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

František Zigo, Milan Vasiľ, Zuzana Farkašová, Silvia Ondrašovičová, Martina Zigová, Jana Maľová, Jana Výrostková, Jolanta Bujok, Ewa Pecka-Kiełb

ABSTRACT

Given growing concerns about the use of antibiotics in the treatment of animals, identifying organic alternatives as feed additives to improve animal health and the development of immune responses has become of interest in dairy farming. Humic acids (HA) seem to be a suitable alternative with a favorable impact on the health and production parameters of animals. This study aimed to determine the effects of an HA supplemented diet on milk parameters as well as the effects on somatic cell count (SCC) and the occurrence of mastitis in dairy cows during the peripartum period. Twenty dairy cows in the last stage of pregnancy were selected from a herd of 140 cows. The selected cows were randomly divided into two groups: control (C) and experimental (E). The two groups were fed the same feed mixture and group E was additionally supplemented with HA at a total dose of 100 g per cow per day during the last 50 days of pregnancy. The milk parameters (dry matter, lactose, fat, crude protein, casein and milk urea) and SCC of every cow, and the presence of mastitis, were checked on days 10 and 30 during the first month of lactation. The results of the study show that dietary supplementation with HA significantly reduced the milk urea (MU) content and SCC on the 10th day after calving but did not affect the other milk compositions. In addition to the decreased MU and SCC, the number of positive quarters detected by the California Mastitis Test was reduced by 20.0% and the occurrence of mastitis caused by coagulase-negative staphylococci (CNS). Based on the obtained results we can conclude that the addition of HA stabilizes the nutrient digestion, as was confirmed by a reduced MU content in the supplemented group. Their indirect beneficial effects improved the development of immune responses, resulting in decreased SCC and the occurrence of mastitis caused by CNS.

Keywords: dairy cows; supplementation; humid acid; mastitis; milk urea

INTRODUCTION

The health-safety and nutritional quality of raw milk are influenced by many factors. One of the main factors that affect 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 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, Shaohua and Lixia, 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 **(Trckova et al., 2005; Galip, Polat and Biricik, 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 **(Marcinčáková 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)**.

Scientific hypothesis

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 the occurrence of mastitis in dairy cows during the peripartum period.

MATERIAL AND METHODOLOGY

Animal care

The practical part of the study was carried out in a 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 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 fed twice a day with feed mixture formulated for a 650 kg 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 $c.min^{-1}$ and milking was automatically terminated when the milk flow dropped to 0.2 $L.min^{-1}$. 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.

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 before 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 the 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 per cow per day.

The experimental group (E) was supplemented into the diet with humid acids at a dose of 100 g per cow per day. The humic acids (product Humac Nature AFM) used in the experiment were obtained from Humac ltd. the 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 before 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 calving the cows from the control and experimental group were milked twice a day together with all lactating cows.

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 the 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 Cockroft (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.

Analytical methods

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)**.

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.

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.

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.

Statistical analysis

A one-way ANOVA with an 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.

RESULTS AND DISCUSSION

Table 2 illustrates the effect of the 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 per cow per day. Changes of MU content (Figure 2) 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).

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 the bloodstream. Thus, a 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, Schumacher and Gropp, 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.

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, the control group shoved increased SCC value above the legal limit on the 10th day after calving (Figure 2). 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. The 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, Singh and Bhadwal, 2011)**.

In particular, differences were found by comparing the positive quarters 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 and Faust (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, Shaohua and Lixia (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, Singh and Bhadwal, 2011)**.

The addition of HA at a dose 100 g per 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 (Figure 3). 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)**. *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%.

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 **(Islam, Schumacher and Gropp, 2005)**.

Table 1 Chemical composition of feed mixture.

