOD activities as well as a significant increase ($P < 0.05$) in MDA concentration in CM cows. SOD and GPx activities in the blood of SM cows did not differ from those of control animals.



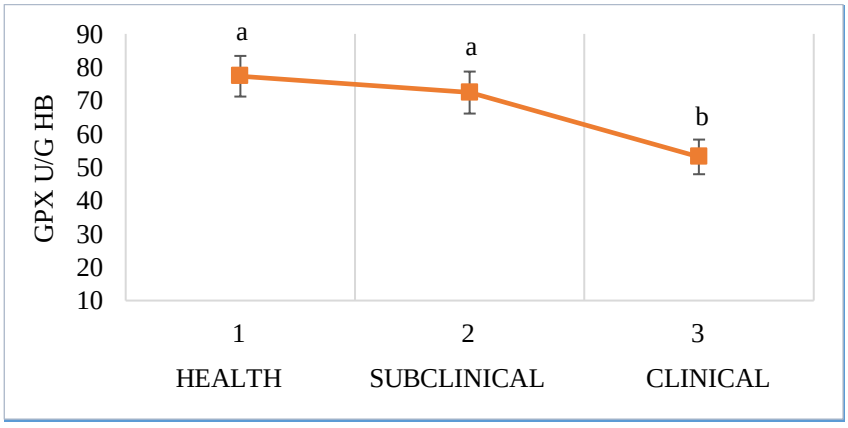
Graph 1. Milk malondialdehyde (MDA) concentrations (nmol.ml^{-1}) **separated by severity of mastitis**

Note: $^{A-C}$Different superscripts indicate that means of MDA differed significantly ($P < 0.01$).



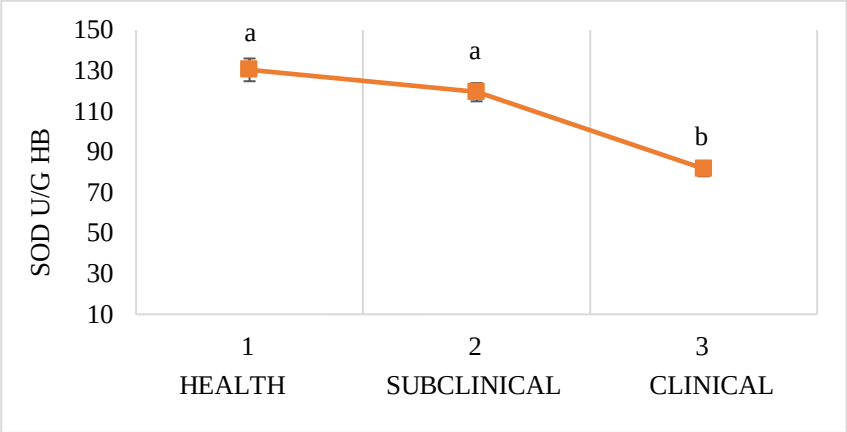
Graph 2. Comparison of malondialdehyde (MDA) concentrations (nmol.ml^{-1}) **from healthy and quarters with positive CMT score**

Note: Statistical analysis by Post Hoc Test were used to compare milk MDA concentrations from negative (healthy) quarter milk samples with SCC 94.5 x 10^3 per ml and positive quarter milk samples with CMT score 1 – 4 in SCC 505.1, 861.6, 1695.5 and 5.724 x 10^3 per ml, respectively. Variable MDA probabilities for Post Hoc Tests - error: between MS = 5.97, df = 45.000 were used. $^{A-D}$Different superscripts indicate that means of MDA concentrations differed significantly ($P < 0.01$).



Graph 3. Comparison of erythrocyte glutathione peroxidase (GPx) activity separated from healthy and affected cows

Note: [a,b]Different superscripts indicate that activity of GPx differed significantly (P < 0.05).



Graph 4. Comparison of erythrocyte superoxide dismutase (SOD) activity separated from healthy and affected cows

Note: [a,b]Different superscripts indicate that activity of SOD differed significantly (P < 0.05).

1.5 Discussion

The prevalence or number of infected cows (quarters) is often unknown to most farms. As opposed to clinical mastitis, SM is characterized by no obvious signs and can only be detected by the microbiological examination of milk or determination of other milk abnormalities. The most common methods are evaluation CMT with SCC in milk (Turk et al., 2017).

In Europe, the ECC directive 92/46 from 1992 states that milk with SCC over 4 x 10^5 cells ml^{-1} cannot be used for human consumption. Generally, the SCC greater than 2 x 10^5 cells ml^{-1}, has been used as an indicator for udder inflammation and oxidative damage. In the present study, we noted a negative CMT score in 488 from 606 investigated quarter milk samples (80.5%) with SCC under 100 x 10^3 cells ml^{-1} in healthy lactating cows. The SCC over the regular limit with the positive CMT scores 1 - 4 was present in 92 quarter milk samples (15.2%) ranging from 505.1 to 5724 x 10^3 per ml.

Increased SCC may be associated with the generation of larger amounts of ROS which are considered the main factors responsible for lipid peroxidation. The products of lipid peroxidation thus may be utilized as indicators of oxidative stress in tissues and secretions. In our experiment, the increased SCC in milk was positively associated with milk MDA concentration. Milk with higher SCC was shown to have more infiltrated PMNs causing an increase in a rate of oxidative reactions and apoptosis. Previous studies have shown the relationship between SCC and MDA concentration in both quarter milk and bulk tank milk samples (Suriyasathaporn et al., 2006, 2010).

Moreover, a recent study showed that milk MDA concentration was a marker of a decreased milk yield in cows with CM and high SCC (Sharma et al. 2011). In our study, not only milk from

cows with CM but also with SM had higher MDA concentrations as compared to milk from healthy udders. Therefore, it could be a useful indicator of subclinical mastitis. The mean concentrations of MDA were higher in SM milk when compared with normal milk, and MDA would be considered as an indicator of SM udders. Similar results were reported by Suriyasathaporn et al. (2012), who found the increased milk MDA levels in cows with SM and CM infected with staphylococci or streptococci.

High-producing dairy cows are highly susceptible to IMI caused by *Staphylococcus aureus,* CNS or *Streptococcus* spp., and losses in milk yield are related to an increase in composite milk SCC. Shaheen et al. (2016) reported that Staphylococci were the predominant organisms isolated from mastitis milk samples followed by Streptococci, *E. coli Pseudomonas* spp. and *Klebsiella* spp. The isolated pathogens from milk samples in our study are in accordance with previous findings. From infected quarter milk samples were the most frequently found positive for CNS (47.4%), *S. aureus* (11.8%), Streptococci (11.0%) and for other bacteria (11.8%). In many countries, the problem of environmental mastitis caused by CNS has gradually increased since 2005. More and more frequently, CNS have been acknowledged as a cause of SM and CM in dairy cattle (Contreras et al., 2007; Pyörälä and Taponen, 2009; Lange et al., 2015). The results of bacteriological evaluation of milk in our experiment revealed predominance of CNS such as *S. chromogenes, S. haemolyticus S. warneri,* and *S. xylosus,* and are in accordance with the previous reports. The pathogenicity of the different CNS species varies widely. Generally, these bacteria as major mastitis-causing pathogens in cows are rather associated with a low-grade infection, a subclinical state, which affects 10 - 15% of lactating animals and is characterized by an increase in SCC, reduction in milk production and high bacterial content in milk (Monday and Bohach, 1999).

Although CNS usually cause relatively mild clinical signs and their pathogenicity is lower than that of *S. aureus,* in many cases persistent SM or chronic mastitis caused by *S. chromogenes* and *S. warneri* lead to a decrease in antioxidant potential and is associated with accumulation of ROS and oxidation products (Castillo et al., 2006; Sharma et al., 2011; Zigo et al., 2019).

Synthesis of reactive oxygen spec and their accumulation are controlled by antioxidant defense systems. Several mechanisms are available to prevent oxidative damage including enzymatic scavengers as GPx and SOD (Andrei et al., 2011; Celi, 2011).

According to Bernabucci et al. (2005) the decreased erythrocyte SOD and GPx activities after calving and during the lactation in dairy cows indicate higher oxidative stress and lower antioxidant status. In our study, the SOD and GPx activities were decreased in cows with CM, which was probably caused by the depletion of antioxidant enzymes resulting from increased ROS generation rate by the inflamed gland. A significant decrease in SOD and GPx activity was also observed in infected milk in the study by Sharma et al. (2010) on bovine staphylococcal mastitis. In our study

SOD and GPx activities in blood from cows with SM were similar to those in healthy controls probably due to the low severity of inflammation in the affected mammary glands in SM. However, induction of oxidative stress was detected in cows with SM (Turk et al., 2012).

In the studies of Castillo et al. (2006) and Sharma et al. (2011) the activity of GPx has been confirmed to associate closely with the antioxidant capability of the body. However, the enhanced plasma GPx activity, which was accompanied by the increase of plasma MDA level, was observed in cows from 30 days after calving as compared to cows from 30 days before calving. On the other hand, decreased activity of GPx and SOD were observed in cows with CM compared to healthy cows at the same stage of lactation.

1.6 Conclusion

The results of the present work showed that bacteria such as CNS, *S. aureus, Str. uberis*, and *E. coli* were the principal factors causing IMI leading to increased SCC and lipid peroxides in the milk of affected cows. On the other hand antioxidant enzyme activities were lower only in cows with CM compared to health cows. In conclusion, CM leads to systemic oxidative stress with depletion of enzymatic antioxidant mechanisms and lipid peroxidation products accumulation, while in SM oxidative stress seems to be more restricted to the mammary gland. In both forms of mastitis milk quality is affected by the accumulation of oxidation products, which positively correlates with the SCC. Therefore the measurement of milk MDA concentration could be a potential biomarker for better screening of udder health in early diagnosis of mastitis.

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2 Chapter

DAIRY COWS' UDDER PATHOGENS AND OCCURRENCE OF VIRULENCE FACTORS IN STAPHYLOCOCCI

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Abstract: This chapter includes a study in which were investigated 960 Slovak and Czech spotted cattle from four different conventional (non-organic) dairy herds located in Eastern Slovakia and Czechia during early lactation (14–100 days after calving). Dairy cows were examined clinically; milk from fore-stripping of each udder quarter was subjected to sensory examination and assessed by the California mastitis test (CMT), and laboratory analyses of bacterial pathogens in milk, including virulence factors, were conducted. Positive CMT score (1–3) for one or more quarters were detected in 271 (28.2%) of the examined animals. Out of 230 infected milk samples, representing 24.0% of all dairy cows, staphylococci (59.1% of positive findings) were the most commonly isolated organisms, followed by *E. coli* (11.3%), streptococci *Str. uberis* (9.1%) and *Str. agalactiae* (3.4%), and enterococci (6.1%). From 136 isolates of *S. aureus* (38 isolates) and non-aureus staphylococci (NAS; 98 isolates), virulence factors and their resistance to 14 antimicrobials were detected using the disk diffusion method, with PCR detection of the methicillin resistance gene, *mecA*. An increased incidence of clinical and chronic forms of mastitis has been reported in mastitic cows in which staphylococci, especially *S. aureus* and NAS *(S. chromogenes, S. warneri* and *S. xylosus),* have been detected and compared to other isolated udder pathogens. From those species, *S. aureus* and isolates of NAS mentioned above showed multiple virulence factors that are more likely to hydrolyze DNA, hemolysis, produce gelatinase and biofilm, and have multi-drug resistance as compared to other less virulent staphylococci. Generally, the isolated staphylococci showed 77.2% resistance to one or more antimicrobials, in particular to aminoglycosides, β-lactams, macrolides, or cephalosporins. Especially, isolates that showed the ability to form a biofilm were more resistant to more than one antimicrobial than isolates without biofilm production. Multi-drug resistance to three or more antimicrobial classes was recorded in 16 isolates (11.7%), and the presence of the *mecA* gene was also confirmed in two isolates of *S. aureus* and two species of NAS.

2.1 Introduction

Dairy farming and milk production are among the basic pillars of livestock production and comprise the second biggest agricultural sector in the EU, representing more than 12% of total

agricultural outputs. In European countries, dairy farmers produce around 170 million tons of milk per year, 97% of which is cows' milk and 3% milk from ewes, buffalo, and goats. The EU rules emphasize that raw milk must come from healthy animals because the safety of dairy products is important in decreasing the risk of foodborne diseases (Augère-Granier, 2018).

Despite efforts by farmers to improve cows' breeding environments and hygiene levels during milking, inflammation of the mammary gland, known as mastitis, is still one of the main health problems for animals and causes economic losses for breeders. Infectious and non-infectious influences can cause mastitis. Infectious influences are mostly of microbial origin, and up to 95% of mastitis is caused by pathogenic bacteria that penetrate the teat canal into the mammary gland. In comparison with most other animal diseases, mastitis differs in the fact that several diverse kinds of bacteria can cause the infection. These pathogens are capable of invading the udder, multiplying there, and producing harmful, inflammation-causing compounds (Tančin et al., 2020; Čobirka et al., 2020).

The most common forms of mastitis are caused by agents from two groups of bacteria. The group of contagious pathogens (e.g., *Staphylococcus aureus, Streptococcus agalactiae*, and *Streptococcus dysgalactiae*) includes bacteria that survive and grow within the mammary gland (MG), and thus the infection can spread from infected to uninfected quarters and from cow to cow. This occurs most frequently during milking (Dufour et al., 2019). The group of environmental pathogens consists of a variety of species, including some staphylococcal species. They prosper in the environment, especially in the presence of cow feces and skin (Zigo et al., 2021a).

Of this group, *Str. uberis*, non-aureus staphylococci (NAS), and *E. coli* are the most important, with multiple strains of varying pathogenicity for animals and humans (Zigo et al., 2021b). Cobirka et al. (2020) reports that many mastitis pathogens, such as *S. aureus*, *Str. agalactiae* and *Str. uberis*, can be classified as contagious or environmental pathogens because they can be transmitted through multiple routes not only through contagious milk from infected cows or poor hygiene during milking, but also via bedding, urine, feces, and other contaminants.

In general, each mastitis case is believed to be caused by one primary pathogen, as only one bacterial species is identified in the milk samples from affected udders. However, it is not rare to detect simultaneous infections by two different pathogen species, and even three pathogens have been found in a small proportion of samples (Singha et al., 2021).

In recent years, *S. aureus* and NAS belong to the most common microorganisms causing mastitis in dairy cows (Condas et al., 2017; Monistero et al., 2018; El-Diasty et al., 2019). The manifestations of the inflammatory process caused by staphylococci are different, as they depend on the degree of reaction of the udder tissue to injury or infection. The clinical or subclinical manifestations of mammary gland infection, as well as its further course, depend on the interplay of

immunity with the immune response of the dairy cow and the concentration and virulence of the staphylococcal strains (Monistero et al., 2018).

The signs of clinical mastitis (CM) are sudden onset with redness and swelling of the udder, with altered milk from the affected quarter, containing flakes or clots with higher somatic cell count (SCC), or having a watery consistency. On the other hand, subclinical mastitis (SM) is characterized by a lack of visible signs in the udder or the milk, but the infection is still present. Affected animals with SM are associated with reduced milk production in the range of 60–140 liters per cow annually, with an increased SCC of over 200,000 cells/mL (Čobirka et al., 2020).

The main complications associated with the treatment of intramammary staphylococcal infection include the fact that many strains can exhibit multiple virulence factors, some of them related to the severity of mastitis, which is controlled by a complex network of transcriptional regulatory factors. The ability of staphylococci to form biofilms and resistance to antimicrobials is one of the key factors in virulence that facilitates the adherence and colonization of these pathogens in the mammary gland epithelium, with ineffective antimicrobials treatment (Zigo et al., 2021b).

The increase in resistance also occurs because, in addition to treating clinical cases of intramammary infection (IMI), the common routine on farms is to dry dairy cows across the board with antimicrobials. The largest proportion is administered for the intramammary treatment of CM and dry cow therapy. According to a study by Ferroni et al. (2020) management practices are associated with increased antimicrobials used in dairy cows. The authors analyzed 101 beef and dairy cattle farms in central Italy and compared the overall average antimicrobial consumption during one year. The total course of administered antimicrobials was three times higher in the case of dairy cows than in beef cows. The increased number was mainly related to the treatment of lactating and drying cows with antimicrobials.

The studies Holko et al. (2019), Idriss et al. (2013), and Zigo et al. (2021b), performed on Slovak dairy farms, confirmed the increased resistance of mainly udder pathogens (*S. aureus, S. uberis and S. agalactiae)* as well as NAS to those antibiotics that are part of the intramammary applicators used to treat dry cows.

On the other hand, NAS is considered to be minor pathogens in dairy mastitis; however, there are studies published by several authors that emphasize their role in the development of MG inflammation (Grinberg et al., 2004; Nascimento et al., 2005; Vasil' et al., 2012; De Buck et al., 2021; Fergestad et al., 2021). This heterogeneous group of bacteria consists of 54 species, of which at least 42 have been isolated from bovine-associated habitats such as quarter milk, teat apices, and/or rectal feces from dairy cows. They are also abundantly present in the cow's environment, with every habitat and niche having its specific NAS distribution. Research is still ongoing to unravel the species-

specific ecology and epidemiology and to study the host–microorganism interaction (De Buck et al., 2021).

An increase in the occurrence of NAS on farms was observed after a decrease in the incidence of mastitis caused by the main pathogens; the causative NAS showed increased resistance to common antibiotics and disinfectants. Compared to *S. aureus*, NAS usually exhibits a lower number of virulence factors. The essential factor of pathogenicity of NAS is biofilm formation, which allows them to survive the application of disinfectants and other sanitation procedures (Fergestad et al., 2021). Nascimento et al. (2005) reported that the NAS (*S. epidermidis*, *S. saprophyticus*, *S. hominis* and *S. aerletae*) isolated from mastitic cows were resistant to the antimicrobials used to treat cows during lactation and were able to produce some staphylococcal enterotoxins.

In particular, multi-resistant strains of staphylococci associated with resistance to more than one antimicrobial class are a serious risk to public health (Condas et al., 2017). Recent studies also suggest that multi-resistant staphylococci, especially β-lactams antibiotics indicate the presence of methicillin-resistant staphylococci (MRS), which have been identified in raw milk and dairy products, including cheeses (De Buck et al., 2021; Regecová et al., 2021). According to the WHO, the opportunistic ability of MRS strains to cause mastitis is a threat to public health. They may become a source of zoonotic infections, serving as a potential source of antimicrobial resistance genes for humans in contact with dairy cows (Fergestad et al., 2021); however, the opposite may also occur with humans being a source of MRS to cows (Grinberg et al., 2004). Of the MRS of concern, *Staphylococcus aureus* (MRSA) is the species most widely reported; however, in several studies, NAS was also identified as MRS isolates (Haveri et al., 2007; De Buck et al., 2021; Fergestad et al., 2021).

In addition to the increased antibiotic resistance of staphylococci, Haveri et al. (2007) and Vasil et al. (2012) confirmed biofilm formation and hemolysis from infected milk samples and considered them as important virulence factors involved in the development of mastitis.

2.1.1 Objectivites of this chapter

Previous studies indicated the importance of staphylococci and their virulence factors in the pathogenesis of mastitis and its clinical presentation. Therefore, the study aimed at determination of the occurrence and etiology of mastitis in four dairy herds, with the detection of selected virulence factors such as the production of gelatinase, hemolysis, and biofilm, the ability to hydrolyze DNA, and resistance to antibiotics, with the detection of the methicillin resistance gene *mecA* in isolated staphylococci.

2.2 Materials and Methods

2.2.1 Monitored Dairy Farms

The practical part of the study was carried out using four different dairy herds located on farms in Slovakia and Czechia. The four farms were selected because they practiced dairy cow breeding with conventional (non-organic) farming using nationals breeds of cattle, to facilitate the detection of contagious and environmental udder pathogens. Slovak and Czech spotted cattle are breeds of combined utility, used both for dairy and meat production, with milk yields of 6.5 – 7.5 kg x 10^3 per lactation. Both breeds are part of the worldwide population of the Simmental breed, which is widespread in Slovakia and the Czech Republic. Two dairy herds from eastern Slovakia (Presov region) were investigated, ranging in size from 250 to 350 dairy cows of Slovak spotted cattle between the 1st and 4th lactation, with an average daily milk yield of 21.6 +/- 2.4 L and 23.2 +/- 3.1 L, respectively. In Czechia (Moravian-Silesian region) two dairy herds of Czech spotted cattle were investigated, ranging in size from 200 to 300 cows between the 1st and 4th lactation, with an average daily milk yield of 18.7 +/- 2.8 L and 22.1 +/- 3.9 L, respectively.

The dairy cows investigated on all four farms were kept in a free housing system on straw litter, with ad libitum access to water. They were fed a mixed feed based on silage, hay, and concentrate, in agreement with the nutritional requirements of dairy cattle (NRC) (2001). The exact amount of feed was determined by lactation performance, and the rations met the nutritional requirements of cows weighing 650 kg and with average milk yield of 20–30 L per day. All cows were milked twice daily in parallel (Boumatic, USA) or herring bone (DeLaval, Sweden) parlors.

2.2.2 Dairy Cow Selection and Udder Health Examination

A total of 270 cows from the first and 215 cows from the second Slovak farm, and 250 cows from the first and 225 cows from the second Czech dairy farm were monitored in the same time. The dairy cows were selected on the basis of the formation of production groups according to the stage of lactation (early lactation 14–100 days of lactation) and the phase nutrition, which were compiled by the zootechnician on each farm. The selected dairy cows of the same performance class (early lactation) were housed in individual husbandry groups, which included 45–90 animals on each farm. Each dairy cow from the selected husbandry group was comprehensively examined on the basis of a clinical examination, with sensory examination and palpation of the udder, and milk from the fore-stripping of each udder quarter was subjected to sensory examination and assessed by the California mastitis test (CMT) (Indirect Diagnostic Test, Krause, Denmark) (Tančin, 2013) with the collection of raw milk samples from positive cows (Holko et al., 2019). Subsequently, from the 960 examined cows, 689 cows had a negative CMT score, and 271 cows, based on clinical manifestations and with a CMT score indicating trace or positive (score of 1–3) were chosen for aseptic collection of 12 mL

mixed quarter milk samples for laboratory analyses of bacterial pathogens, according to Holko et al. (2019). The samples were cooled to 4 °C and immediately transported to the laboratory and were analyzed on the following day.

Each mastitis case was assigned a corresponding mastitis grade according to the National Mastitis Council (2001), with the mastitis grade categorized into severity levels. Subclinical mastitis (SM) was detected by high somatic SCC using CMT evaluation without any visible abnormalities of the milk and apparent signs of local inflammation or systemic involvement. Clinical mastitis (CM) was classified as mild mastitis (CM1), being characterized by visible changes in secretion, moderate mastitis (CM2), additionally showing local signs of inflammation of the mammary gland, and severe mastitis (CM3), also showing general signs such as fever, low temperature, loss of appetite or inability to stand. Chronic mastitis or persistent mastitis was detected based on history (previous treatment) of clinical examination of the udder with a positive CMT score.

2.2.3 Cultivation and Determination of Bacterial Pathogens

The 10 µL aliquots of all milk samples were inoculated onto plates with esculin blood agar (Oxoid, Hampshire, UK) and MacConkey (MAC) agar (Oxoid, Hampshire, UK), and the plates were cultivated aerobically at 37 °C and checked after a 24-hour and 48-hour incubation period. If more than 2 phenotypically different colony types were present, the milk sample was considered contaminated and rejected. The primocultivated colony from blood agar and identification of *Staphylococcus* spp. were sub-cultured onto different selective bacteriological media (No. 110, Baird-Parker agar, Brilliance UTI Clarity Agar, Oxoid, Hampshire, UK) and incubated at 37 °C for 24 hours. The esculin hydrolysis, pigment formation, catalase positivity (3% H_2O_2), Gram positivity, and creation of free or coupled coagulase were determined according to studies by Holko et al. (2019) and Vasiľ et al. (2012). All presumptive *S. aureus* and NAS were identified by a matrix-assisted laser desorption/ionization (MALDI-TOF) biotyper (Bruker Daltonics, Leipzig, Germany). Mass spectrometry measurements were carried out on bacterial extracts prepared according to manufacturer′s instructions. MALDI-TOF analysis started with spotting one colony onto a ground steel target (Bruker Daltonik GmbH, Leipzig, Germany), followed by air drying for 15 min. Each sample spot was then overlaid with 2 µL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid), and again subjected to air drying for 15 min. The identifying of the relevant microorganisms consisted of importing the raw spectra obtained for individual isolates into the BioTyper software, version 2.0 (Bruker Daltonik GmbH, Leipzig, Germany). They were then analyzed without any further intervention by the user [24]. As a control for good quality, standards *S. aureus* CCM 4750 and *S. chromogenes* CCM 3386 (Czech Collection of Microorganisms, Brno, Czech Republic) were used.

The streptococci were determined on the basis of minute transparent colonies on blood agar and sub-cultured on Edward's agar medium (Oxoid, Hampshire, UK). The pure colonies were described based on their growth, with color, classic morphological and hemolytic characteristics. Suspected streptococci microscopically appeared as Gram-positive cocci, either in long or short chains. Standard biochemical tests, including catalase, sodium hippurate, and esculin hydrolysis, were carried out according to El-Aziz et al. (2021).

The presence of enterococci from primocultivation was confirmed by Gram-staining and sub-cultivation on MAC agar and SlaBa-plates agar (Slanetz & Bartley, Medium, Oxoid Ltd., Basingstoke, UK), with the growth and color of typical colonies. Confirmed colonies of *Streptococcus* spp. and important strains of family *Enterobacteriaceae* were biochemically identified to the species level using the STREPTOtest 24 and ENTEROtest 24 (Erba Lachema, Brno, Czech Republic) and evaluated according to the manufacturer's instructions by the software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic) with a probability of correct designations of the species above 90%.

2.2.4 DNase Test and Detection of Biofilm and Hemolysis in Staphylococci

Confirmed staphylococci based on MALDI-TOF analysis was exposed to deoxyribonuclease (DNase test) according to Hiko (2019). An overnight cultured colony was inoculated in the form of lines on DNase agar (OXOID Ltd., Basingstoke, Hants, UK) and incubated for 18–24 hours at 37 °C. The culture was over-flushed with 1 mL 1 mole/mL hydrochloric acid. The strains were determined as DNase positive, based on a DNA digestion zone of clear transparence surrounding the culture (Figure 1). The formation of biofilm was determined by phenotypic method by growth on Congo Red agar (CRA) according to Vasil' et al. (2017). The production of slime by all strains was compared by cultivation of the staphylococcal strains on CRA plates consisting of 0.8 g of CRA and 36 g of saccharose in 1 L of brain-heart infusion agar after incubation at 37 °C for 24 h and subsequently overnight at room temperature. The slime-producing strains form black colonies on CRA, whereas the non-producing strains develop red colonies (Figure 1).

The ability of staphylococci to produce of hemolysins was also determined. According to Moraveji et al. (2014), types of hemolysis were phenotypically characterized based on the lysis zone of each staphylococcal isolate on plates of blood agar base supplemented with 5% sheep blood after 24 and 48 h incubation at 37 °C.

2.2.5 Detection of Sensitivity to Antimicrobials in Staphylococci

The susceptibility of staphylococci (n = 136) isolated from milk from the investigated cows was tested *in vitro* against 14 antimicrobial agents. The susceptibility tests of isolates were carried

out on Mueller Hinton agar using a standard disk diffusion procedure (CLSI, 2018). The antibiotic discs used in the current study were penicillin (PEN;10 µg), ampicillin (AMP; 10 µg), amoxicillin (AMC; 10 µg), amoxicillin+clavulanic acid (AXC; 20/10 µg), ceftiofur (CEF; µg), oxacillin (OXA; 1 µg), cefoxitin (CFX; 30 µg), ciprofloxacin (CPR; 5 µg), lincomycin (LNC; 15 µg), neomycin (NMC; 10 µg), novobiocin (NVB; 5 µg), rifaximin (RFX; 15 µg), streptomycin (STR; 10 µg) and tetracycline (TET; 30 µg). The zone of inhibition was recorded in millimeters, and results were interpreted as previously described. The determined diameters of the respective inhibition zones were evaluated (susceptible, intermediate, resistant) according to CLSI breakpoints (CLSI, 2021). In the tests, the tribes *S. aureus* CCM 4750 and *S. chromogenes* CCM 3386 (Czech Collection of Microorganisms, Brno, Czech Republic) were used as a control. The choice of antimicrobials reflects the range contained in a number of intramammary products to treat mastitis, which are available in Slovakia and Czechia.

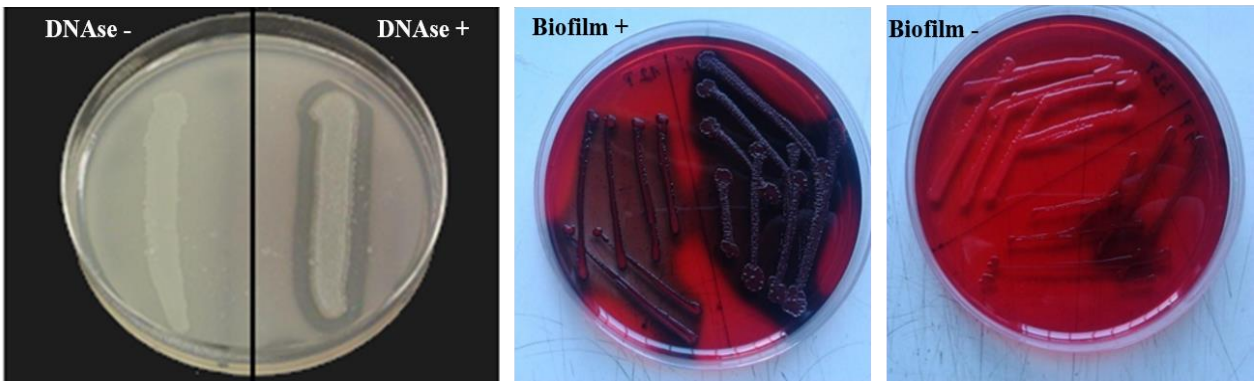

Figure 1. DNase test and biofilm production on Congo Red agar for staphylococcal detection

Source: Zigo et al. (2021a).

2.2.6 Detection of the mecA gene from Isolated Staphylococci

Phenotypical positive *S. aureus* (18 isolates from 38) and NAS (29 isolates from 98) based on their antimicrobial resistance to β-lactams antimicrobials were subjected to PCR to test for methicillin resistance. Total genomic DNA was isolated according to Hein et al. (2005). DNA quality was checked using a BioSpec spectrophotometer (SHIMADZU, Japan).

The source of DNA obtained as supernatant with centrifugation was used in PCR reactions using primers MecA1 (GGGATCATAGCGTCATTATTC) and MecA2 (AACGATTGTGACACGATAGCC) (Amplia s.r.o, Bratislava, Slovakia) for detection of the *mecA* gene according to Poulsen et al. (2003). Confirmation of the identity of the PCR products (527 bp) with the selected primers was in accordance with the instructions specified by GATC Biotech (AG, Cologne, Germany) using Sanger sequencing. The similarity of the DNA sequences obtained from

the isolates with those available from the GenBank–EMBL (the European Molecular Biology Laboratory) database were determined using the BLAST program (NCBI soft-ware package). *S. aureus* CCM 4750 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a reference strain for PCR in this study.

2.3 Statistical Analysis

Data were entered into Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) and analyzed using Excel, State 11, and SPSS version 20 (IBM Corp., Armonk, USA). The dependence of the production of virulence factors on the most frequently isolated staphylococci from clinical, chronic and subclinical mastitis was statistically analyzed using the chi-squared test with the significance level $\alpha = 0.05$; critical value $\chi^2 = 2.206$; and testing value - G. Statistical independence between isolates with virulence factors and isolates without virulence factors within each species was confirmed when $G > \chi^2$; the independence was not statistically significant when testing values of $G < \chi^2$.

2.4 Results

An examination of four dairy herds showed that, of 960 dairy cows examined during the early lactation phase (14–100 days of lactation), 689 cows (71.7%) had a negative CMT score and 271 cows (28.2%) had a CMT score of trace or 1–3 for one or more quarters. Of the mixed quarter milk samples taken from each examined cow based on the anamnesis, a CMT score of trace or 1–3 was identified as bacterial agents causing a clinical or subclinical mastitis in 230 (84.8%) samples, and 41 samples (15.1%) were identified as negative or contaminated. Based on the clinical examination of the MG, assessment of CMT and laboratory diagnosis of milk samples, the prevalence of CM in the monitored first and second Czech dairy farms was 7.2% and 12.1%, respectively. In the monitored first and second Slovak dairy farms, the prevalence of CM was 5.9% and 10.1%, respectively. An increased incidence of chronic mastitis of 4.2% and 5.6% was reported in the second Slovak and Czech dairy farms, with a high prevalence of clinical forms of intramammary infection (Figure 2).

The results of the culture and identification of udder pathogens are shown in Table 1. Of the 960 samples taken from the four dairy farms, 230 were positive for udder bacterial pathogens. Of the positive sample, 136 cases (59.1% of the infected samples) contained the most commonly isolated staphylococci. The NAS represented the most commonly detected bacteria (42.6% of positive findings); *S. aureus* (16.5 %) were the second most abundant pathogens, followed by *E. coli* (11.3%), streptococci (*Str. uberis*: 9.1%; *Str. agalactiae:* 3.4%) and enterococci (6.1%). The most common

form of intramammary infection was subclinical mastitis, which accounted for 46.9% of all the positive samples.

In addition, 37.8% of the positive samples were clinical cases of mastitis, classified as mild mastitis, characterized by visible changes in secretion with a high score of CMT (24.7% of all positive cases), moderate mastitis (10.5%), additionally showing local signs of inflammation of the mammary gland, and severe mastitis (2.6%), with general signs on the body and udder. NAS (16.5% of all CM), *S. aureus* (7.8% of all CM) and *Str. uberis* (3.8% of all CM) contributed the most to the occurrence of clinical mastitis. Based on previous anamnesis and actual examination of udder health, chronic mastitis (15.2% of all positive cases) was caused primarily by *S. aureus* (4.7%), NAS (3.5%), *Str. uberis* (3.0%), *Str. agalactiae* (1.3%) and mixed infections (1.7%).

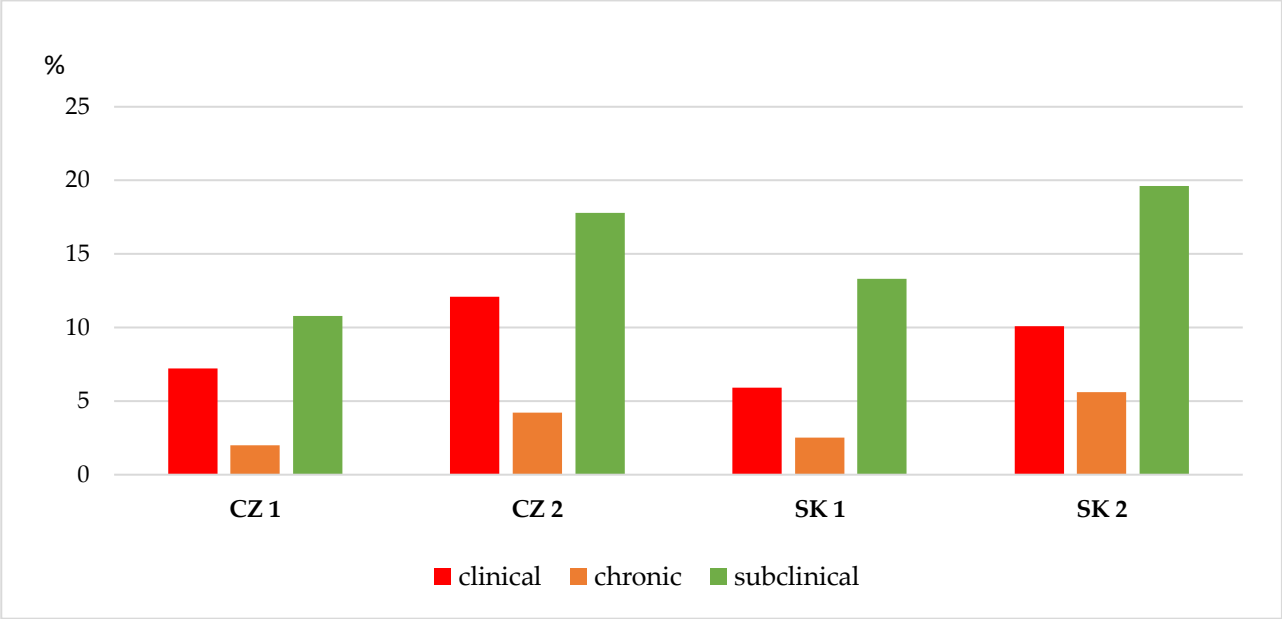

Figure 2. Prevalence of mastitis in monitored dairy herds during early lactation

Note: CZ 1-2: dairy farms situated in Czechia; SK 1-2: dairy farms situated in Slovakia. Subclinical mastitis: no signs are observed, the udder and milk appears normal, but an infection is still present with a positive CMT score and an increased SCC. Clinical mastitis: signs that are mild, moderate or severe. Chronic mastitis: detected based on history (previous treatment) of clinical examination of the udder and positive CMT score.

Table 2 summarizes, in descending frequency, the isolated strains of *Staphylococcus* spp., and indicates their role in the type of mastitis and the occurrence of selected virulence factors. *S. aureus* was isolated from 18 clinical, 11 chronic and 9 subclinical cases of mastitis and thus appeared to be the causative agent most frequently isolated with the highest ability to report virulence factors in our study. Isolated strains of *S. aureus* from clinical, chronic and subclinical forms showed hemolysis in blood plates, production of gelatinase, biofilm, and the ability to hydrolyze DNA. In two isolates of *S. aureus* from clinical mastitis, the *mecA* gene was detected. Eight species were isolated from NAS, with the following recorded as the most numerable species: *S. chromogenes* (22.4%), *S. warneri*

(20.4%), *S. xylosus* (18.4%), *S. epidermidis* (9.1%), *S. haemolyticus* (7.1%), *S. hyicus* (10.2%), *S. capitis* (4.4%) and *S. piscifermentans* (4.4%). The representation of NAS in the individual forms of IMI was different. Most frequently, cases of subclinical mastitis (39.0%) were detected, caused predominantly by *S. xylosus, S. hyicus, S. warneri. S. hyicus* and *S. epidermidis*. Clinical and chronic mastitis were detected in 37 cases (27.2%) and 8 cases (5.9%), respectively, caused in particular by *S. chromogenes, S. warneri* and *S. xylosus.*

Table 1. Udder pathogens isolated from milk samples of four monitored dairy herds

Pathogens	**Number of isolates**	**% n=230**	**Clinical IMI[1]** n/%			**Chronic IMI[2]**	**Subclinical IMI[3]**
			CM1	CM2	CM3	n/%	n/%
NAS	98	*42.6*	28/*12.2*	7/*3.0*	2/*1.3*	8/*3.5*	53/*23.0*
S. aureus	38	*16.5*	10/*4.3*	5/2.2	3/*1.3*	11/*4.7*	9/3.9
Escherichia coli	26	*11.2*	5/*2.2*	2/*0.9*	0/*0*	2/*0.9*	17/7.4
Streptococcus uberis	21	*9.1*	4/*1.7*	4/*1.7*	1/*0.4*	7/*3.0*	5/2.2
Streptococcus agalactiae	8	*3.4*	0/*0*	3/*1.3*	0/*0*	3/1.3	2/0.9
Streptococcus spp.	10	*4.3*	4/*1.7*	0/*0*	0/*0*	0/*0*	6/*2.6*
Enterococcus spp.	14	*6.1*	2/*0.9*	1/*0.4*	0/*0*	0/*0*	11/*4.8*
Mixed infection[4]	15	*6.5*	4/*1.7*	2/*0.9*	0/*0*	4/*1.7*	5/2.2
Total	**230**	***100***	**57/*24.7***	**24/*10.5***	**6/*2.6***	**35/*15.2***	**108/*46.9***

Note: Clinical IMI[1] - clinical intramammary infection (IMI), including mild (CM1), moderate (CM2) and severe forms (CM3) of mastitis; Chronic IMI[2] - chronic or persistent intramammary infection; Subclinical IMI[3] - subclinical intramammary infection. Mixed infection[4] - include a mix infection of two bacterial pathogens.

From all CM and chronic mastitis with NAS, 30 cases involved the production of hemolysins, 9 the hydrolysis of DNA, 7 the production of gelatinase, and 18 involved biofilm production. The production of biofilm was also found in 9 isolates of NAS from subclinical cases of mastitis. The significance level of $\alpha = 0.05$ was confirmed in the isolated stafylococci *S. aureus, S. chromogenes, S. warneri* and *S. xylosus* from CM and chronic mastitis, which had the most numerous representation of virulence factors (production of hemolysins, gelatinase, the ability to hydrolyze DNA and biofilm) in comparison to less virulent strains. In addition, the *mecA* gene was confirmed from one chronic case of mastitis in *S. chromogenes* and one CM case in *S. warneri*.

In 136 isolates of staphylococci, in vitro resistance to 14 antimicrobials was tested by the standard disk diffusion method (Table 3). Generally, low resistance was shown to tetracycline, amoxicillin reinforced with clavulanic acid, rifaximin and cephalexin. In three and two isolates of *S. aureus*, intermediate sensitivity to tested aminoglycoside and β-lactams antimicrobials was observed,

respectively. The intermediate sensitivity to aminoglycoside antimicrobials was observed in three isolates of *S. chromogenes*, two isolates of *S. warneri* and one isolate of *S. xylosus*. In two isolates of *S. chromogenes* and one isolate of *S. warneri* and S. sylosus, intermediate sensitivity to β-lactams antimicrobials was observed.