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

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

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

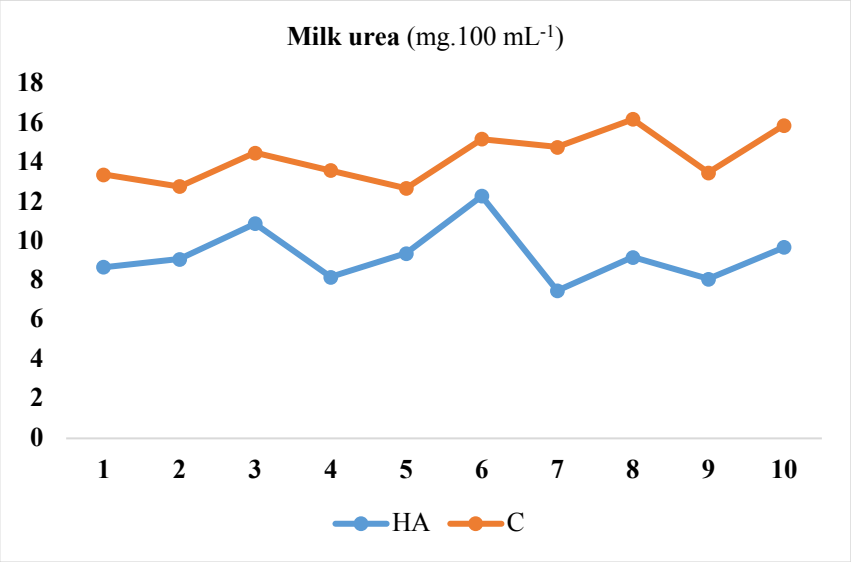

Figure 2 Comparison of milk urea content in supplemented by humic acid (HA) and control (C) group on the 10th day of lactation.

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 M ±SD	Experimental M ±SD	*p**	Control M ±SD	Experimental M ±SD	*p**
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 – sandard deviation; * p <0.05 – significant difference; * p >0.05 – no significant difference.

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 (negat.)	0 – 200	Healthy quarter	37.5	35.0	42.5	47.5
T (trace)	200 – 400 (±50)	Healthy/latent mastitis[1]	25.0	22.5	20.0	22.5
1	400 – 650 (±150)	Subclinical mastitis[2]	20.0[a]	32.5[b]	17.5	12.5
2	850 – 1.200 (±200)	Subclinical/clinical mastitis[3]	10.0	10.0	12.5	15.0
3	1.500 – 5.000 (±300)	Clinical mastitis	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; Latent mastitis[1] – normal milk consistency, but infection is present in samples of raw milk without changing the SCC and a negative CMT score; Subclinical mastitis[2] – no symptomps are observed, the udder and milk appear normal, but infection is still present with positive CMT score and increased SCC; Clinical mastitis[3] – 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.