Table 2. The role of *S. aureus* and NAS in the form of mastitis and their virulence factors

Staphyloc. *spp.*/number	IMI[1]/number	Hemolysins[2]	DNAse[3]	Gelatinase	Biofilm	*mecA* gene	Testing value
S. aureus (38)	clinical (18)	6α/4δ/1β	14	15	9	2	5.447*
	chronic (11)	3α/2δ/2β	8	7	7	0	
	subclinical (9)	3α/1β	6	7	5	0	
S. chromogenes (22)	clinical (11)	4β/3δ	3	4	4	0	3.204*
	chronic (4)	3β	1	1	2	1	
	subclinical (7)	2β/2δ	1	1	2	0	
S. warneri (20)	clinical (9)	4δ/2β	2	2	4	1	2.688*
	chronic (3)	3β	0	0	1	0	
	subclinical (8)	3β/1δ	2	0	2	0	
S. xylosus (18)	clinical (7)	2δ/2β	2	0	3	0	2.255*
	chronic (1)	0	0	0	0	0	
	subclinical (10)	4β/1δ	0	0	2	0	
S. epidermidis (9)	clinical (2)	1δ	0	0	1	0	1.012
	subclinical (7)	2δ	0	0	2	0	
S. haemolyticus (7)	clinical (4)	2β/1δ	1	0	2	0	0.742
	subclinical (3)	0	0	0	0	0	
S. capitis (6)	clinical (2)	2δ	0	0	0	0	0.401
	subclinical (4)	0	0	0	0	0	
S. piscifermentans (6)	clinical (2)	1β	0	0	1	0	0.851
	subclinical (4)	2δ	0	0	0	0	
S. hyicus (10)	clinical (0)	0	0	0	0	0	0.332
	subclinical (10)	1δ	0	0	1	0	

Note: IMI[1]—number of isolates and their influence on type of mastitis; hemolysines[2]—production of hemolysin type α, β or δ; DNAse[3]—ability of staphylococci to hydrolyze DNA; *Chi-squared test significance level $\alpha = 0.05$; critical value $\chi^2 = 2.206$; Testing value (G) and statistical independence of virulence factors in isolated staphylococci was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$.

The obtained results presented in Table 3 show that the resistance of *S. aureus* and NAS, in particular to aminoglycosides and β-lactams, indicates the trend of increasing multi-antimicrobial-

resistant pathogens in monitored dairy farms. High resistance to streptomycin, neomycin, ampicillin, penicillin, amoxicillin, and oxacillin was observed in tested strains of *S. aureus* and NAS. Tested *S. aureus* (5 isolates) and NAS (10 isolates) demonstrated resistance to oxacillin. Resistance in 37 isolates was observed to other β-lactam antimicrobials.

Table 3. Antimicrobial resistance of *S. aureus* and NAS isolated from mastitic milk samples

Bacterial strains (n=136)	***S. aureus*** (n=38)		***S. chromogenes*** (n=22)		***S. warneri*** (n=20)		***S. xylosus*** (n=18)		**Other NAS*** (n=39)	
Antibiotic/resistance	*%*	n	*%*	n	*%*	n	*%*	n	*%*	n
Penicillin	*18.4*	7	*13.6*	3	*20.0*	4	*11.1*	2	*10.3*	4
Amoxicillin	*18.4*	7	*18.1*	4	*15.0*	3	*11.1*	2	*10.3*	4
Ampicillin	*23.6*	9	*18.1*	4	*20.0*	4	*16.7*	3	*15.4*	6
Amox. + clav.	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0
Oxacillin	*13.2*	5	*13.6*	3	*15.0*	3	*11.1*	2	*5.1*	2
Cefoxitin	*10.5*	4	*9.0*	2	*10.0*	2	*0*	0	*0*	0
Cephalexin	*10.5*	4	*9.0*	2	*0*	0	*0*	0	*0*	0
Ciprofloxacin	*10.5*	4	*9.0*	2	*10.0*	2	*11.1*	2	*0*	0
Lincomycin	*13.2*	5	*9.0*	2	*5.0*	1	*11.1*	2	*7.7*	3
Neomycin	*29.0*	11	*18.1*	4	*20.0*	4	*16.7*	3	*15.4*	6
Novobiocin	*18.4*	7	*18.1*	4	*10.0*	2	*77.8*	14	*7.7*	3
Rifaximin	*5.3*	2	*0*	0	*0*	0	*0*	0	*0*	0
Streptomycin	*29.0*	11	*27.3*	6	*25.0*	5	*11.1*	2	*17.9*	7
Tetracycline	*5.3*	2	*0*	0	*0*	0	*0*	0	*0*	0

Note: Other NAS*: *S. epidermidis, S. haemolyticus, S. capitis, S. piscifermentans, S hyicus*; n—number of tested isolates.

Table 4 shows the phenotypic resistance profile of 136 isolates of *Staphylococcus* spp. from infected milk samples. Of the tested staphylococci, 105 isolates (77.2%) showed resistance to one or more antimicrobials. To one antimicrobial, 51 isolates (37.5%) were resistant. To two or more antimicrobials, 54 (39.7%) isolates of all tested staphylococci were resistant. Multi-drug resistance to three or more antimicrobial classes were recorded in 16 isolates (11.7%). Tested staphylococci showed multi-resistance to a combination of antimicrobial classes, such as aminoglycosides, β-lactams, macrolides and cephalosporins.

Table 4. Phenotypic resistance profile in isolates of Staphylococcus spp. (n=136) **from mastitic cows**

Number group of antimicrobials	Phenotypic resistance profile	Number of isolates	% of isolates
0		30	22.1
1	STR	11	8.1
1	PEN	9	6.6
1	NMC	8	5.9
1	AMX	7	5.1
1	NVB	6	4.4
1	AMP	6	4.4
1	LNC	4	2.9
2	NMC, STR	9	6.6
2	AMP, NVB	4	2.9
2	CPR, NVB	2	2.2
2	LNC, NVB	2	1.5
3	PEN, AMX, OXA	4	2.9
3	AMP, AMX, OXA	3	2.2
3	PEN, LNC, NVB	2	1.5
3	AMP, OXA, NMC	3	2.2
3	AMP, AMX, NVB	4	2.9
3	PEN, AMX, AMC,	2	1.5
3*	NVB, LNC, STR	4	2.9
4	AMP, CEP, FOX, OXA	3	2.2
4*	RFX, CPR, STR, TET	2	1.5
4*	CPR, LNC, NMC, NVB	3	2.2
4*	NVB, CPR, NMC, STR	2	1.5
4*	AMP, CEP, FOX, PEN	3	2.2
5*	OXA, FOX, AMP, NMC, STR	2	1.5
Total ATBs resistant isolates		**105**	**77.2**

Note: *MDR- multi drug resistant isolates to three or more antimicrobial classes; AMX - amoxicillin, AMC - amoxicillin+clavulanat acid, AMP - ampicillin, CEP - cephalexin, CPR - ciprofloxacin, FOX - cefoxitin, LNC - lincomycin, NMC - neomycin, NVB - novobiocin, OXA - oxacillin, PEN - penicillin, RFX - rifaximin, STR - streptomycin, TET - tetracycline.

In particular, staphylococci with biofilm-forming ability were in most cases resistant to the tested antimicrobials. Figure 3 is comparison of resistance to more than one antimicrobials in isolates of staphylococci forming or non-forming biofilm at a time. Isolates *S. aureus* and NAS that showed the ability to form a biofilm were more resistant (66.7% in *S. aureus* and 51.8% in NAS) than isolates without biofilm production (29.4% in *S. aureus* and 25.7% in NAS).

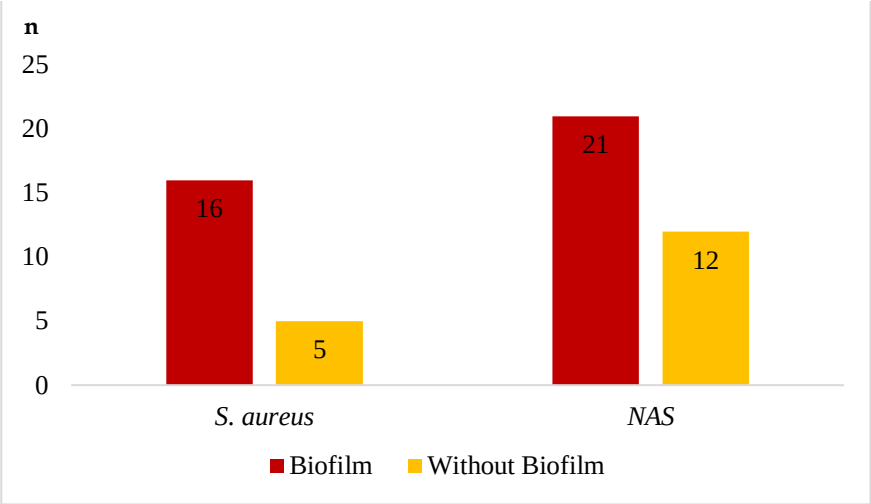

Figure 3. Comparison of resistance to more than one antimicrobials in isolates forming or non-forming biofilm at a time

Note**:** n – number of resistant isolates to more than one antimicrobials

The 47 isolates (34.6% of all isolated staphylococci) in which phenotypic resistance was confirmed to β-lactam antimicrobials were tested by PCR for methicillin resistance with the detection of the *mecA* gene (Figure 4). The presence of *mecA* gene was confirmed in four isolates of staphylococci (two isolates of *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri*), which at the same time showed resistance to both cefoxitin and oxacillin. Based on the results of our study, these isolates were considered as methicillin–resistant staphylococci (MRS).

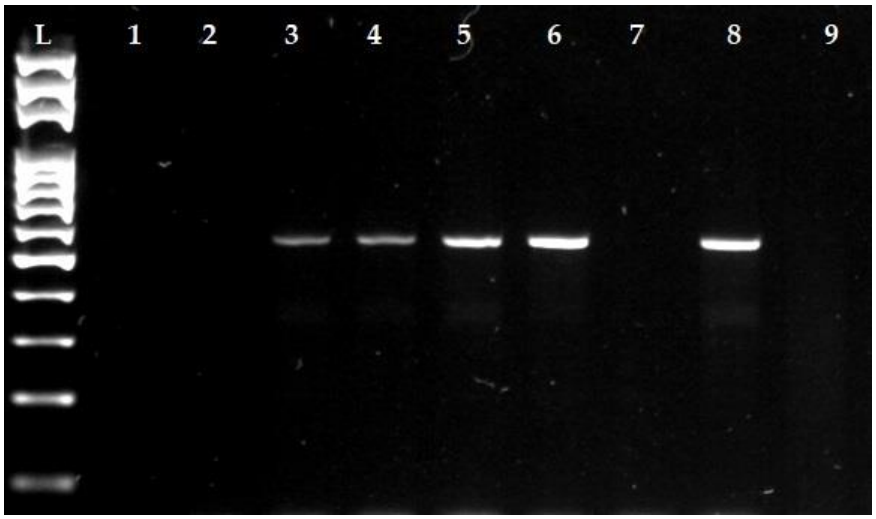

Figure 4. Detection of *mecA* gene in *Staphylococcus* spp. isolates isolated from infected raw milk samples using the PCR method (527 bp)

Note: L: 100 bp ladder; Line 1: isolate *S. xylosus* without *mecA* gene; Line 2: isolate *S. capitis* without *mecA* gene; Line 3: isolate *S. chromogenes* with *mecA* gene; Line 4: isolate S. *warneri* with *mecA* gene; Lines 5 and 6: isolate *S. aureus* with *mecA* gene; Line 7: isolate *S. haemolyticus* without *mecA* gene; Line 8: reference strain CCM 4750 *S. aureus* (positive control); Line 9: water (negative control).

2.5 Discussion

Mastitis is currently one of the main health problems of dairy cows, despite the increasing advances in technology and veterinary measures. The incidence of mastitis is, of course, highly dependent on the lactation stage (Singha et al., 2021; Nitz et al., 2021). In our study, we monitored the prevalence and etiology of mastitis in four dairy farms during the early lactation phase. Cows in this lactation stage (14–100 days after calving) represent the largest group in farms because milk production depends on them. The dairy cow produces a quantity of milk representing 42–45% of the total milk produced during the first 100 days of lactation. With such an enormous milk production burden, cows are exposed to stress factors, such as hormonal changes associated with lactogenesis, reduced dry matter intake (which is in contrast to the desired increasing milk yield), increased lipomobilization of body reserves with a negative energy balance and a change in body score (Nitz et al., 2021).

All the above-mentioned risk factors affect the non-specific and specific immune system, in particular, the MG, through which pathogenic microorganisms penetrate more easily from the external environment. With the onset of intramammary infection, one of the indicators is an increased SCC (Tančin, 2013) which was confirmed in our study. Of the 960 examined dairy cows, 689 (71.7%) cows, based on anamnesis, clinical examination, and evaluation of CMT, were negative and 271 cows (28.2%) showed trace or positive CMT, with a score of 1–3. Cows with a high SCC were in 84.9% cases (230 positive cows from 271 examined cows) positive for the presence of an udder pathogen, which poses a significant risk to the health of the individual and the spread of infection to the environment. Generally, IMI begins when pathogens pass through the teat canal, interact with the mammary tissue cells, multiply and disseminate in the cisterns and throughout the duct system. The manifestation of mastitis depends mainly on the degree of reaction of the udder tissue to injury or infection (Čobirka et al., 2020). Of the 230 infected cows, 46.9% were diagnosed with subclinical mastitis, 37.8% clinical (mild (24.7%), moderate (10.5%) or severe (2.6%) signs) or chronic (15.3%) mastitis (Table 1). The prevalence of CM in the present study was 5.9% and 10.1%, and 7.2% to 12.1%, in the monitored first and second Czech and Slovak dairy farms, respectively (Figure 3). The prevalence of clinical mastitis in the monitored Czech and Slovak dairy farms was approximately at the same level, from which we can conclude that the farms apply generally accepted procedures related to the breeding and milking of Simmental cattle.

According to Singha et al. (2021), CM represents a serious health problem that can result in the reduction of milk yield, milk quality deterioration, treatment costs, involuntary culling, death, increased risk of antimicrobial resistance, and reduced animal welfare. Therefore, the prevalence of CM should be at the lowest level in lactating cows. Our results indicate that the prevalence of CM in

monitored farms is in contrast with the studies of Silva et al. (2021) and Rahman et al. (2010), who reported the prevalence of clinical forms from 2.3% to 4.1% in lactating cows.

Wentz et al. (2001) reported that many cases of CM are caused by Gram-positive microorganisms (*Staphylococcus* spp. or *Streptococcus* spp.), which was also confirmed in our study. However, bacteremia develops in a substantial proportion of cows with coliform mastitis, and about 20% of udder infections are caused by Gram-negative microorganisms, depending on the farm structure and hygiene status. This is consistent with our results, whereas SM and CM caused by *E. coli* accounted for 11.2% of infections from all infected cows.

In a Finnish study focused on the detection and etiology of mastitis, Pyörälä and Taponen (2009) point to a much higher risk of CM caused by *S. aureus* and NAS, which was also confirmed in all monitored dairy herds. Of the 230 infected samples, NAS (42.6% of positive findings) and *S. aureus* (16.5%) were the most frequently detected, in 136 cases (59.1%). The isolates of *S. aureus* and NAS accounted for 7.8% and 16.5% of CM; however, *S. aureus, S. chromogenes, S. warneri* and *S. xylosus* often caused chronic mastitis due to persistent IMI. As the results of our study show, the incidence of clinical and chronic mastitis in the investigated Slovak and Czech farms caused by *S. aureus* and some NAS is higher compared to other udder pathogens (Table 2).

Consistent with the results of Persson Waller et al. (2009), we assumed that the chronic IMIs caused by *S. aureus* (4.7%) and NAS (3.5%) in our study are predominantly persistent rather than new infections. It has been shown that many cows with *S. aureus* IMI in early lactation were already positive at drying off, which can precipitate a persistent, subclinical infection into CM in immunocompromised animals after calving.

High detected incidence of staphylococci in our results is consistent with the study Holko et al. (2019), who recorded a high incidence of NAS and *S. aureus* isolated from infected milk samples during the examination of 42 dairy farms in the west of Slovakia. The NAS represented 35.9% of positive findings and were the most commonly detected bacteria. On the other hand, the authors confirmed high resistance to aminoglycosides and β-lactam antimicrobials but without the presence of methicillin resistance genes, which contradicted our study.

In recent years, increasing studies have reported *S. haemolyticus*, *S. chromogenes, S. warneri*, and *S. xylosus* as the dominant strains of NAS isolated from mastitis in dairy cows (Supre et al., 2011; Hosseinzadeh et al., 2014; Zigo et al., 2019). In addition to subclinical forms of IMI, NAS has been largely isolated from CM (Supre et al., 2011), which was confirmed in our study. The CM mastitis with mild or moderate signs caused by NAS was associated with increased SCC, ability to form a biofilm, and resistance to aminoglycosides and β-lactam antimicrobials, especially to penicillin, amoxicillin, and oxacillin.

The increased incidence of staphylococcal infection in dairy cows also encourages the highest degree of pathogenicity in the production of more virulence factors, which are of crucial importance in persistent and CM cases (Fredheim et al., 2009; Pérez et al., 2020). These include, among others, cell wall-associated factors, different enzymes, and exotoxins that facilitate the infection pathway. For the individual virulence factors, co-production of hemolysins ß and δ in five species and single hemolysin δ in eight species of staphylococci were observed. Hydrolysis of DNase was detected in *S. aureus, S. chromogenes*, *S. warneri, S. xylosus,* and *S. haemolyticus* as well as the production of gelatinase. The staphylococci *S. aureus, S. chromogenes,* and *S. warneri* had the most numerous representation of virulence factors resulting in the increasing incidence of CM and persistent cases in comparison to strains with no virulence factors (Table 3).

The formation of biofilms is considered an important virulence factor of staphylococcal strains, as it facilitates their adhesion to biotic and abiotic surfaces (Fredheim et al., 2009). The ability of these pathogens to form biofilms adhering to the MG epithelium helps them to evade immunological defenses and causes recurrent or persistent infections. From our results, the ability to form a biofilm was attributed mainly to *S. aureus* as well as seven species of NAS isolated from CM and chronic mastitis. In addition to *S. aureus*, the NAS that caused CM and chronic mastitis demonstrated the production of hemolysins, the ability to hydrolyze DNA, and resistance to antimicrobials as other important virulence factors.

According to Perez et al. (2020), the interaction between the production of hemolysins and biofilm can increase adherence of staphylococci to bovine mammary epithelial cells and their survival during the body's immune response and antibiotic treatment. Our results confirmed the fact that the bacteria carrying this typical peculiarity are highly resilient to antimicrobials. In isolates of staphylococci with the ability to biofilm-producing confirmed by the phenotypic method on Congo red agar were more resistant to more than one antimicrobials compared to the isolates without biofilm formation at a time (Fig. 4).

Melchior et al. (2011), in their study of staphylococci isolated from mastitis milk in cows, reported that biofilm production and resistance to antimicrobials were the most frequent virulence factors in strains isolated from CM. Increasing biofilm production was evident in strains from CM and repeat cases of mastitis after previous unsuccessful treatment. The IMI caused by *S. aureus* or NAS is difficult to treat, even with intramammary antimicrobials, so proper consideration should be given to the infections produced by biofilm-producing bacteria.

The resistance to one or more antimicrobials in our study was detected in 105 isolates (77.2%) of staphylococci. Multi-resistant isolates for three or more groups of antimicrobial classes accounted for 11.7% (16 isolates). The tested staphylococci phenotypically showed multi-resistance to a combination of antimicrobial classes, such as aminoglycosides, β-lactams, macrolides, and

cephalosporins (Table 4). In addition, the presence of β-lactam-resistant strains in our results indicates the presence of methicillin-resistant staphylococci (MRS) which was confirmed in 47 isolates (34.6%), and PCR was used to confirm the *mecA* gene in two isolates of *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri*. All positive staphylococci (n=4; 2.9%) with the *mecA* gene showed resistance to oxacillin and cefoxitin and were considered MRS. In a study by Khazandi et al. (2021), when the whole genome was sequenced, they identified the presence of a *mecA* homologue in four oxacillin-resistant *S. sciuri* isolates. The homologue was not detected by conventional *mecA* PCR or cefoxitin susceptibility testing. However, in our study, MRS were also phenotypically confirmed, so we do not assume the presence of a false positive *mecA* homologue.

In the Czech study by Vyleťelova et al. (2011), who investigated 1729 bulk milk and individual samples from dairy cows, ewes and goats were the most frequent bacterial pathogens *S. aureus* and NAS (n = 634; 36.7%) from all tested samples. The species were also examined for antimicrobial susceptibility by using the disk diffusion method and the presence of the *mecA* gene by the PCR method. Among the resistant staphylococci, *S. aureus* (51%) was found the most frequent, followed by *S. epidermidis* (34.7%) and *S. chromogenes* (12.2%). The presence of the *mecA* gene was confirmed from 13 isolates of resistant staphylococci to β-lactam antimicrobials that occurred mainly in cow's milk.

A similar study by Bogdanovičová et al. (2014) monitored the prevalence and antimicrobial resistance of *S. aureus* from 50 dairy farms situated in the Czech Republic. From 261 raw milk and filtered milk samples, the authors detected positivity to *S. aureus* in 58 samples, 37 (14.2%) of which were isolated from raw milk and 21 (8.1%) from filtered milk. Resistance to β-lactam antimicrobials, especially to amoxicillin and oxacillin, was detected in the largest proportion (17.8%) of raw milk *S. aureus* isolates, followed by the isolates resistant to macrolides and tetracycline. Using the PCR method, methicillin-resistant *S. aureus* (MRSA) with the presence of the *mecA* gene was detected in four isolates obtained from raw milk samples and two isolates from filtered milk. Based on the previous two studies (Vyleťelova et al., 2011; Bogdanovičová et al., 2014) and our findings, we can confirm that the occurrence of IMI caused by staphylococci mainly *S. aureus* with increased resistance to β-lactam antimicrobials is still a major problem in Czech and Slovak dairy farms. The occurrence of MRS with the presence of the *mecA* gene is also worrying which is in the range of 3 – 6% from isolates strains.

The WHO classified *S. aureus* as a high-priority pathogen, and it has gained the most attention among resistant staphylococci. However, methicillin resistance has also been described in several species of the NAS group (Corti et al., 2003; Seixas et al., 2014). In our study, we confirmed that two isolates of *S. aureus* demonstrated the *mecA* gene as well as one isolates each of *S. chromogenes* and *S. warneri*. According to Vinodkumar et al. (2017), the NAS is thought to be a reservoir for numerous

resistance genes, which could be transferred into the more pathogenic *S. aureus*. In particular, the presence of the antimicrobials and their metabolites in the environment may be the reason for the selection and spread of resistant isolates. This negative impact of the massive use of antimicrobials, especially in cows drying with a combination of an ineffective antibiotic (without antibiogram before application) and slow degradation in the udder, may potentially be a cause of increasing resistance and MRS in veterinary medicine.

MRS is resistant to almost all β-lactam antimicrobials, and infections caused by these pathogens result in unsuccessful or repeated treatment, increased SCC, and poorer outcomes. When it comes to MRSA as the cause of mastitis, Norway may be regarded as a naive country, as MRSA has been associated with bovine mastitis on only one occasion (Fergestad et al., 2021). This contrasts with our results and the current situation in Belgium, where Bardiau et al. (2013) found a similar occurrence of MRSA in 4.4% of milk samples from clinical cases of mastitis and Vanderhaeghen et al. (2010) found MRSA in 9.3% of milk samples from farms experiencing *S. aureus* mastitis comparing to our findings. Although our results showed a higher resistance of the tested staphylococci to β-lactam antimicrobials than in previous studies (Bardiau et al., 2013; Fergestad et al., 2021) we can state that the occurrence of MRS in the monitored farms was approximately at the same level.

Due to the increasing resistance of bacterial pathogens and the occurrence of MRS in veterinary medicine, the European Union plans to reduce livestock antimicrobial sales by 50% by 2030, based on the strategy "*Farm to Fork*" (EU, 2021). The urgently required or necessary administration of antimicrobials in veterinary medicine and the dairy sector remains possible in the future, but only if justified primarily on the results of targeted diagnostics, which, by means of anamnestic data, clinical examination, SCC from utility control and culture of samples with antibiogram, reveal the health status of the udder and its physiological functions in each dairy cow.

5. Conclusions

The present study clarifies that more than half of the IMIs were caused by staphylococci (59.1%), especially NAS (42.6%), followed by *S. aureus* (16.5%) in monitored Slovak and Czech dairy farms. In addition to *S. aureus*, *S. chromogenes*, *S. warneri* and *S. xylosus* isolated from CM and chronic mastitis indicated a high degree of pathogenicity in the production of more virulence factors in comparison to other strains of NAS (*S. epidermidis*, *S. haemolyticus*, *S. hyicus*, *S. capitis*, and *S. piscifermentans)*. Resistance to aminoglycoside and β-lactam antimicrobials were frequently detected in the tested staphylococci, possibly because these are the antimicrobials most frequently used in the drying and mastitis treatment of dairy cows. Especially isolates with the ability to form a biofilm were more resistant to more than one antimicrobials compared to the isolates without biofilm production at a time. Based on the phenotypic manifestation of antimicrobial resistance, detection of

the presence of the *mecA* gene was confirmed in MRS (2.9%) in two isolates of *S. aureus* and two isolates of NAS (one isolate each of *S. chromogenes* and *S. warneri*).

We can state that *S. aureus* still comes on top in the number of chronic or severe mastitis cases, as well as the number of virulence factors but some NAS species could have the same aggressive potential based on their production of gelatinase, hemolysis, biofilm, hydrolyzed DNA and multi-drug resistance. Knowledge regarding the virulence of both *S. aureus* and NAS species associated with bovine mastitis, especially in combination with resistance patterns and the presence of MRS isolates, is important for designing efficient prophylaxis and treatment guidelines to minimize the negative effects on milk yield and culling hazards in dairy cows.

2.5.1 Institutional Review Board Statement

For this study, the clinical examination of the cows and the collection of milk samples were approved by the Ethics Committee at the University of Veterinary Medicine and Pharmacy in Košice no. EKVP 2022/05 following EU legislation 2010/63/EU, article 1:5 (practices not likely to cause pain, suffering, distress, or lasting harm equivalent to or higher than, that caused by the introduction of a need to follow good veterinary practice).

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Disclaimer

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Prevention and control of mastitis

The second part of the scientific publication presents a set of four studies dealing with the causes of the occurrence, control and prevention of dairy mastitis. The variety of causes that lead to this disease is very complex due to many potential combinations, so mastitis is referred to as polyfactorial disease. Due to the polyfactorial and poly-aethological nature of mammary gland inflammation, the first study presents a wide spectrum of issues of crucial importance for influencing all environmental factors and the relationship between the infectious agent and the dairy cow organism.

The other two studies focused on the prevention of mastitis by supplementation of minerals, vitamins and organic additives in the form of humic acids. Their aim was to evaluate and improve the level of nutrition during the dry period which is characterized by limited feed intake and deficiency of "immunostimulatory and antioxidant" nutrients, such as selenium compounds (Se) and vitamin E (vit. E). The knowledge obtained during the studies bring new insights into the best way of achieving an increase in Se and vit. E levels in the blood of dairy cows during the dry period, which affects directly the pro-oxidative – antioxidant balance of the body and the occurrence of mastitis at the beginning of lactation.

Supplementation of organic additives in the form of humic acids is one of the prophylactic nutritive measures for increasing the body's immunity and reducing the incidence of mastitis in the peripartum period. The studies indicate the positive role of the addition of humic acids to dairy cow feed rations during the dry period as well as their effect on ammonia utilization by means of determination of urea in milk.

The system and hygiene of housing are among the most risky factors with respect to udder contamination. Appropriate control and mastitis prevention measures at the level of dairy housing are discussed in the last study. This study presents new technological procedure for cleaning and putting down bedding into the lying boxes used in free (loose) housing systems. The purpose of the study was to prepare a suitable type of bedding by mixing alternative materials (separated solids, limestone, straw and water) and to evaluate their effect on hygienic quality, contamination of bedding by faeces and the occurrence of environmental mastitis in dairy cows.

3 Chapter

PREVENTIVE METHODS IN REDUCTION OF MASTITIS PATHOGENS IN DAIRY COWS

František Zigo, Juraj Elečko, Zuzana Farkašová, Martina Zigová, Milan Vasiľ and Lenka Kudělková

Abstract: The aim of this chapter was analyze designed preventive and control methods focused to reduction of mastitis in herd of Slovak pied cattle in the east of Slovakia during two years of experiment. From 180 cows at quarterly intervals in the 3rd, 6th, 9th and 12th month was performed a complex examination of health udder including an assessment clinical signs of mammary gland, abnormal udder secretions, Californian Mastitis Test (CMT) with subsequent collecting of milk samples for bacteriological examination. In the first year during the first two complex examinations, treatment of mastitis caused by coagulase positive staphylococci (*S. aureus),* coagulase negative staphylococci (*S. haemolyticus, S. warneri, S. epidermidis)* and *Str. agalactiae,* a reduction in prevalence from the original 41.3 % to 32.1 % was achieved. During the last two complex examinations in the first year the prevalence decreased to 25.2 % and then at the end to 21.1 %, respectively. The reduction of mastitis during the second year is characterized by a 22.1 %, 19.2 %, 12.2 % to 7.3 % mastitis, when the prevalence dropped by 5.5 %, respectively. Coagulase negative staphylococci and *Str. agalactiae* were the most numerous in each case during the second year and their occurrence subject to a proportional reduction. Proposed antimastitis methods and their implementation of continuous mastitis control system during two years, significantly reduced prevalence of mastitis by 34.0 % and influenced the occurrence of the most common pathogens of the mammary gland in monitored herd of dairy cows. Recorded reduction of mastitis in monitored dairy herd is an example of using available scientifically validated methods in a rationally compiled mastitis control program for the specific conditions of each dairy farm in the long period.

3.1 Introduction

Milk products from cows, sheep and goats are unique, especially in the field of trational nutrition of consumers. Many of milk products and specialties can be included among the functional foods for their nutritional value. The economic value of dairy cows is determined mainly by their milk yield and longevity, because milk is the main source of income on dairy farms. The main important factors affecting the quantity and quality of milk produced is the occurrence of production diseases, especially mastitis (Vršková et al., 2015).

Worldwide, mastitis (intramammary infection) is known as a multifactorial disease, and it is closely related to the production system and the environment that the cows are kept in (Tančin et al., 2006). The most recent estimates from the National Mastitis Council (2001) suggest that mastitis affects one third of all dairy cows and will cost the dairy industry over 2 billion dollars annually in the United States in lost profits. The incidence of mastitis increases when defense mechanisms of the mammary gland are impaired. Dairy cattle are exposed to numerous genetic, physiological, and environmental factors associated with the host, pathogens that can compromise host immunity and increase the incidence of mastitis. Among the most infectious agents causing mastitis and reduction of milk yield belongs bacteria (Pecka-Kiełb et al., 2016), viruses, fungi and algae (Tančin and Uhrinčať, 2014).

Bacteria are the major source of mastitis. For an intramammary infection (IMI) to occur it is necessary for the teat skin to be contaminated with pathogens, the pathogens to penetrate the teat duct and the infection to be established in the sinuses, ducts or tissues of the udder (Figure 1). With the inflammation follows an increase in the level of white blood cells or leukocytes, and this causes an increase in the somatic cell count (SCC) of the milk.

The leukocytes are produced as a response to the injury or infection, and they are a crucial part in fighting the damage of tissue and infective agents (Zadoks et al., 2001; Vasiľ et al., 2016). The prevalence of the IMI varies with the breed, age of the cows, milk yield and the stage of lactation. More than 50% of mastitis cases occurring during peripartal period or in the first two months after calving (Huijps et al., 2008; Sharif et al., 2009; Zadoks et al., 2011).

Based on the intensity and severity of clinical signs, mastitis is usually divided into subclinical and clinical disease (Sztachańska et al., 2016). In clinical mastitis (CM), signs range from mild to severe and can be systemic, local, or milk related, whereas in subclinical mastitis (SM), no signs are observed. During SM the udder and milk appears normal, but infection is still present. Due to the lack of symptoms, SCC can be used to indicate the prevalence of mastitis. In CM the clinical signs are clear. The most prominent symptoms of CM are swelling, heat, hardness, redness or pain of the udder. The milk of a cow with CM has a watery appearance, and flakes, clots or pus is often present. Subclinical cases of mastitis are more common than clinical cases of mastitis. On average in the herd there are 15 - 40 undetected cases of SM for every CM in cows. There is an increase SCC what leads to reduced milk production to the tune of 60 to 140 liters per cow per year in subclinical mastitic animals (Hameed et al., 2008; Sinha et al., 2014).

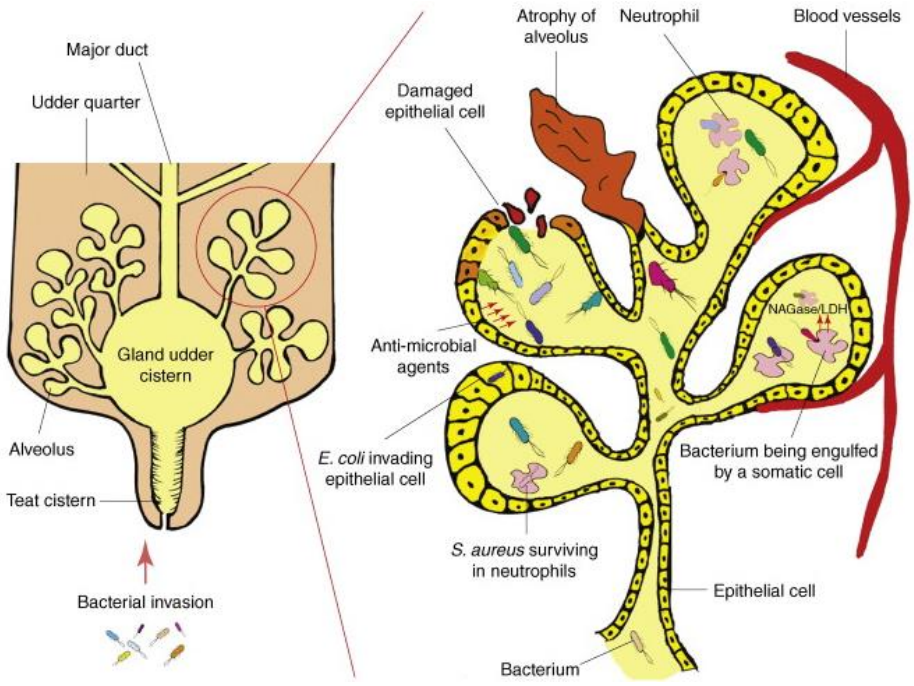

Figure 1. Environmental and contagious microorganisms invade the udder though the teat cistern

Note: Microorganisms are multiply within the alveolus where they are attacked by neutrophils (white blood cells) while damaging the milk-producing epithelial cells of the bovine udder

Source: Viguier et al. (2009)

Treatment of CM incurs costs that vary with the severity of the case and the response of the farmer. Determination of the cost of a case of CM can be complex given that losses can result not only from decreased production and discarded milk, but labor and medication costs, premium loss and penalties, culling and replacement costs, mortality and even impaired reproduction (Table1). Unlike subclinical mastitis, the largest losses due to clinical disease are from discarded milk. Research regarding economic losses associated with mastitis differ greatly depending on the study and have been variously estimated at between $50 and $400 per case. As a rule of thumb, however, losses related to a single case of mastitis are typically put at somewhere between $100 and $200 (Hillerton and Berry (2005).

Procedures across multiple versions of programs for prevention and control mastitis known as Mastitis Control Program, in principle, are designed to ensure a standard level of nutrition, hygiene of environment, and the proper functioning of the milking machine, targeted antibiotic treatment of inflammation during lactation, non-selective treatment of the udder at the beginning of drying-off, as well as the elimination of dairy cows with chronic mastitis from next breeding (Pyörälä and Taponen 2009). The diversity of the aetiology, the current sensitivity to antibiotics, rearing technology and breeding status, however, often requires the modification (Bradley and Green, 2007).

Table 1. Cost of an average case of clinical mastitis and bacterial pathogens in a dairy cow producing 7000 kg milk per lactation

Factor	**Cost** (£)	**Bacterial pathogens**	**Positive identifications** (%) **from 100 clinical cases of IMI**
Labour, 2 h at £6	12	**Coliforms**	43
Treatment, drugs and vet	3 - 11	***Streptococcus* spp**.	33 - 36
Discarded milk	26	*Str. uberis*	30 - 33
Production loss (10%)	135	*Str. dysgalactiae*	1 - 3
Reduced food intake	- 56 - 25	***Staphylococcus* spp.**	16 - 18
Fatality (1%)	3	*Staphylococcus aureus*	10 - 14
		CNS*	2 - 4
Total	**131**	***Trueperella pyogenes***	1 - 2

Note: CNS* - coagulase negative staphylococci, IMI – intramammary infection

Sources: Berry et al. (2004) and Hillerton and Berry (2005).

3.1.1 Objectivites of this chapter

The aetiology and prevalence of the mastitis in a dairy herd of Slovak Pied cattle were analysed with the application of continued methods of control and prevention to reduction of IMI caused by pathogens bacteria during two years.

3.2 Material and methods

3.2.1 Animals and milking

The study was realized in herd of 180 Slovak pied dairy cattle (Zemplin) with standard zootechnic and zoohygienic conditions during two years. On the farm there are two brick stables where cows were kept on deep litter with free housing system. The zootechnicians office and the cloakroom were in the building, along with a milking parlour, which was followed by a covered waiting room. All cows were fed total mixed ration based on grass silage, maize silage, hay and concentrate according to international standards (NRC, 2001) to meet the nutritional requirements of a 600 kg cow, yielding 15 - 25 kg of milk/d and were allowed *ad libitum* access to water. Dirty litter was cleared and exchanged 2 times a week using a UNC mechanism.

The cows were milked twice a day in tandem milking parlor DeLaval 2x5 (Tumba, Sweden), with the first milking starting at 4.30 h in the morning and the second afternoon at 16.30 h. First, the wet toilet was performed with water to remove impurities from udder and teats. Subsequently, the udder was thoroughly wiped disposable paper wipes. The first milk from each quarter were hand-drawn into a dark-bottomed pot, and the milk was sensitively assessed. Milking and pulsation vacuum was set at 42 kPa. Pulsation ratio was 60:40 at a rate of 52 c/min and termination was automatically signalled when the milk flow dropped to 0.2 l/min. After milking process, the teats were disinfected in the form of teat-dipping. Milk was stored in refrigerating milk tanks at + 5 °C and removed daily around 11.30 hrs.

3.2.2 Examination of health status and milk samples collection

A complex examination of all lactation cows were carried out quarterly in 3rd, 6th, 9th, and 12th of the month. After a veterinary history, cow udders, including sensory evaluation of the first milk, were examined. This was followed by an examination of all quarters using CMT (Indirect Diagnostic Test, Krause, Denmark) according to Jackson et Cockcroft (2002) (Table 2 and Figure 2). The CMT score interpretation in table 2 is expressed as the average percentage of individualy mastitis forms from all four investigations during first and second year. Mixed quarter samples of cow's milk (10 ml) were then collected by aseptic techniques in accordance with the guidelines of the National Mastitis Council (2001). The cooled samples were immediately transported to the laboratory of University of Veterinary Medicine and Pharmacy in Kosice. Based on clinical signs, CMT score and bacteriological examination of milk samples, IMI were classified as latent, subclinical and clinical cases according to Vasil et al. (2009).

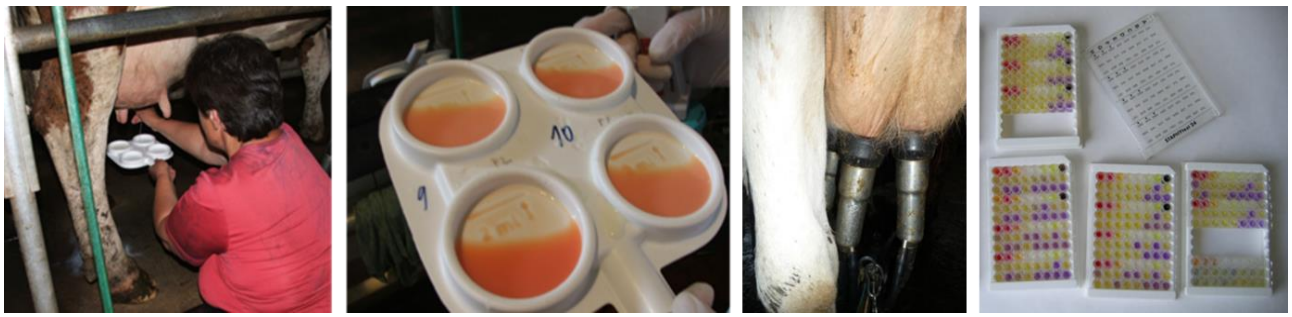

Figure 2. From left: **Assessment of the first pre-milked samples with the evaluation of CMT score, machine milking and laboratory identification of bacteria *Staphylococcus* spp. using biochemical STAPHY-test**

Photo by Zigo (2019).

3.2.3 Laboratory analysis

Bacteriological examinations and identification were performed according to generally accepted principles Malinowski et Kłosowska (2002). Milk samples (10 µl) were inoculated on Petri plates with Columbia Blood Agar Base (Oxoid, UK) with 5% of defibrinated ram blood and incubated for 48 h at 37° C; the plates were examined after 24 and 48 h of incubation. A milk sample was classified as positive if at least two colony of *Staphylococcus aureus* or *Streptococcus agalactiae* was identified. For other bacteria, the presence of at least five to seven typical colonies was required for positive classification. Suspected colony were inoculated and cultured on selective nutrient soils such as Staphylococcus medium N°110 (Fig. 3), Baird-Parker agar, Brilliance^{TM} UTI Clarity Agar, Edwards Medium, Mac Conkey Agar (Oxoid, OXOID Ltd., Basingstoke, Hants, UK).

Figure 3. From left: ***S. aureus*** **and** ***S. warneri*** **cultured on blood agar base with 5% of defibrinated blood and Staphylococcal medium N° 110**

Photo by Zigo (2019).