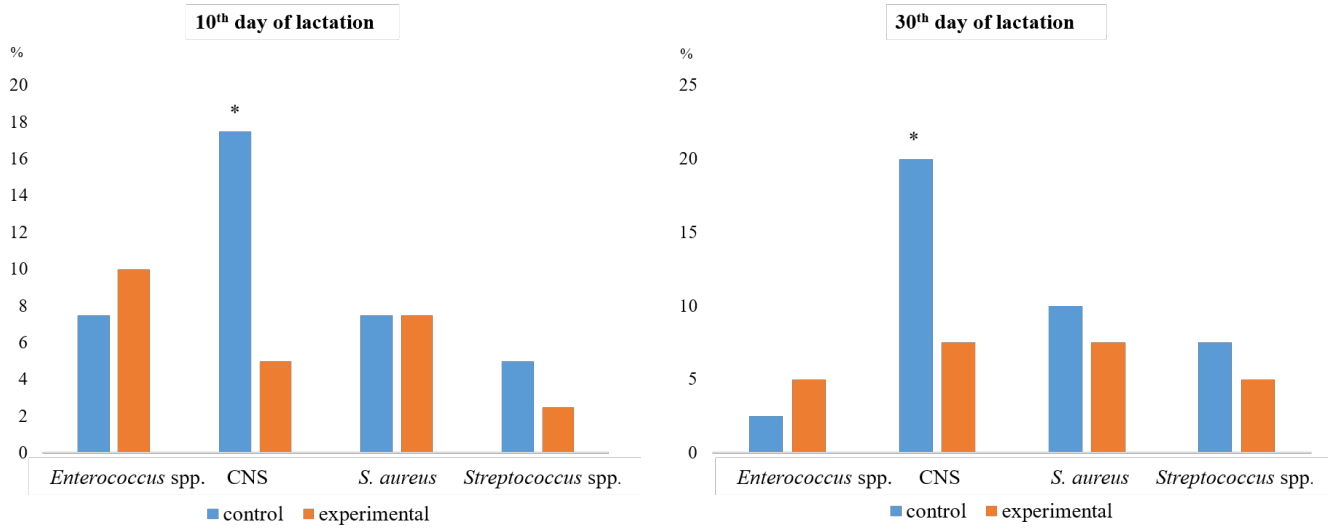

Figure 3 Distribution of bacterial pathogens causing mastitis in monitored groups (%). Note: Experimental – experimental group supplemented with HA, control – group without supplementation of HA, CNS – coagulase negative staphylococci, *Significant difference p <0.05 when significance level α = 0.05 (5%); critical value $\chi 2$= 5.99.

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 after 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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Contact address:

*DVM. František Zigo, PhD., University of Veterinary Medicine and Pharmacy, Department of Animal Husbandry, Komenského 73, 041 81 Košice, Slovakia, Tel.: +421908689722,
E-mail: frantisek.zigo@uvlf.sk
ORCID: https://orcid.org/0000-0002-2791-166X

Doc. DVM. Milan Vasiľ, CSc., University of Veterinary Medicine and Pharmacy, Department of Animal Husbandry, UVLF, Komenského 73, 041 81 Košice, Slovakia, Tel.: +421905385671,
E-mail: milan.vasil@uvlf.sk
ORCID: https://orcid.org/0000-0002-3607-818X

DVM. Zuzana Farkašová, PhD., University of Veterinary Medicine and Pharmacy, Department of Animal Husbandry, UVLF, Komenského 73 041 81, Košice, Slovakia, Tel.: +421905201646,
E-mail: zuzana.farkasova@uvlf.sk
ORCID: https://orcid.org/0000-0003-1953-1840

DVM. Silvia Ondrašovičová, PhD., University of Veterinary Medicine and Pharmacy, Department of Physiology, UVLF, Komenského 73, 041 81 Košice, Slovakia, Tel.: +421915984702,
E-mail: silvia.ondrasovicova@uvlf.sk
ORCID: https://orcid.org/0000-0002-8027-6693

DVM. Martina Zigová, PhD., University of Veterinary Medicine and Pharmacy, Department of Pharmacology, Pavol Jozef Šafárik University of Košice, Šrobárova 1014/2, 040 01 Košice, Slovakia, Tel.: +421908689722,
ORCID: https://orcid.org/0000-0001-7486-8249
E- mail: chripkova.martina@gmail.com

DVM. Jana Maľová, PhD., University of Veterinary Medicine and Pharmacy, Department of Milk Hygiene, UVLF, Komenského 73 041 81, Košice, Slovakia, Tel.: +421915986955,
E-mail: jana.malova@uvlf.sk
ORCID: https://orcid.org/0000-0002-5547-9149

DVM. Jana Výrostková, University of Veterinary Medicine and Pharmacy, Department of Milk Hygiene, UVLF, Komenského 73 041 81, Košice, Slovakia, Tel.: +421908360406,
E-mail: jana.vyrostkova@uvlf.sk
ORCID: https://orcid.org/0000-0002-3117-8915

DVM. Jolanta Bujok, Wrocław University of Environmental and Life Sciences, Department of Animal Physiology and Biostructure Norwida 31, 50-375 Wroclaw, Poland, Tel.: +48509735696,
E-mail: jolanta.bujok@upwr.edu.pl
ORCID: https://orcid.org/0000-0003-1630-3893

DVM. Ewa Pecka-Kiełb, Wrocław University of Environmental and Life Sciences, Department of Animal Physiology and Biostructure, Norwida 31, 50-375 Wroclaw, Poland. Tel.: +48506227929,
E-mail: ewa.pecka@upwr.edu.pl
ORCID: https://orcid.org/0000-0002-8703-8268

Corresponding author: *