Table 2. Evaluation of milk samples and interpretation of CMT score

CMT score	SCC	Interpretation	Monitored herd	
			First year (%)	Second year (%)
N (negative)	0 – 200.000	Healthy quarter	67.6	82.2
T (trace)	200.000 – 400.000 (±50,000)	Latent mastitis*	3.9	2.3
1	400.000 – 650.000 (±150,000)	Subclinical mastitis*	14.8	7.9
2	850. 000 – 1.200.000 (±200,000)	Subclinical mastitis Serious mastitis*	5.6	3.5
3	1.500.000 – 5.000.000 (±300,000)	Serious mastitis	5.3	2.5
4	Over 5.500.000	Serious mastitis	2.8	1.6

Note: CMT - The Californian Mastitis Test, SCC- somatic cell count, Latent mastitis* - milk appears normal, but infection is still present in samples of raw milk without changing of SCC with negative CMT score, Subclinical mastitis* - no signs are observed, the udder and milk appears normal, but infection is still present with positive CMT score and increased SCC. Serious* or clinical mastitis – signs range from mild to severe with positive CMT score, bacteriological

cultivation, high level of SCC, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production with clinical signs.

Parameters such as colony size and appearance, pigment production and coagulase, catalase activity, hemolysis, Gram staining have also been taken into account in the determination of bacterial species. Colonies of *Staphylococcus* spp. were selected for a test for coagulase in a tube (Staphylo PK, Imuna Pharm, SR). Growth-confirmed colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were detailed identified biochemically using the STAPHYtest 24, STREPTOtest 24, resp. ENTEROtest 24 (Erba-Lachema, CZ) and identification by software TNW Pro 7.0 (Erba-Lachema, CZ). Colonies morphologically compatible with *Trueperella.pyogenes* were subjected to a conventional phenotypic assay API Coryne strips (BioMe'rieux, France).

3.2.4 Suggested methods of damping mastitis during two years in monitored herd

Good routines for hygiene and treatment is the most important step for prevention of mastitis. From the anamnesis and analysis of the first examination of the herd of dairy cows, was designed the Mastitis Control Program which consist from:

a) renew the bedding materials frequently, preferable daily and do not keep cows in dirty paddocks
b) maintain good foot health
c) pre-milking hygiene - udder toilet: washing udder (have ready the udder cloths, basket preparation for dirty udder cloths), assessment of the first pre-milked samples into the vessel - cup with double bottom and control of milk consistency for presence of flakes, pre-dipping (disinfection of teats before milking with Valiant (ABS, CZ), drying of udder before milking
d) after milking - disinfection of all teats with IODERM 5000 (Hypered, CZ)
e) keeping the cows out of lying areas for 30 minutes after milking
f) cows with mastitis must be separated from healthy cows and individually milked as the last
g) all acute and subacute cases of mastitis treat according to actually sensitive to antibiotics process
h) monitoring milk quality and composition of the treated animals after inclusion in the milking
i) dairy cows with chronic mastitis or atrophy of secretory tissue in udder quarters after unsuccessful treatment must be rejected because they represent a constant reservoir for infection for the other cows in the herd
j) unselective treatment of udder with antibiotics, on the start dry period
k) new dairy cows (predominantly gestative) can be integrated to herd after completely control of health status

l) keep the milking machine serviced

m) control of right observance of designed methods.

3.3 Results and discussion

A diagnosis of mastitis is based on clinical observations or direct or indirect measures of the inflammatory response to infection, whereas a diagnosis of an intramammary infection is based on identification of the infectious agent. SCC is a common diagnostic tests for the detection of SM, as well as the use of CMT. Culture and PCR can be useful in the diagnosis of an IMI. However, both have their advantages and disadvantages. Diagnosing the bacterial agent causing the intramammary infection can help to determine treatment and prevention strategies on the farm, which in turn can help to reduce incidence and prevalence (Adkins and Middleton, 2018).

The prevalence and aetiology of mastitis from mixed milk samples of 180 dairy cows during two years in eight examinations of experiment are described in Tables 3 and 4. In first year were 32.4 % cows positive of CMT reaction (Table 2) and from 29.6 % of cows` mixed milk samples were cultivated bacterial pathogens. During the first planned screening was prevalence of mastitis 41.3 % (55 positive cows` mixed milk samples) in the first year. The results of first examination (Table 3) are reflection of detected anamnesis of herd and its status of completely breeding activity.

After reading the results of the first examination by a breeder, the herd began to apply measures such as: the creation of groups of dairy cows under production and stage of lactation, ensuring the implementation of appropriate disinfectants and devices for milking, treating during lactation and mammary gland treatments at the beginning of drying-off. In particular the treatment of clinical mastitis already after three months to reach the reduction of the prevalence from 41.3 % to 32.1 %, and preferably treated dairy cows with the findings of *S. aureus*, CNS, *Str. agalactiae* and *Trueperella pyogenes* from positive milk samples. In the first year, after the third examination, the prevalence decreased (25.2 %), and then at the end of the first year of the experiment with the standardization of hygiene in milking the value of 21.1 % was recorded. In second year were 17.8 % cows were positive of CMT reaction (Table 2) and from 15.3 % of cows` mixed milk samples were cultivated bacterial pathogens.

Effect of applied controlling methods during the second year (Table 4), reduced the incidence of the apparent mastitis 22.8 %, 19.2 %, 12.2 % and 7.3 %, respectively, while there was a decline in the prevalence of 5.5 % in the course, or for the entire experiment of 34.0 %.

Table 3. Prevalence and etiology of mastitis from cow's milk samples in four examinations during the first year of monitoring

First year (sequentially accepted methods of prevention and reduction of mastitis)								
Examination/Milk samples	**I./133**		**II./137**		**III./143**		**IV./147**	
Isolated bacteria	n	%	n	%	n	%	n	%
***Staphylococcus* spp.**	**41**	**30.9**	**20**	**14.5**	**16**	**11.2**	**16**	**10.9**
S. aureus	*9*	*6.8*	*4*	*2.9*	*1*	*0.7*		
S. haemolyticus	*4*	*3.0*	*7*	*5.1*	*3*	*2.1*	*6*	*40.1*
S. warneri	*7*	*5.3*	*1*	*0.7*	*3*	*2.1*	*2*	*1.4*
S. epidermidis	*2*	*1.5*	*1*	*0.7*			*3*	*2.0*
S. chromogenes			*3*	*2.2*	*2*	*1.4*		
S. sciuri	*11*	*8.3*						
S. schleiferi					*6*	*4.2*	*5*	*3.4*
S. xylosus			*4*	*2.9*	*1*	*0.7*		
S. lentus	*8*	*6.0*						
Other pathogens bacteria								
Streptococcus agalactiae	7	5.3	5	3.6	10	7.0	8	5.4
Trueperella pyogenes	2	1.5	7	5.1	4	2.8	3	1.4
mixed infection*	5	3.7	12	8.8	6	4.2	4	2.7
Prevalence of mastitis	***55***	***41.3***	***44***	***32.1***	***36***	***25.2***	***31***	***21.1***

Note: n – number of samples with positive cultivation of bacterial pathogens, mixed infection* - mixed infection caused two or more bacteria (*Enterococcus spp., E. coli* and *Bacillus spp*.)

Different types of IMI are caused by different bacterial species. Some bacteria prefer environmental niches, others are contagious, and many are opportunistic. According to the authors Sharif et al. (2009), the most contagious mastitis pathogens causing IMI are *Staphylococcus aureus*, *Str. agalactiae* and *Streptococcus uberis*. The main reservoirs of contagious pathogens are bovine tonsils, rumen, rectal, genital regions and mammary gland itself. Intramammary infections caused by contagious pathogens can vary from subclinical to clinical mastitis. Subclinical infection often goes unnoticed. Long lasting subclinical infection can sometimes progress to a clinical mastitis with drastic changes in milk (clotting, hemorrhage) and in the udder (pain, swelling), as well as systemic signs (fever, loss of appetite). *S. aureus* with *Str. agalactiae* were observed to be the most common cause of CM in our study.

Their transmission in the herd is thought to be strictly contagious, i.e. from cow to cow, due to insufficient hygiene in the milking parlor, allowing multiple animals to come into contact with equipment, hands or towels that are contaminated by milk from an infected cow. Frequency of

contagious pathogens among CM cases is greater. The use of dry cow therapy, post milking teat disinfectants and effective pre-milking hygiene are effective control procedures for most contagious mastitis pathogens (Sori et al., 2005).

The treatment of mastitis by antimicrobials produces residues in milk, which is an important aspect to consider. CM should in general be treated with narrow-spectrum antimicrobial, and first choice for treating infections caused by streptococci and penicillin-susceptible staphylococci are β-lactam antimicrobials. It is recommended to treat both systemically as well as intramammary for at least three days (Pyörälä, 2009).

Table 4. Prevalence and etiology of mastitis from cow's milk samples in four herd examinations during the second year of monitoring

Second year (application of mastitis control program)								
Examination/Milk samples	**V./149**		**VI./151**		**VII./156**		**VIII./151**	
Isolated bacteria	n	%	n	%	n	%	n	%
Staphylococcus spp.	**14**	**9.5**	**10**	**6.6**	**13**	**8.3**	**6**	**4.0**
S. aureus	*1*	*0.7*			*1*	*0.6*		
S. chromogenes	*3*	*2.0*			*3*	*1.9*	*2*	*1.3*
S. epidermidis	*4*	*2.7*					*1*	*0.7*
S. haemolyticus			*1*	*0.7*	*5*	*3.2*	*3*	*2.0*
S. warneri	*5*	*3.4*	*3*	*2.0*				
S. capitis					*4*	*2.6*		
S. sciuri			*2*	*1.3*				
S. schleiferi			*4*	*2.6*				
S. xylosus	*1*	*0.7*						
Other pathogens bacteria								
Streptococcus agalactiae	6	4.0	9	6.0	1	0.6	3	2.0
Trueperella pyogenes	4	2.7	3	2.0	2	1.3	1	0.7
mixed infection*	10	6.7	7	4.6	3	1.9	1	0.7
Prevalence of mastitis	***34***	***22.8***	***29***	***19.2***	***19***	***12.2***	***11***	***7.3***

Note: n – number of samples with positive cultivation of bacterial pathogens, mixed infection* - mixed infection caused two or more bacteria (*Enterococcus spp., E. coli* and *Bacillus spp*.)

Among environmental pathogens, the most common bacteria are *Str. dysgalactiae*, coliforms such as *E. coli* and *Klebsiella*. Coagulase negative staphylococci are also the environmental bacterial pathogens of increasing importance in udder infections. Their are normal inhabitants of the skin and teat canal and may be frequently isolated from milk samples. In recent decades, CNS have become among the most common mastitis-causing agents in well-managed dairy farms in many countries

(Orwin et al., 2001; Taponen et al., 2006; Sameer et al., 2018). *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri* and *S. epidermidis* are the most common mastitis-causing CNS species (Pyörälä et Taponen, 2009).

Particularly *S. chromogenes, S. epidermidis, S. haemoyticus* and *S. warneri* may have pathological significance of IMI with increase in mean milk SCC (Zigo et al., 2017). Bacteria of *Staphylococcus* spp. (Tab. 3 and 4) were the most numerous and each time of experiment they were subject to a proportional reduction. For the duration of the experiment in addition to the coagulase-positive *S. aureus* were isolated other 10 types of CNS. From these *S. haemolyticus* and *S. warneri* which have been isolated on a regular basis, especially in the first year, along with other species such as S. *chromogenes* and *S. epidermidis* caused clinical and subclinical mastitis most frequently. Sporadic findings of *S. schleiferi, S. xylosus, S. lentus, S. sciuri* and *S. capitis* were isolated usually in cases of latent mastitis which are characteristic only with the presence of bacterial pathogens in samples of milk without changing its consistency. The last examination on the end of second year was characterized by finding only 4.0 % of the staphylococci (*S. haemolyticus, S. chromogenes* and *S. epidermidis).*

Mastitis caused by CNS usually displays relatively mild clinical signs, and these bacteria can therefore affect milk quality for a long period before being noticed. In contrast, *Streptococcus uberis* is a widely distributed environmental pathogen causing more severe signs. The environmental pathogens are more difficult to eradicate due to their ubiquitous presence and they remain a major challenge to the dairy industry (Hogan and Smith, 2012). They can be controlled by reducing exposure and by increasing immune resistance of the cow by post milking teat dipping with a germicidal and treatment of all quarters with antibiotics during drying off (Fox and Gay, 1993).

In the studies conducted by Monday et Bohach (1999) and Thorber et al. (2009) were CNS the most prevalent pathogens causing SM in dairy cows and ewes. Although less pathogenic than *S. aureus*, CNS can also produce persistent subclinical or clinical mastitis. After infection of CNS is significantly increased SCC, CMT, cause CM as well as producing thermostable enterotoxins. Nevertheless, despite the accepted role of these bacteria as major mastitis causing pathogens in cows and ewes. The pathogenicity of the different CNS species varies widely.

The most frequently isolates from CNS species in subclinical and chronical mastitis are *S. epidermidis, S. caprae, S. simulans, S. chromogenes* and *S. xylosus* (Bergdoll and Lee Wong, 2006). Thorberg and colleagues (2009) confirmed and demonstrated one or two types of *S. epidermidis* in two monitored herds of dairy cows. The dominant types of *S. epidermidis* from milk were also isolated from skin of the people who were responsible for milking cows because isolation of *S. epidermidis* from human skin is more common than isolation from bovine skin. The authors conclude that humans who are daily in contact with animals are probably the main source of infection for cows.

Other types of bacteria in our study were represented by *Str. agalactiae, Trueperella pyogenes, E coli, Enterococcus* spp. and *Bacillus* spp. during monitored period The importance of using the results of diagnostics is manifested even in control of owns cows, and its inclusion into the herd rearing. Designed by continuous year lasting mastitis control system significantly reduced the incidence and the prevalence of the most common pathogens affected mammary gland. Given the variety of factors causing the IMI milk production and economic prosperity will depend on the expertise of the farmers to quickly implement preventive anti-mastitis methods to their own dairy production.

The study done by Hillerton and Berry (2005) confirmed that the implementation of the mastitis control program does not completely exclude IMI from the herd, but it is effective in keeping the incidences at a low level. However, advances in detection systems have not brought efficient cow-side methods to achieve this better care. Usually annually, in a resting or dry period, all dairy animals must spend anywhere from 6 to 10 weeks prior to caving in a non-lactating phase. The cow remains especially susceptible to the contraction of IMI soon after carving, and the secession of milking or "drying off" period.

3.4 Conclusion

A unique and continuous progress of the reduction of mastitis (in the first of a partial, or total in the second year) by implementation of the mastitis control program is the result of the interplay of effects applied to the methods of prevention that provide protection against the emergence of new infections and disease control methods (treatment and rejection), which drastically reduce the duration of the infection. Constant observance of the hygiene practices in milking, treatment of dairy cows by making effective therapies in cows with clinical mastitis, and disposal of cows with the chronic form has been reduced the incidence of clinical mastitis in the herd to minimum. Rich knowledge of systematic research into the problem of reducing the presence of mastitis at home and in the world confirm the need to take into account the polyetiological and multifactorial character of IMI in the everyday practice of farmers. It is therefore necessary to implement new knowledge and technological processes in dairy farming in order to achieve the highest quality of produced milk.

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4 Chapter

COMPARISON OF EFFECT OF PARENTERAL AND ORAL SUPPLEMENTATION OF SELENIUM AND VITAMIN E ON SELECTED ANTIOXIDANT PARAMETERS AND UDDER HEALTH OF DAIRY COWS

Milan Vasiľ, František Zigo, Zuzana Farkašová, Ewa Pecka-Kielb, Jolanta Bujok and Josef Illek

4.1 Introduction

Healthy cows are the foundation of sustainable milk production. However, mastitis and other infectious diseases are common problems in dairy herds, resulting in increased costs and decreased production. Most diseases in dairy cows occur at, or just after, calving, which is a period associated with immune suppression, resulting in increased susceptibility to infections. Prepartum immune suppression is multifactorial but results from endocrine changes and decreased intake of critical nutrients. Among the most important nutrients often deficient in compound feeds, involved in the biological functions and antioxidative activity are vitamin E (VTE) and selenium (Se) (Waller et al., 2007; Kafilzadeh et al., 2014).

Vitamin E is a term used to describe all tocol and tocotrienol derivatives that exhibit biological activity similar to a-tocopherol. There are eight naturally occurring VTE isomers. Of these eight isomers, a-tocopherol has been proven to be most effective in preventing VTE deficiency syndromes, and is the most biologically active isomer (O'Rouke, 2009).

Selenium is one of the essential trace elements which protect organisms from oxidative damage. The presence of Se in soil on the territory of the EU is very variable, from an average of 0.05 to 0.1 ppm. Se deficient soils are found on the territory of Nordic countries, France, the Balkans and England. In recent years, it has been confirmed that the Central Europe is an area with very low concentrations of Se in the soil as well (Gresakova et al., 2013).

There are many biological functions of Se, mainly as the component of various selenoproteins. The most important of these is glutathione peroxidase (GPx). Different forms of this enzyme are present in all tissues which are exposed to oxidative stress. The biological functions of selenium are complemented by VTE, which also functions as a cellular antioxidant. Antioxidants are involved in the prevention of many disorders in female reproduction, such as mastitis, retained placenta, infertility, endometritis, and ovarian cysts (Table 1). The target site of GPx activity is cell cytosol, and VTE is corporated into lipid membranes. Both systems participate in the protection of membrane

polyunsaturated fatty acids (PUFA), which are very sensitive to the effects of reactive oxygen species activity. Vitamin E deficiency in animals is associated with PUFA metabolism alteration, which may subsequently lead to impaired function of many cells, among others polymorphonuclear cells, which provide the main protective mechanisms against infections (Pechová et al., 2008; Hoque et al., 2016).

Table 1. Overview of Se and vitamin E deficiency syndromes in ruminants

Species	Syndrome	Affected system, resp. organ
Cattle	Nutritional myodystrophy of calves	Skeletal muscle, myocardium
	Retained placenta	Placental connection with the uterus
	Fetal death, reabsorption	Embryonic vascular system
	Ovarian cysts	Ovaries
	Decreased production, mastitis	Udder, mammary gland
	Immune system disorders	Decreased Th lymphocyte production and phagocytic activity
	Anemia	Erythrocytes
Sheep/goat	Nutritional myodystrophy	Skeletal muscle, myocardium
	Stiff lamb disease	Striated muscle
	Infertility	Loss of uterine tone
	Fetal death, reabsorption	Embryonic vascular system
	Decreased production, mastitis	Udder, mammary gland
	Immune system disorders	Decreased Th lymphocyte production and phagocytic activity

Source: modified table according to Zigo et al. (2021)

Selenium and VTE supplementation given to cows around calving has been reported to prevent suppression of blood neutrophil and macrophage function during the early post-parturition period. Vitamin E supplementation also enhances the rise in specific antibodies titres after vaccination, and increases *in vitro* T and B cell mitogenesis, interleukin production, and phagocyte activity (Khatti et al., 2016).

According to the nutrient requirements of dairy cattle (NRC; 2001), dietary recommendations for VTE and Se intake are 1000 IU VTE/head/day and 0.3 mg Se/kg of DM for dry cows. Diets containing less than 0.2 mg Se/kg of DM and 500 IU of VTE/head/day do not provide antioxidant and immunostimulating effects in the transition period. Fresh green forages are excellent sources of VTE, usually containing 80-200 IU VTE/kg of DM. However, concentrates and stored forages (hays, haylages, and silages) are generally deficient in VTE.

Deficiencies of Se and VTE are frequently diagnosed also on farms in central and northern Europe, which requires treatment by administration of products containing Se and VTE. Generally, farmers apply Se and VTE products by giving dietary supplements, injections, salt licks, drenches and bolus. There are two different sources of Se in nutritional supplements available: mineral, such as sodium selenite or selenate, and organic present in selenium-rich yeast (selenomethionine, selenocysteine) (Mehdi and Dufrasne, 2016).

Application of synthetic injectable forms of Se and a-tocopherol seems to be the most effective way to meet the organism needs for both antioxidants, especially in the case of dry cows, when oral supplementation fails to increase the reduced concentration in the blood plasma of dairy cows (Pavlata et al., 2002; Spears and Weiss 2008).

4.1.1 Objectivites of this chapter

The aim of this study was to compare the effect of parenteral and oral supplementation of Se and VTE on selected antioxidant parameters in the blood and colostrum of dairy cows and indicate their effect on the occurrence of mastitis during the peripartum period.

4.2 Materials and Methods

4.2.1 Description of farm and animals

This study was carried out in a herd of 280 Slovak Pied cattle in the east of Slovakia. The animals were kept in a free housing system with a separate calving barn and equipped with individual boxes with bedding. The cows were fed a total mixed ration (TMR) containing grass hay, corn silage, clover-grass silage, triticale grain, soybean meal and concentrate according to actual demand during the dry period and lactation (Table 2). The mean daily intake during the dry period and on the 5^{th} day after calving was 10 kg and 18 kg of DM, respectively. Milking took place in a parallel parlor Boumatic 2 x 10 Xpressway (Wisconsin, USA). The average daily milk production was 19.3±1.06 kg. Before drying, intramammary antibiotic preparation Orbenin Dry cow *a.u.v.* (Pfizer, IT) was applied to every quarter of the udder. The calves were separated immediately after birth (within 15

minutes) and received 2 L of colostrum followed by feeding of another 2 L in 8 – 10 hours via a nursing bottle tube.

Table 2. Composition of the feed rations fed *pre partum* and *post partum*.

	Composition	
Parameter	**Pre partum**	**Post partum**
DM (g/kg)	473	456
CP (g/kg DM)	128.03	148.15
Fat (g/kg DM)	25.4	28.62
NDF (g/kg DM)	348.12	332.3
ADF (g/kg DM)	229.02	211.16
NSP (g/kg DM)	374.12	418.03
Starch (g/kg DM)	257.25	307.5
NDP (g/kg DM)	24.2	15.6
NE, MJ/kg	6.18	6.67
Se mg/kg DM	0.2	0.2
[b]Se mg/kg DM	0.5	-
Vitamin E IU/kg	56	60
[c]Vitamin E IU/kg	102	-

DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, ADF – acid detergent fibre, NSP – non-starch polysaccharides, NDP – non-degraded protein, NE – net energy; [a]Composition – analysed values; [b]Se – analysed value with addition of 0.3 mg Se/kg of DM in form of Na_2SeO_3, [c] Vitamin E – analysed value with addition of 50 IU vit. E/kg of DM in form d-l-α-tocopherol acetate (IU) – international unit of vitamin E defined as 1 mg (±) α-tocoferol acetate.

In total, 36 cows (between the 2nd and the 4th lactation) in the final period of pregnancy based on e sonographic examination were randomly assigned into three groups (C, D1, and D2). Six weeks prior to the expected parturition cows in groups D1 and D2 received one of the treatments:

D1 - an experimental group of 12 animals with a median age of 4.3±0.3 years to which the injectable products Selevit inj. a.u.v. (sodium selenite 2.2 mg, dl-α-tocopherol acetate 25 mg in 1 ml of the solution), and Erevit sol. inj. (dl-α-tocopherol acetate 300 mg in 1 ml of the solution) were administered intramuscularly (IM) twice during the dry period (6 and 3 weeks before expected

parturition). The totals dose of sodium selenite and dl-α-tocopherol acetate were 88 mg and 2 000 mg, respectively.

D2 – an experimental group of 12 animals with a median age of 3.9±0.2 years was orally supplemented with 0.3 mg of Se/kg as Na_2SeO_3 and 50 mg of dl-α-tocopherol acetate/kg of DM given daily for six weeks before parturition (total doses: 5 mg of Se/head and 1020 mg of dl-α-tocopherol acetate/head per day).

The control group (C) with a median age of 4.6±0.3 years did not receive antioxidants. The animals' diet contained 0.2 mg of Se and 56 mg of VTE/kg of DM.

4.2.2 Collection of samples and laboratory examination

Blood samples were collected into 12 ml heparinized test tubes from the jugular vein of cows six weeks before the expected time of calving (before the supplementation period), on parturition day, and on the 14th day after calving. Samples of the first colostrum were also collected into 10 ml tubes. The health status of the mammary gland was assessed based on comprehensive examination on the 14th day according to the National Mastitis Council (2001). It consisted of a clinical examination, evaluation of milk from each quarter of the udder by California mastitis test (CMT, Jackson and Cockcroft, 2002), and collection of two 10 ml milk samples at a 45° angle for the microbiological examination and determination of malondialdehyde (MDA) concentration. The TMR nutritional values as well as concentrations of selected mineral elements were assessed in a 1 kg comprehensive sample of TMR from feed troughs according to Bujnak et al. (2011).

The blood plasma was obtained by high-speed centrifugation of heparinized blood at 3000 rpm for 15 min. Plasma from each sample was divided into two 3 ml tubes for determination of Se and a-tocopherol concentrations. All samples of blood plasma, milk and colostrum, and 2 ml of heparinised whole blood samples were stored at -54 °C until analysis.

The concentrations of Se in the samples of feed, plasma and colostrum were determined, after wet mineralization in a closed system using a microwave (Milestone MLS 1200) and digestion with HNO_3 and H_2O_2, by atomic absorptive spectrometer Zeman 4100 (Perkin Elmer, USA) equipped with a generating system, according to the procedure Pechova et al. (2005).

The GPx activity in the samples was measured using the method developed by Paglia and Valentine (1967), using a commercial kit (Randox RS 505) and an automatic analyzer (Cobas Mira), and expressed as units per gram of hemoglobin (U/g of Hb). Hemoglobin was analyzed using Drabkin's method using a commercial reagent **(**Randox, UK).

The content of α-tocopherol in plasma and colostrum was determined using the HPLC method developed by Hess et al. (1991) after extraction of the samples in N-heptane, their evaporation, and subsequent dissolution in methanol. Determination of α-Toc from the homogenized sample of TMR

after saponification and extraction was carried out using the HPLC method according to Smith et al. (1997).

Milk samples (0.05 ml) were inoculated onto blood agar (Oxoid, UK) and cultivated at 37 °C for 24h. Based on the colony morphology, *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspected colonies of *Staphylococcus* spp., *Streptococcus* spp., and *Enterobacteriaceae* spp. were isolated onto blood agar, cultivated at 37°C for 24h and identified biochemically using the STAPHY-test, STREPTO-test, ENTERO-test, and TNW Pro 7.0 sotfware (Erba-Lachema, CZ). Dry matter was acquired by drying the samples at 105 °C for 48 h. The nutritional values of TMR were determined using AOAC methods (2001).

4.2.3 Malondialdehyde (MDA) determination

The selection of milk samples from all groups for the measurement of MDA concentration was based on the veterinary medical record, CMT results, and clinical examination. In each experimental group two subgroups of quarter milk samples were chosen. In the first subgroup milk came from udder quarters without clinical signs of mastitis or other illnesses, milk samples from the second subgroup were positive in CMT (score 1 - 4) and bacteriological cultures. Lipid oxidation products expressed as MDA concentration were measured using the method based on a reaction of lipid peroxides with thiobarbituric acid and analyzed by UV-VIS spectrophotometry at 532 nm, as described by Andrei et al. (2016). The results were expressed in $nmol \cdot ml^{-1}$ of milk.

4.2.4 Statistical analysis

One-way analysis of variance (ANOVA) with the *post hoc* Dunett´s Multiple Comparison Test was used to compare the experimental groups with the control group. Comparison of MDA in healthy and mastitis milk samples in the individual experimental groups was done using a paired t-test. Differences between the mean values of the different treatment groups were considered assuming significance levels of 0.05 and 0.01. Values in the tables are expressed as means (M) and standard deviation (SD).

4.3 Results

Plasma and colostrum Se concentrations are shown in Tables 3 and 4. Plasma Se and VTE concentrations in cows were similar before oral and repeated parenteral supplementation. In both experimental groups, plasma concentrations of Se and VTE directly after calving were higher compared to the control group. Moreover, in the plasma of animals supplemented orally (D2) both parameters (Se and VTE) had higher values on the 14th day after calving. Similarly, the colostrum of cows from the D2 group was richer in Se and VTE compared to D1 and C groups.

A comparison of enzymatic antioxidant activity in the examined groups is shown in Table 5. A significant decrease of GPx activity was detected in the control group after calving in comparison with the orally supplemented group D2. When comparing the activity of GPx on the 14th day after calving, no statistical differences were observed among the studied groups of cows.

Table 3. Effect of oral and parenteral supplementation of selenium and vitamin E on selenium concentrations in blood plasma and colostrum (μg/L).

Period		C	D1	D2
		M±SD	M±SD	M±SD
42th day a.p.	Cows	75.5±6.8	74.1±6.5	72.1±6.8
Parturition	Cows	69.4±6.7[a]	81.3±6.7[b]	88.1±9.1[b]
	Colostrum	30.5±4.4[a]	38.2±6.8	44.7±5.6[b]
14th day p.p.	Cows	71.6±6.1[a]	75.7±8.7	82.3±8.1[b]

Note: D1 – parenterally supplemented group on the 42nd and the 21st day before parturition; D2 – group after 42 days of oral supplementation of Se and vitamin E, C – control group; a. p. – *ante partum*; p.p – *post partum*; M±SD – mean ± standard deviation; **[a,b]** significance level $p < 0.05$.

The evaluation of the effect of oral and parenteral supplementation of Se and VTE on the incidence of mastitis is shown in Table 6. On the 14th day after calving, a 25% reduction in mastitis incidence was observed in the orally supplemented dairy group (D2) compared to the control group. Both subclinical and clinical mastitis incidence decreased. The number of infected udder quarters was lower by 10.

Table 4. Effect of oral and parenteral supplementation of selenium and vitamin E on α-tocopherol concentrations in blood plasma and colostrum (μg/mL)

Period		C	D1	D2
		M±SD	M±SD	M±SD
42th day a.p.	Cows	5.5±0.58	5.1±0.62	5.3±0.56
Parturition	Cows	4.4±0.76[a]	7.4±0.86[b]	8.2±0.82[b]
	Colostrum	9.8±1.74[a]	12.4±3.1	18.1±2.3[b]
14th day p.p.	Cows	4.6±5.8[a]	5.1±0.76	6.4±0.68[b]

Note: D1 – parenteral supplemented group on the 42nd and the 21st day before parturition; D2 – group after 42 days of oral supplementation of selenium and vitamin E, C – control group; a. p. – *ante partum*; p.p – *post partum*; M±SD – mean ± standard deviation; **[a,b]** significance level $p < 0.05$.

Table 5. Effect of oral and parenteral supplementation of selenium and vitamin E on the activity of glutathione peroxidase (U/g of Hb) **in blood of dairy cows**

Period	C	D1	D2
	M±SD	M±SD	M±SD
42nd day a. p. cows	497 ± 45.3	481 ± 41.6	475 ± 46.7
Parturition	384 ± 32.8[a]	406 ± 37.5	452 ± 46.2[b]
14th day p. p. cows	357 ± 36.4	364 ± 41.2	382 ± 37.4

Note: D1 – parenterally supplemented group on the 42nd and the 21st day before parturition; D2 – group after 42 days of oral supplementation of selenium and vitamin E, C – control group p; a. p. – *ante partum*; p.p – *post partum*; M±SD – mean ± standard deviation; **a,b** significance level $p < 0.05$.

Bacteriological milk cultures from the infected quarters revealed coagulase-negative staphylococci, *Staphylococcus aureus*, and *Streptococcus uberis*, which are most often associated with subclinical and clinical mastitis. There were no differences in pathogens isolated from various disease courses among groups.

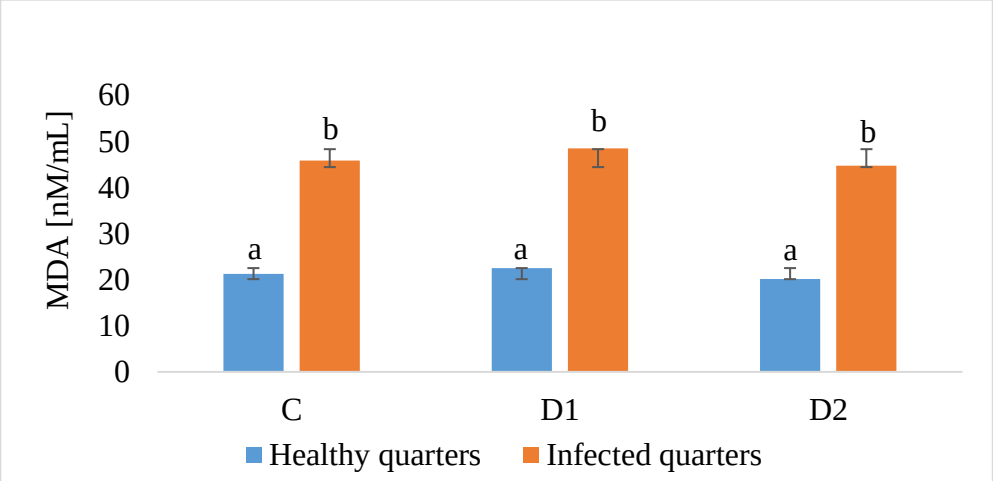
Figure 1 compares milk MDA concentrations from healthy and infected quarters in the examined groups. The milk MDA concentrations were evaluated in samples taken on the 14th day postpartum. In all groups, MDA concentration was higher in mastitic milk compared to milk from healthy udders. No statistical differences in milk MDA concentrations were observed when comparing the three groups of cows.

Table 6. Occurrence of mastitis in the studied groups two weeks after calving

Group	Total/healthy cows			Mastitic cows		Heathy/ infected quarters	Mastitis forms in infected quarters %		
	n_T	n	%	n	%		SC	SA	A
C	12	5	41.7	7	58.3	30/18	14.6	18.8	4.7
D_1	12	6	50.0	6	50.1	34/14	12.5	12.5	4.2
D_2	12	8	66.7	4	33.3	40/8	6.3	10.4	1.0
Total	36	19	52.7	17	47.3	104/40	16.4	14.6	6.94

Note: n_T- number of cows included in the group, n - number of healthy/infected dairy cows from each group, SC - subclinical mastitis, SA - subacute mastitis, A - acute mastitis, D1 – parenterally supplemented group on the 42nd and the 21st day before parturition; D2 – group after 42 days of oral supplementation of Se and vitamin E, C – control group.

Figure 1. Comparison of milk malondialdehyde (MDA) concentrations (nM.mL^{-1}) **in healthy and infected quarters**



Note: D1 –group supplemented parenterally on the 42nd and the 21st day before parturition; D2 – group after 42 days of oral supplementation of Se and vitamin E, C – control group; **a,b** significance level between columns at p <0.05.

4.4 Discussion

4.4.1 Assessment of oral and parenteral supplementation of Se and VTE

The peripartum period is one of the most critical phases of the reproductive and lactation cycles of cows. During this period, it is important to supply the cows with mineral-vitamin supplements containing Se and VTE for proper immunostimulation and antioxidative protection. Estimated daily nutritional requirements of dairy cows for Se and VTE are 300 µg/kg DM and 20 to 60 international units (IU), respectively. By analyzing the feed ration during the dry period, the Se content 0.2 mg/kg DM and 56 IU/VTE per kg/DM were determined (Table 2). Although the content was too low according to the recommendations of NRC (2001) both antioxidant nutrients have similar functions and dietary Se consumption is influenced by VTE. The amount of Se should be increased when a diet low in VTE is consumed. Therefore, Se deficiency could be partially compensated by an adequate intake of VTE and vice versa (Liu et al., 2016). Pavlata et al. (2004a) recommend determining Se and VTE not only in the TMR, but also in the blood of animals, because those concentrations often do not correlate. In assessing the blood Se status the same authors recommend three basic ranges: adequate (> 100 µg of Se/L), borderline (70–100 µg/L) and deficient (< 70 µg/L).

Scholz and Stober (2002) also considered a blood concentration of Se greater than 100 µg/l as adequate in cows. At the beginning of the studied period, the concentration of Se in the blood plasma of dairy cows ranged from 72.1 to 75.5 µg of Se/L, which can be considered the borderline concentration. The animals from the D1 and D2 groups had significantly higher blood Se concentration on the parturition day than the control animals (p <0.05). However, increased plasma

concentration ($p<0.05$) on the 14th day after parturition was recorded only in the orally supplemented group (D2) (Table 3).

The VTE status of dairy cows is one important component of a well-functioning immune system because of its antioxidant effects on cows and young dairy calves (Pavlata et al., 2004b).

NRC (2001) recommends 40 - 60 IU/kg DM of VTE for optimal antioxidant action of this vitamin in feed and to ensure sufficient plasma concentrations in dairy cows during the peripartum period. In our analysis, the content of VTE in TMR was 56 IU/kg DM, which may be considered adequate for dairy cows during the drying period.

According to the recommendation of Cohen et al. (1991) on optimal VTE supplementation in TMR, serum a-tocopherol concentration should be higher than 4.0 mg/mL. Canadian researchers tested ten clinically normal cows from five different herds and found mean serum VTE concentrations to range from 3.19–5.3 mg/mL when cows were fed a diet with a VTE dose in TMR up to 50 IU/kg DM. Our findings indicate that plasma levels of VTE (5.1 – 5.5 mg/mL) at the beginning of the study were adequate in all monitored groups (Table 4).

Bouwstra et al. (2010) reported that long-term oral administration of VTE to cows in the dry period has a better effect on alpha-tocopherol plasma concentration than parenteral supplementation. Similarly, in our study, in parenterally supplemented cows (group D1), the plasma concentration of VTE was increased only on the day of parturition while no changes were observed in the colostrum and blood plasma VTE on the 14th day after calving compared to the control group. Nutrient deficiencies during pregnancy in cows often result in metabolic disorders and increased incidence of related diseases (mastitis, nutritional myopathy, retained placenta, and other reproductive disorders). The lowest plasma concentrations of Se and a-tocopherol are typically detected between one week prepartum and two weeks postpartum (Kafilzadeh et al., 2014).

Meglia et al. (2006) and Pavlata et al. (2004a) found decreased plasma concentrations of VTE and Se on the parturition day, because of the reduction in DM intake at calving, and an increased need for antioxidants during this time. We observed a similar phenomenon in our study. The concentrations of Se and VTE were lower in the control group after calving compared to the supplemented groups D1 and D2.

Pregnant cows with a low intake of Se and VTE in the feed during the drying period will likely have low concentrations of these antioxidants in the colostrum and give brith to calves deficient in these elements. Se and VTE deficiencies in calves are associated with a significantly increased risk of myodegeneration and illness, mainly due to immune function compromise. Therefore, determining the concentration of these antioxidants in TMR and colostrum should be a part of preventive diagnostics of increased calve morbidity (Pavlata et al., 2004b).

In our study, colostrum examination revealed a trend towards higher Se and VTE concentrations ($p < 0.01$) in the D2 group after 42 days of oral supplementation of Se and VTE during the dry period. The orally supplemented group tended to have higher blood plasma Se and VTE concentrations than the control group, which probably translated to higher transfer ($p < 0.05$) to colostrum. It shows that oral supplementation is more efficient and stabilizes the monitored antioxidants in blood plasma and colostrum. Our data are similar to those found in Czech Red-and-White cattle, in which there was a very close correlation between colostral and blood Se and VTE contents. Concentrations of both antioxidants in colostrum may be higher in combined breeds than in dairy breeds due to lower colostrum production (Pavlata et al., 2004a).

4.4.2 Evaluation of glutathione peroxidase activity

Synthesis of reactive oxygen species (ROS) and their accumulation during parturition or inflammation are controlled by antioxidant enzyme systems. Several defense mechanisms prevent oxidative damage in living organisms. These include scavenging enzymes such as GPx, superoxide dismutase (SOD), and catalase (CAT) (Andrei et al., 2011). Blood GPx activity reflects long-term Se supply since the regeneration of the enzyme is related to the erythrocyte lifespan. However, it is a matter of debate how fast the changes in GPx activity follow alterations in Se status.

For practical use, Pavlata et al. (2002) recommend the lower limit of the reference value of GPx in whole blood of cattle of 250 U/g of Hb. In our study, the activity of GPx throughout the study period in all experimental groups of cows was adequate. During calving, increased ROS accumulation results in a faster decrease in GPx activity. We observed this effect as significantly lower GPx activity in the control group after calving than in orally supplemented group D2 (Table 5). Decreased GPx activity in the control group is also closely related to the long-term reduced intake of Se in the TMR at a level of 0.2 mg/kg DM. In contrast, with a sufficient long-term supplementation of Se in the feed ration, it is possible to incorporate this element into the erythrocyte GPx and thus ensure sufficient antioxidant protection against ROS, which was also seen in our study in the orally supplemented group.

The antioxidant status does not only depend on the long-term Se supply and current status of GPx activity. Glutathione peroxidase action is complemented by vitamins and other redox systems in the elimination of ROS. Consequently, the consumption of one antioxidant may affect the concentration of the others, since the action of antioxidant enzymes also depends on their sparing effect and target tissue.

4.4.3 Evaluation of the occurrence of mastitis

The current content of Se and VTE in the feed ration and blood in dairy cows are closely linked to the optimal immune function and mammary gland health. Selenium and VTE deficiencies in dairy cows have been often associated with intramammary infection (Mehdi and Dufrasne, 2016).

Table 6 shows that after oral supplementation of the selenium-vitamin supplements in group D2 a 25% reduction of mastitis was observed and 10 less infected quarters, compared to control cows. Table 6 shows that in our study oral supplementation of the selenium-vitamin supplements led to a 25% reduction of mastitis incidence and ten fewer infected udder quarters than in control cows. Kommisrud et al. (2005) carried out their study on 254 dairy cows of the Norwegian Red breed to highlight the importance of determining Se in the feed ration. They found that offering diets low in Se <0.1 μg/g of DM for a long time was associated with an increased incidence of mastitis by 1.3 to 1.4 times 30 days after calving.

Pavlata et al. (2004a) compared the influence of different doses of parenteral Se and VTE in dairy cows prior to parturition on selected metabolic parameters, colostrum quality, and occurence of mastitis. Cows injected two times during the dry period, 8 and 4 weeks before expected parturition, (88 mg of sodium selenite and 1 000 mg of a-tocopherol acetate) had significantly ($p < 0.05$) higher concentrations of Se and VTE in the colostrum collected on the day of parturition. Moreover, no clinical cases of mastitis were reported in the parenterally supplemented group compared to 5 incidents of treated mastitis in the control group during the first month of lactation. In our study, repeated injections increased a-tocopherol acetate and Se concentrations in blood plasma compared to the control group. However, we did not observe statistically significant differences in the occurrence of mastitis 14 days after calving.

Similar results were described by Smith et al. (1997) after repeated intramuscular injection of 2 mg/kg body weight of α-tocopherol acetate and 0.1 mg/kg body weight of Se on the 42nd and the 21st day prepartum in combination with oral supplementation of 740 IU of vitamin E and 3 mg of Se per day. In early lactation, the occurrence of intramammary gland infections decreased from 63% to 37% compared to the control group supplemented with 100 IU of vitamin E/day and on a low selenium diet (< 0.10 mg/kg DM).

Intramammary infections are most commonly of microbial origin, as up to 95% of mastitis is caused by pathogenic bacteria that penetrate the mammary gland through the teat canal. The bacteria causing the most common forms of mastitis may be divided into two groups. The first group consists of contagious pathogens (e.g. *Staphylococcus aureus, Streptococcus agalactiae, and Streptococcus dysgalactiale*). They survive and grow within the mammary gland and so transmission of infection from infected to uninfected quarters and from cow to cow most likely occurs during milking (Zigo et

al., 2021). In the second group, we are environmental pathogens found in the environment, such as *Streptococcus uberis*, *E. coli*, non-aureus staphylococci (NAS), and *Corynebacterium spp.* (Hawari et al., 2008).

Analyzing the milk samples from the infected udder quarter, we confirmed NAS (S. *epidermidis, S. chromogenes* and *S. xylosus)* bacteria *Streptococcus uberis* and *Staphylococcus aureus,* which are most often associated with the subclinical and clinical forms of mastitis. There were no differences in the bacteria species or course of mastitis among the studied groups of cows.

4.4.4 Comparison of milk malondialdehyde concentrations

Intramammary infections are among to the main causes of depletion of antioxidant enzymes resulting in excess ROS accumulation and lipid peroxidation. Malondialdehyde (MDA) is generated during lipid peroxidation and is considered a biomarker of oxidative stress. The results of our study show that MDA concentrations from affected quarter milk samples were increased compared to samples from healthy cows in each monitored group. The comparison of infected milk samples among individual groups did not reveal changes in concentration of MDA (Fig. 1).

The changes in MDA concentration from affected quarter milk samples and changes in blood GPx in the present study are in accordance with the previous report (Turk et al., 2017) that showed a significant increase in MDA level in mastitis cows as compared to healthy cows.

Castillo et al. (2006) and Sharma et al. (2011) observed a higher MDA concentration in the milk samples from healthy udders and those with mastitis, as well as in the blood of cows in early lactation as compared to the cows during the dry period or mid-lactation. The activity of GPx has been confirmed to closely correlate with the antioxidant capability of the organism. Our results indicate a close relationship between inflammatory response and oxidative stress in mastitis, which can be interpreted as an important role of the antioxidant defense system in maintaining the health of the mammary gland. In order to prevent oxidative stress and moderate inflammatory response as well as to improve the immune status of dairy cows, it is important to meet their nutritive needs and balance them with an appropriate ratio of vitamin-mineral supplements involved in antioxidative defense mechanisms.

4.5 Conclusion

Both routes of Se and VTE administration to pregnant dairy cows positively influence the concentration of a substances in the blood plasma of animals on the parturition day. However, only prolonged oral supplementation is associated with high concentrations of Se and α-tocopherol in colostrum and the blood plasma of cows on the 14th day after calving. Therefore, we conclude that oral supplementation of Se and VTE is more efficient and has a better stabilizing effect on the

antioxidant enzymes in blood plasma and colostrum, thus leading to the reduced incidence of mastitis. The data obtained in this study also show that the duration of higher plasma α-tocopherol and Se concentrations is relatively short after parenteral injections. Therefore we recommend a combination of the injectable and oral vitamin E supplements to quickly increase and then maintain a high concentration of VTE and Se in cows during the postpartum period.

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5 Chapter
IMPACT OF HUMIC ACID AS AN ORGANIC ADDITIVE ON THE MILK PARAMETERS AND OCCURRENCE OF MASTITIS IN DAIRY COWS

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5.1 Introduction

The health-safety and nutritional quality of raw milk is influenced by many factors. One of the main factors that affects the health of and milk production by dairy cows is mastitis. Mastitis is an inflammation of the mammary gland characterized by physical, chemical, bacteriological and cytological changes in the milk. Changes in the quality and quantity of milk, as well as pathological changes in the glandular tissues of the udder have been observed (Pyörälä and Taponen, 2009).

Mastitis is mainly caused by microorganisms. These are usually bacteria, including gram-negative and gram-positive bacteria, mycoplasmas, yeasts and algae (Zadoks et al., 2011). The majority of mastitis cases are caused by a few common bacterial pathogens involved: *Staphylococcus* spp. (*S. aureus, S. warneri* and *S. chromogenes*), *Streptococcus* spp. (*Str. agalactiae, Str. dysgalactiae, Str. uberis* and *Str. bovis*), coliforms (mainly *E. coli* and *Klebsiella pneumoniae*) and *Actinomyces pyogenes* (Idriss et al., 2013). Although some pathogens from the group of coagulase negative staphylococci (CNS) and *Corynebacterium bovis*, are historically considered to be of limited importance and are therefore often described as minor pathogens. In the last decade the impact of CNS has increased probably because the prevalence of major pathogens has decreased (Pyörälä and Taponen, 2009).

Mastitis and other diseases are common problems in dairy herds, resulting in increased costs and decreased production. Most diseases in dairy cows occur at or just after calving, which is a period associated with immune suppression, resulting in an increased susceptibility to infections. Prepartum immune suppression is multifactorial but is associated with endocrine changes and destabilization of intestinal flora, leading to impaired digestion and utilization of nutrients from animal feed (Xiaowang et al., 2010; Zigo et al., 2014).

During the past few decades the use of organic feed additives to improve health, wellbeing and production has been investigated in some areas of animal husbandry. Humic substances (HS) are one such additive (Marcinčáková et al., 2015; Semjon et al., 2020).

Humic substances (HS) are geological deposits made of a complex mixture of acids that arise from the natural decomposition of plant and animal material by soil microorganisms occurring in water, soil, carbon and other sources. They are heterogeneous high molecular weight organic substances and their composition differs according to the geographic region (Jaďuttová et al., 2019; Mudroňová et al., 2020).

A yellow to brown-coloured seam (brown seam) may contain high concentrations of fulvic acid, whereas a dark brown to black-coloured seam (black seam) may contain high amounts of humic acid and humin. Humic acids (HA) are considered to be adsorbent, because of various binding sites present in their structure. It has been assumed that humic acids could reduce the absorption and systemic availability of bacterial endotoxins, which could be of great importance in the protection of animal and human health (Trčkova et al., 2005; Galip et al., 2010).

Moreover, many positive effects on the performance and health of animals have been attributed to humic acids. They inhibit the growth of pathogenic bacteria and moulds and decrease the level of mycotoxins and thus may lead to improved gut health (Marcinčákova et al., 2015).

Humic acids stabilize the intestinal flora, and in this way, improve the utilization of nutrients from animal feed, which affects the composition of dairy cows' and goats' raw milk (Potůčková and Kouřimská, 2017).

5.1.1 Objectivites of this chapter

Previous studies reported that the addition of HA to the diet of cows stimulated the fermentation products with improved nutrients digestion, growth and development of immune responses, but there is no data on the effect of its use on cow milk parameters and mammary health. Therefore, the aim of this work was to evaluate the effects of a humic acid supplemented diet on the main milk parameters and composition as well as occurrence of mastitis in dairy cows during the peripartum period.

5.2 Material and methodology

5.2.1 Animal care

The practical part of study was carried out in dairy herd of 140 crossbred Slovak Pied cattle x Red Holstein. Dairy cows from the monitored herd were kept in a free housing system with a separate calving barn, equipped with individual boxes with bedding and were allowed *ad libitum* access to water. Cows were fed twice a day with feed mixture formulated for a 650 kg cow according to the Nutrient Requirements of Dairy Cattle (NRC, 2001).

During the lactation period cows were milked twice a day at 4:30 a.m. and 4:30 p.m. in the fishing-milking parlour (FarmTec) 2x10 pcs (Figure 1). First, water was used to remove impurities from the udder and teats. Subsequently, the udder was thoroughly wiped with disposable paper wipes. The first milk from each quarter was hand-drawn into a dark-bottomed pot, and the milk was subjected to sensory analysis. During the milking process, the pulsation ratio was 60:40 at a rate of 52 c/min and milking was automatically terminated when the milk flow dropped to 0.2 l/min. After milking, the teats were disinfected by teat-dipping. Before drying an intramammary antibiotic preparation Orbenin Dry Cow a.u.v. (Pfizer, IT) was applied to every quarter of the udder in pregnant cows.

Figure 1. Dairy cows fed with TMR, assessment of CMT and milking process

5.2.2 Experimental design, animals and diets

The experimental conditions were designed in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). Twenty gravid cows in the last stage of pregnancy were selected from the herd. Fifty days prior to the expected calving date the cows were randomly divided into two groups, control (C) and experimental (E). The 10 animals per group were housed on a deep litter divided into two separate stables with ad *libitum* access to water and feed

Selected cows from each group were fed twice a day with a total mixed ration (TMR) containing corn silage (65%), grass hay (12%), barley straw (10%), bean (11.7%) and concentrate (1.3%) with content of extracted rape and soy meal according to the current NRC (2001) during the dry period (Table 1). The mean daily intake for the dry period under study was 9.6 kg of DM/cow per day.

The experimental group (E) was supplemented into diet with humid acids at a dose of 100 g/cow per day. The humic acids (product Humac Nature AFM) used in the experiment were obtained from Humac ltd. company, SR. According to the producer, Humic Nature as an organic additive in the diet of animals contains: total humic acids 65% and minerals 15%, of which accounted for free humic acids 60%.

The experimental period lasted 50 days prior to the expected parturition and ended immediately after calving. Subsequently, the calves were separated from cows that were then milked into individual containers. Five days after were milked twice a day together with the all lactating cows.

5.2.3 Udder health examination and milk sampling

Udder health was evaluated and milk samples were taken from each selected cow on days 10 and 30 of the first month of lactation. A thorough evaluation of udder health included clinical examination, sensory analysis of milk from forestripping of each udder quarter followed by assessment of CMT (Indirect Diagnostic Test, Krause, Denmark). Milk from every quarter was mixed with the reagent and the result was scored as negative, trace or positive (score 1–3) depending on the formation of gel in the milk sample according to Jackson and Cockeroft (2002).

Next, we collected a milk sample from one quarter for bacteriological cultivation and two mixed milk samples for measurement of the milk components and SCC from each cow aseptically in accordance with the guidelines of the National Mastitis Council (2001). The samples were cooled to 4 °C and immediately transported to the laboratory and analysed on the following day.

5.2.4 Analytical methods

5.2.4.1 TMR chemical composition

A 1 kg sample of TMR was analysed for dry matter (DM), crude protein, crude fat, ash neutral detergent fibre (NDF) and acid detergent fibre (ADF) according to AOAC methods (2012). The net energy (NE) contents were obtained by calculation (NRC, 2001).

Table 1. Chemical composition of feed mixture

Component	Content
DM (g/kg)	408.8
CP (g/kg DM)	53.3
Fat (g/kg DM)	14.5
NDF (g/kg DM)	182.4
ADF (g/kg DM)	129.4
Ash (g/kg DM)	38.9
Starch (g/kg DM)	40.8
NE[1], MJ/kg	5.65

Note: DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, ADF – acid detergent fibre, NE[1] - net energy, obtained by calculation.

5.2.4.2 Determination of milk parameters

The raw milk samples were analysed for dry matter (total solids), non-fat dry matter (SNF; solids non-fat), lactose, fat, proteins content and pH using the Milk analyzer Lactoscan MCCW (Milkotronic, Bulgaria) according to Potůčková and Kouřimská (2017). Milk urea (MU) content was measured on a CHEMSPEC apparatus (Bentley Instruments Inc.) according to Pecka et al. (2012). All measurements were performed twice for each sample.

5.2.4.3 Determination of SCC

The somatic cell count (SCC) is one of the internationally recognized standards for milk quality control and is also a useful indicator of mastitis presence. The Somatic Cell Counter Lactoscan SCC (Milkotronic, Bulgaria) is based on direct fluorescent, low magnification microscopic somatic cell counting. Lactoscan SCC uses a very sensitive fluorescent dye (Sofia Green) and LED optics (CCD technologies) in order to make the cell analysis more accurate, reliable and fast.

5.2.4.4 Laboratory analyses

Bacteriological examinations were performed according to commonly accepted rules (Malinowski et al., 2006). Milk samples (10 μL) were cultured at the respective veterinary practice according to routine procedures, usually employing Columbia Blood Agar Base with 5% of defibrinated blood, Staphylococcal medium N° 110, Baird-Parker agar, Edwards Medium, Mac Conkey Agar (Oxoid, OXOID Ltd., Basingstoke, Hants, UK), and incubation at 37 °C for 24 h.

As well as evaluating bacterial growth characteristics other assays were used to identify bacterial species: pigment and coagulase production, catalase activity, haemolysis, Gram staining and other virulence factors. Staphylococcus spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspected colonies of *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were isolated on blood agar and cultivated at 37 °C for 24 h and identified biochemically using the Staphy test, Strepto test and resp. Entero test using the software TNW Pro 7.0 (Erba-Lachema, CZ) according to the manufacturer's instructions.

5.2.4.5 Statistical analysis

A one-way ANOVA with F-test on the arithmetic means (M) from 10 parallel measurements with standard deviation (SD) of dry matter (total solids), SNF (solids non-fat), lactose, fat, protein content, pH, MU and SCC was performed by Microsoft Excel 2003. Statistical significance was set at $p < 0.05$. The differences in the prevalence of mastitis and distribution of bacterial pathogens among monitored groups of cows were statistically analysed using the Chi-square test. The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value = 5.991.

5.3 Results and discussion

Table 2 illustrates the effect of supplementation of HA on the milk components and SCC in dairy cows. The two groups of animals, control and experimental, were fed with TMR (Table 1) during the 50 days prior to parturition. In addition to this feed, the experimental group was supplemented with HA at a dose of 100 g/cow per day. Changes of MU content (Graph 1) and SCC on the 10th day after calving in HA supplemented group was reported. No changes in the composition of dry matter, SNF, lactose, fat, protein content or pH in raw milk were noted during the monitoring period (Table 2).

The observed decreased level of MU in cows fed diet with HA can be explained by lower blood urea nitrogen (BUN) level which resulted from lower ruminal ammonia nitrogen (NH_3-N) concentration indicated by more efficient utilization of dietary crude protein (CP).

Table 2. Effect of supplemental humic acid on SCC and milk parameters

Parameters	Groups comparison after 10th day of lactation			Groups comparison after 30th day of lactation		
	Control	Experimental	P*	Control	Experimental	P*
	M±SD	M±SD		M±SD	M±SD	
SCC x 10^3	425.30 ±53.8^a	358.14 ±41.5^b	p <0.05	384.42 ±40.02	331.60 ±36.3	p >0.05
MU (mg.100 ml^{-1})	14.26 ±1.21^a	9.31 ±1.35^b	p <0.05	12.3 ±1.56	13.61 ±1.42	p >0.05
DM (g.100 g^{-1})	12.68 ±1.23	12.56 ±0.74	p >0.05	12.44 ±0.86	12.68 ±1.13	p >0.05
SNF (g.100 g^{-1})	8.63 ±0.64	8.41 ±0.74	p >0.05	8.32 ±0.55	8.60 ±0.51	p >0.05
Fat (g.100 g^{-1})	4.05 ±0.35	4.15 ±0.56	p >0.05	4.12 ±0.28	4.21 ±0.60	p >0.05
Protein (g.100 g^{-1})	3.47 ±0.36	3.51 ±0.42	p >0.05	3.38 ±0.31	3.55 ±0.38	p >0.05
Lactose (g.100 g^{-1})	4.71 ±0.23	4.90 ±0.18	p >0.05	4.84 ±0.37	4.76 ±0.30	p >0.05
pH	6.61 ±0.09	6.64 ±0.12	p >0.05	6.58 ±0.18	6.65 ±0.16	p >0.05

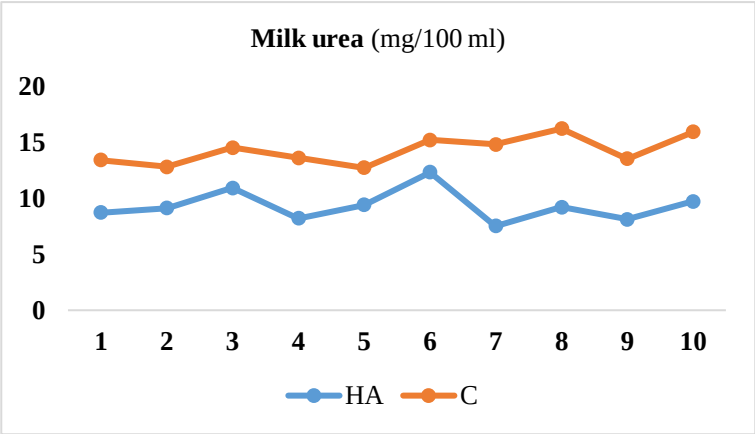
Note: SCC - somatic cell count; MU – milk urea; DM - dry matter (total solids); SNF - non-fat dry matter (solids non-fat); M – mean; SD - standard deviation; *P <0.05Significant difference; *P >0.05 - no significant difference.

The same trend was observed in the study by Van Soest (1994) who supplemented dairy cows with HA during lactation. In HA treated cows, lower blood urea nitrogen (BUN) value was indicated by more efficient utilization of dietary CP for microbial protein synthesis associated with nitrogen-binding capabilities of HA. This low value of BUN is usually associated with a lower ruminal NH_3-

N flux to bloodstream. Thus, reduction in MU result can be explained with the reduction of BUN supported by lower ruminal NH_3-N concentration.

Similar results were observed by Degirmencioglu (2014) after supplementation of HA to ruminants for 90 days of lactation, with no improvements in milk composition (non-fat dry matter, lactose, fat and protein content). The effect on milk yield was inconsistent. Other studies have reported positive effects of HA on milk production, milk fat (Thomassen and Faust, 2000) and milk protein (Potůčková and Kouřimská, 2017) in dairy cows. Another study showed that the use of HA as an animal feed supplement leads to increased milk production and increased butterfat percentage in dairy cows (Islam et al., 2005).

However, it is difficult to compare the effects of HS across studies due to the different sources and preparations of HA used, as well as because animals reared in various regions of the world are exposed to different climates and environmental conditions.



Graph 1. Comparison of milk urea content in supplemented by humic acid (HA) and control (C) group on the 10th day of lactation

Milk SCC is a useful tool for measuring milk quality, the health status of the mammary gland and changes in milk composition. In the European Union the legal limit for cows is 400 000 cells ml^{-1} (Zajác et al., 2012) and in the USA the legal limit established by the Food and Drug Administration for cows is 750 000 cells ml^{-1} (Paape et al., 2007).

In our study, control group shoved increased SCC value above legal limit on the 10th day after calving (Graph 1). However, the HA supplemented group had lower SCC values on the 10th day after calving (Table 2) and positive quarters according to the CMT (Table 3).

A score of 1 to 3 in the CMT indicates an increased SCC over the legal limit, most commonly caused by the presence of pathogenic bacteria. Penetration of pathogenic bacteria into the teat canal

irritates the delicate mammary tissue causing an inflammatory response and changes in milk quality and composition (Sharma et al., 2011).

In particular, differences were found by comparing the positive qurters in both groups with CMT score 1 and 3. According to our results the number of positive quarters detected by the CMT on the 10th day after calving was reduced by 20.0% in the group of cows supplemented with HA (Table 3).

Similar to our study, Thomassen et al. (2000) reported that HA supplementation significantly decreased the SCC in milk. They reported that a diet containing 3 g HA kg^{-1} decreased the SCC level in milk dairy by about 50%.

Similarly, Xiaowang et al. (2010) reported that a lower (by 40.1%) SCC was observed in their humate-supplemented group compared to the control. Intramammary bacterial invasion occurs immediately after calving and leads to glandular damage in parenchymatous tissue. The glandular tissue damage leads to increased SCC and reduced milk production. The cellular presence in milk is one of the important protective mechanisms of the mammary gland (Sharma et al., 2011).

Table 3. Milk evaluation per quarter and interpretation of California Mastitis Test (CMT) score

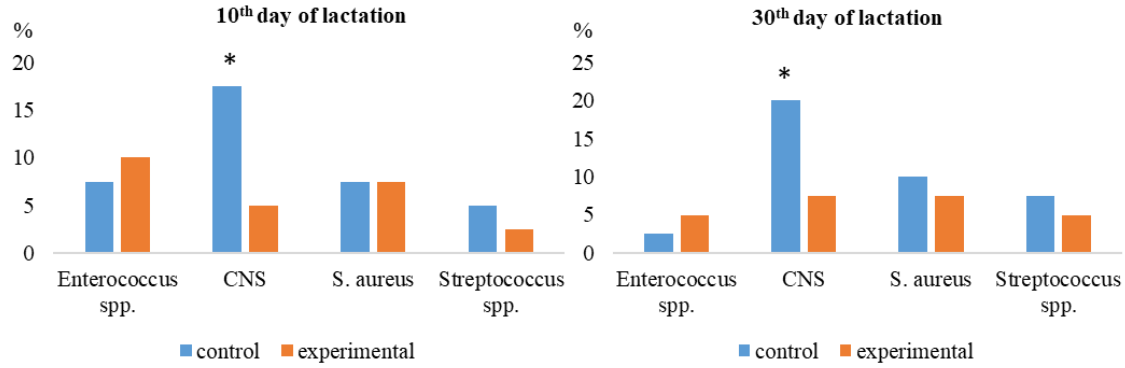
CMT score	SCC* x 10^3	Interpretation	Evaluated quarters in monitored groups			
			10th day of lactation		30th day of lactation	
			Control	Exper.	Control	Exper.
N (neg.)	0 - 200	Healthy quarter	37.5	35.0	42.5	47.5
T (trace)	200 - 400 (±50)	Healthy/LM[1]	25.0	22.5	20.0	22.5
1	400 - 650 (±150)	SM[2]	20.0 [a]	32.5 [b]	17.5	12.5
2	850 - 1.200 (±200)	SM[2]/CM[3]	10.0	10.0	12.5	15.0
3	1.500-5.000 (±300)	CM[3]	7.5[a]	0.0[b]	7.5	2.5

Note: N (neg.) – negative CMT score (healthy quarters), Exper. – experimental group supplemented with HA, [ab]Significant differences; P<0.05; LM[1] - latent mastitis is characterized with normal milk consistency, but infection is present in samples of raw milk without changing the SCC and a negative CMT score; SM[2] - Subclinical mastitis, no symptomps are observed, the udder and milk appear normal, but infection is still present with positive CMT score and increased SCC; CM[3] - clinical mastitis, signs range from mild to severe with a positive CMT score, high level of SCC, positive bacteriological cultivation, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production with clinical signs.

The addition of HA at a dose 100 g/cow per day in our study had a positive nutraceutical effect that stimulated neutrophil activity, which may protect against bacterial pathogens and reduce mortality during acute bacterial infection. The results show that in addition to reducing SCC the number of positive quarters infected with CNS was reduced by 12.5% during the first 30 days of

lactation (Graph 2). On the 10th day of lactation, 15 and 9 quarters were infected in the control and experimental group, respectively. The same trend was observed on the 30th day of lactation. A large proportion of CNS (40%) was noted in the control group from infected quarters.

In recent years CNS have become increasingly important in udder infections. They are normal inhabitants of the skin and teat canal and are frequently isolated from milk samples (Taponen et al., 2006). Bacteria *S. chromogenes, S. haemoyticus* and *S. warneri* were pathologically important in intaramammary infection with increased SCC. The addition of humates to the feed reduced the incidence of staphylococcal infections by 5%.



Graph 2. Distribution of bacterial pathogens causing mastitis in monitored groups (%)

Note: Experimental – experimental group supplemented with HA, control – group without supplementation of HA, CNS – coagulase negative staphylococci, *Significant difference $p < 0.05$ when significance level $\alpha = 0.05$ (5%); critical value.

According to Dabovich et al. (2003) the use of HA increases the body's defenses by stimulating neutrophil activity in response to the onset of inflammation. Testing of milk during field trials often indicates an increase in the number of microbes in the milk, an indication to the dairyman of impending mastitis.

As a result of feeding humates, mastitis cases within the milking herd dropped from an average of 3 to 4 cases daily to 4 cases in a month (Mosley, 1996).

5.4 Conclusion

According to our results dietary supplementation with HA significantly reduced the MU content, SCC and the number of positive quarters detected by the CMT on the 10th day efter calving but did not affect the others milk parameters. Although the mechanism by which HA supplementation affects milk synthesis and mastitis reduction has not been fully described, its indirect beneficial effects could improve the immunity of the mammary gland. Based on the information above, further research into the use of humates for the prevention of mastitis during the peripartum period and early lactation will be needed.

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6 Chapter
NEW TRENDS IN THE USE OF RECYCLED MANURE SOLIDS IN DAIRY HOUSING

František Zigo, Zuzana Lackova, Milan Vasiľ, Silvia Ondrašovičova, Naďa Sasákova, Jana Vyrostkova, Ewa Pecka-Kielb

6.1 Introduction

Housing of cows and ensuring of optimum conditions for achieving their production potential belongs among the basic zootechnical factors. For individual categories of cattle, various housing systems have been introduced in practice, such as free housing in cubicles or housing in group pens. Loose housing in cubicles is used mainly in dairy farming. This housing can also be used successfully for other age categories of cattle. It keeps the animals clean, which is especially important for dairy cows and also addictive to heifers. It provides sufficient comfort for resting and minimizes the risk of development of dominant behaviour as well as mutual interference between animals (Mariano, 2014).

These advantages of lying boxes are achieved only with the right choice of their dimensions according to the body frame of the cattle and sufficient hygiene quality of the litter (Popescu et al., 2014). In the lying cubicles there must be sufficient space not only for comfortable resting but also for getting up and lying down. Otherwise, if the cows do not feel comfortable in the boxes they are looking for another place to lie down, e.g. in the areas of the feeding and manure removal passages or by the drinkers. If this is the case, the cows become excessively dirty and the risk of mammary gland inflammation and hoof disease increases. Boxes with recessed floors are best suited for underlayment (Brouček et al., 2015).

Straw is the most widely used bedding material in boxes, but its durability and hygiene effect are often insufficient. In addition to the use of straw, breeders search for alternative materials that will ensure the comfort of dairy cows and sufficient hygiene. Alternative materials suitable for undercoating include a separate, sand, limestone or zeolite (Bradley et al., 2018).

6.1.1 Objectivites of this chapter

The aim of this study was to investigate the effects of improved composition of bedding used in dairy farm conditions on the indicator microorganisms influencing the level of hygiene, and compare these to the properties of conventional straw bedding.

6.2 Material and methods

6.2.1 Cows and housing

The practical part of the study was carried out in the breeding of 300 dairy cows of the Slovak Spotted Cattle breed in district Stará Ľubovňa. The dairy cows are housed in a newly built high-air stall divided into two sections, between which there is a feeding corridor. Each section is divided into 4 sections. In each section there are 42 cubicles, which are arranged in three rows. Between the rows there are movement areas with automated excrement cleaning, into which dairy cows can be moved as needed to perform technological tasks. The boxes are 2.500 mm long with an active length of the bearing area of 1.700 mm, which is defined by a 200 mm raised concrete threshold above the floor level. The removal of excrements is ensured by automatic collection with hydraulic shovels 24 hours a day.

6.2.2 Production and layering of bedding

Limestone, straw, water and separation (RMS) is used to produce a new type of bedding, which is obtained directly on the farm. The production of RMS is ensured by pumping the collected manure from the homogenization channel to a cylindrical separator where its liquid fraction is separated. Subsequently, the separate is allowed to lie under a shelter for two weeks (Figure 1). For the production of bedding in one bed box the following is required: 200 kg of ground limestone, 40 kg of straw, 10 kg of separate and 110 l of water. The production itself as well as the technological modification of the bedding in the cubicles takes place in three basic steps:

The first step: Approximately half of each component (except water) is taken and placed for mixing in an old feed wagon discarded for this purpose. Straw (20 kg) is added to the mixing space of the car first, which is mixed for 10–15 minutes. Subsequently, limestone (100 kg) with separate (5 kg) is added. After another 15 min, 80 l of water are added to the resulting mixture by stirring. After the addition of water, the whole mixture is mixed for 15–20 minutes to completely mix all components and achieve a slurry (Figure 2). Subsequently, the resulting slurry is dosed from the feed wagon to half the height (10 cm) of the cleaned bed and is evenly distributed. After spreading the mixture on the bed, the mixture is compacted using a vibrating plate.

The second step: In the same way as in the first step, the second half of the straw, limestone and the rest of the separate (5 kg) are mixed in the feed wagon, but only 30 l of water are added to the mixture. The smaller addition of water in the second step will ensure higher elasticity and lower hardness compared to the first layer. After spreading the second layer on the first to a height of 20 cm of the concrete barrier of the bed, this layer is again compacted by means of a vibrating plate.

Figure 1. Separate production by pumping and pressing manure

Note: From left: Manure collection in stall, solid liquid separation, fresh separated manure soil, recycled manure soil. Photo by Zigo F. (2019).

The third step: In the last phase, the remainder of the prepared mixture (2–4 cm) is poured onto the compacted second layer, which is no longer treated with a vibrating plate but only gently spread with rakes (Figure 3). The cubicles thus formed are mechanically held once a day by collecting faeces into the manure passage and, if necessary, a thin layer of limestone is added.

Figure 2. Production of bedding for dairy cows by mixing components

Photo by Zigo F. (2019).

6.2.3 Sampling of cubicles bedded

Samples for microbiological examination were taken from 4 sections according to the time delay of production and layering of bedding. Bedding samples were taken from three sections according to a new recipe with a monthly intervals of 1–3 months after use of improved bedding. A control sample of bedding, consisting of straw, was taken from the last, fourth section. Two cubicles were selected from each section, from which a four samples of bedding to a depth of 20 cm was taken. The samples of bedding were mixed and ground to a particle size of 2–4 mm prior to analysis.

Figure 6. Dosing of the bedding with improved composition and its final adaptation to the cubicles

Photo by Zigo F. (2019).

6.2.4 Microbiological examination

From the bedding sample was taken 10 g and 90 ml of saline was added. Following subsequent homogenization, individual ten-fold dilutions in physiological saline were then prepared from the treated samples. Dilutions from 10-4 to 10-8 according to the method by Fournel et al. (2018) were used for seeding. Various culture media were used to ensure appropriate growth conditions for the individual microorganism strains tested. For total count of bacteria (total viable count, TVC) at 37 °C, Plate Count Agar (Oxoid, UK) was used. Fecal coliform bacteria (FCB) were cultivated on a petri dish containing McConkey agar (Oxoid, UK) at 43 °C. For coliform bacteria (CB) at 37 °C, Endo agar (Hi-Media, India) was used. For fecal streptococci (FS) at 37 °C, M17 agar (Oxoid, UK) was used. The culture media were prepared according to the manufacturer's instructions and poured into 90 mm diameter Petri dishes in parallel. After a specified incubation time for each microorganism, the colony forming units (CFUs) were calculated according to the appropriate formula.

6.2.5 Statistical analyses

For statistical comparison, bacterial counts were also log-transformed and expressed in log CFU/ml. The differences between tested indicator microorganisms of improved bedding and control bedding with straw were analyzed by using analysis of variance (ANOVA) followed by Dunnett's multiple range test. The minimum criteria for statistical significance was set at $p \leq 0.05$ for all.

6.3 Results and discussion

Proper selection of bedding and application of appropriate components to achieve a disinfection effect are the key factors in maintaining the cow's health. The effectiveness of disinfection is affected by the resistance of microorganisms, the selection and use of disinfectant components and the external

environment in which the disinfection takes place (Ismaïl et al., 2013). Under dairy farm conditions, straw is the predominant litter, although other bedding materials may also be used (Wolf et al., 2018).

According to Tančin et al. (2013), it is ideal when cubicles filled with straw are re-bedded twice a day, always after the manure is cleared out from the end of the bed and from the corridors. In some farms the re-bedding procedure is performed only once a day with higher amount of fresh bedding, but the corridors themselves are cleared out twice a day. On farms with a lack of straw, the separated solid fraction of the recycled manure sludge (RMS) has been applied in recent years to bedding in the cubicles (Leach et al., 2015). With a higher dry matter content, it is a suitable material for lining deep bedded cubicles. However, freshly separated sludge has a dry matter content of only about 30% and it is desirable to allowed it to dry before use. The use of RMS as an alternative source for bedding provides a sufficient effect of maintaining a dry and hygienic bedding after application to boxes. Due to its high dry matter content, the required bedding consistency and hygienic effect are achieved only for a short period of time (Fournel et al., 2018).

One of the ways how to achieve the desired consistency of bedding in the long run and ensuring its optimal height and hygienic effect is to mix RMS with straw, limestone and a sufficient amount of water and to carry out the technological treatment directly on the bed. In our study we used separated RMS which was left standing for two weeks before the production of bedding.The sludge was mixed with limestone at a ratio of 1 : 4 to increase the proportion of limestone and the accompanying disinfectant effect of the bedding formed.

The increased disinfection effect in the bedding we produced was confirmed from the samples taken. A reduced total count of microorganisms (TVC) and coliform bacteria (CB) was noted in the improved litter for two months after its loading by the dairy cows compared to straw-filled bed boxes. Comparison of the counts of faecal bacteria (FB) and faecal streptococci (FS) showed that their counts were reduced during the first, second and third month after using the bedding with an improved composition compared to the control group housed on straw (Table 1).

Table 1. Effect of bedding on the level of indicator bacteria (log CFU/ml)

Bedding	Total viable count	Coliform bacteria	Fecal coliform bacteria	Fecal streptococci
Control - straw	8.6[a]	6.6[a]	6.5[a]	9.2[a]
IB 1month	6.9[b]	5.6[b]	4.9[b]	6.2[b]
IB 2 months	7.3[b]	6.0[b]	5.3[b]	6.5[b]
IB 3 months	8.4[a]	6.5[a]	5.7[b]	7.1[b]

Note: IB 1month – one month after use of improved bedding; IB 2 months – two months after use of improved bedding; IB 3 months – three months after use of improved bedding; a, b – values in column with different superscript letters differ significantly at $p < 0.05$.

In addition to the reduction of faecal contamination, a stabilization of the consistency of the litter with an improved composition in the laid beds was observed, which was demonstrated by maintenance of its thickness up to 20 cm.

6.4 Conclusions

Our results show that in the case of classical straw bedding, its height in the bedded cubicles tends to fall below the litter threshold, and the breeders have to resolve this by clearing out and adding new straw. Especially in strawbedded cubicles, at the back (level with the cow‘s rear) the thickness of the bedding layer is often reduced, with an accumulation of dung, urine and an abundance of bacteria, which in favourable conditions rapidly multiply, as demonstrated by their increased numbers in the first two months of our study. Conversely, we found that improved bedding maintained a stable level as well as reduced numbers of TVC, CB, FS and FCB for the same two months. The positive effect of reducing the level of faecal contamination was also reflected in the dairy cows themselves, as they kept a cleaner body and udder.

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7 Summary

Maintaining the health of dairy cows and the quality of milk produced is a very complex daily activity which should be approached with appropriate care. The scientific papers presented in the book bring knowledge about risk factors related to the occurrence of dairy mastitis as well as available innovative scientific methods and practical experience in the field of mastitis diagnosis and control.

In terms of experience gained from the breeding environment, the occurrence of mastitis depends primarily on the possibility of development of new intramammary infections, which arise mainly due to bacterial pressure from the environment, namely:

- errors in the milking hygiene programme and transmission of infection during milking,
- transmission of infection from cows following the ineffective treatment of acute and subacute forms of mastitis which progress to chronic cases,
- the transfer of resistant IMI agents in the herd during lactation and drying of dairy cows,
- during irregular frequency of cleaning housing space and laying the bedding,
- poor or absent management of pregnant and calved heifers,
- the stress load on the body during parturition and several postpartum days,
- during unprofessional handling of animals and due to insufficient staffing.

Effective mastitis control measures include:

- selection of the correct sequence (dairy cow groups) of cows in the milking parlour that should be as follows: calved, start of milking after weaning, productive groups, end of lactation and treated or chronically infected,
- application and adherence to sequence of all steps during milking (predip, first squirts, wiping, correct positioning of the milking equipment, use of postdip).
- regular monitoring of dairy cows during milking. In particular, dairy cows with subclinical and chronic forms of mastitis have a high potential to transmit mammary pathogens throughout the herd. In addition to the evaluation of the CMT test, SCC, and microbiological cultivation of milk samples, one of the innovative methods for the diagnosis of mastitis in dairy cows is the determination of milk malondialdehyde, which can be implemented into mastitis control programs.
- initiation of immediate treatment (in particular *S. agalactiae, S. aureus* and *S. chromogenes*) with appropriate antibiotics, selected on the basis of the susceptibility of the pathogens tested, followed by monitoring of the effectiveness of the treatment,
- selective drying of cows according to the results of milk utility control and bacteriological examination of individual milk samples,

- regular inspection and replacement of bedding. In addition, in resting boxes it is necessary to ensure a sufficient thickness of bedding layer (15 - 20 cm) with the addition of ground limestone in a ratio of 3: 1 (limestone: separated solids) to bedded boxes to optimize pH, remove moisture and eliminate environmental mastitis agents.
- oral supplementation of Se and vit. E with Se content 0.3 mg and 90 IU / vit. E per kg DM to dairy cows throughout the dry period. In addition to supplementation and determination of Se and vit. E in rations, concentrations of these supplements in the blood of animals should be monitored. In case of their long-term reduced or marginal levels, their increase by parenteral administration of preparations based on Se and vit. E should be ensured.
- one of the effective means for increasing body defences and stabilizing rumen microflora is the oral supplementation of humic acids with a daily amount of 100 g per dairy cow minimally for 50 days before the expected birth. One of the beneficial effects of humic acid administration is the reduction of SCC in milk at the beginning of lactation.

The acquired knowledge indicates the need for adapting and updating the antimastitis measures aimed at diagnosing and suppressing mammary gland inflammation on dairy farms during all stages of lactation in a logical context for optimal maintenance of animal health and required milk production.

8 Biography of editors

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Research and Academic Experience: Knowledge and methodological skills in the field in detecting of udder pathogens, their virulence factors and health safety of milk produced. Teaching activities on animal husbandry and technology of animal production.

Research Area: Research activities aimed at solving problems in the field of ruminant breeding and primary milk production, as well as poultry, pigeon and rabbits breeding and diseases.

Number of Published Papers and Citations: The scientific achievement consists from 117 peer-reviewed scientific publications, and 54 of them in SCI/WoS and Scopus, 1 monograph and 6 text books. In databases WoS and Scopus are registered 183 citations and the Hirsh index is 8.

Research Projects: The main researcher of 1 foreign and 3 Slovak projects focused on the detection and prevention of mastitis. Co-solver of 1 foreign NAWA project (Poland) and 4 Slovak projects.

Special Award: Inclusion and publication of a CV in the Encyclopedia of Personalities of the Czech and Slovak Republics, 2018, British Publishing House based on the BP index. Member of the editorial board of the International Journal of Avian & Wildlife Biology.

Silvia Ondrašovičová, DVM., PhD.

Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy, Košice, Slovakia.

Research and Academic Experience: Knowledge and methodological skills in the field in the detection of udder pathogens causing mastitis and health safety of milk produced; teaching activities on veterinary physiology.

Research Area: Research activities aimed at solving problems in the field of milk production in ruminant.

Number of Published Papers and Citations: 181 scientific publications; 16 of them in CC and Scopus journals, 1 monograph and 7 textbooks, 42 registered citations in databases WoS.

Research Projects: Co-author of three projects focused on the detection and prevention of mastitis. The main researcher of one Slovak project.

Pecka-Kiełb Ewa, Assoc. prof., Ing. PhD.

Wroclaw University of Environmental and Life Sciences, Department of Biostructure and Animal Physiology, Norwida 31, Wroclaw 50-375, Poland

Research and Academic Experience: Methodological skills in the field in physico-chemical analysis of colostrum and milk.

Research Area: Changes in physical and chemical properties of milk in cows, ewe with mastitis. Research on the digestive system of ruminants and monogastric animals. In vitro fermentation of rumen, caecum and colon digesta, testing different feeds and feed additives.

Number of Published Papers and Citations: The body of scientific achievement consists of 65 original publications. Presented 95 papers and short communications at meetings and national and international conferences IF (impact factor) is 47.991 and the Hirsh index is 7. In database WoS aare registered 190 citations.

Research Projects: The main researcher of 1 foreign. Co-solver of 1 foreign APPV project (Slovak) and 4 Poland projects.

David Sandor Kiss, Assoc. prof., PhD.

Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest, Hungary

Research and Academic Experience: head of the Neurophysiology, Neuroendocrinology Research Group at the Dept. of Physiology and Biochemistry (University of Veterinary Medicine, Budapest, Hungary); visiting researcher at the MRC Anatomical Neuropharmacology Unit, University of Oxford (Oxford, UK); visiting researcher at the School of Medicine, Section of Comparative Medicine, Dept. Neurobiology and Ob./Gyn., Yale University (New Haven, USA); teaching activities on veterinary physiology and comparative physiology, physiology of extinct animals, in English, German and Hungarian

Research Area: cellular mechanisms of hypothalamic controlling of homeostatic processes; regulation of neuronal energy levels in the hypothalamus; effects of endocrine disruptors on the neuroendocrine regulation; hypothalamic functional asymmetry; role of astrocytes in the development of hepatic encephalopathy

Number of Published Papers and Citations: 81 scientific publications, 26 of them peer-reviewed original research articles or reviews; 113 citations, 67 of them registered in SCI/WoS & Scopus; h-index: 6

Research Projects: Involved in 3 international and 2 national projects

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