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The effect of immunomodulation composition on systemic immune response and udder health in case of bovine subclinical mastitis

G. Gulbe[1,*], S. Doniņa[1], V. Saulīte[1], J. Jermolajevs[1], L. Peškova[1], Š. Svirskis[1] and A. Valdovska[1,2]

[1]Riga Stradins University, A. Kirchenstein Institute of Microbiology and Virology, Ratsupites street 5, Riga, LV-1067, Latvia
[2]Latvia University of Agriculture, Faculty of Veterinary medicine, Institute of Food and Environmental Hygiene, Kr. Helmana street 8, Riga, LV-3004, Latvia
[*]Correspondence: Gundega.Gulbe85@gmail.com

Abstract. Modulation of mammary gland immune response may offer an alternative to antimicrobial therapy in the treatment of subclinical mastitis. The aim of the study is to investigate the systemic immunomodulating effect and the impact of composition LLG which consists of lysozyme, lactic acid and glycopeptides isolated from *Lactobacillus spp.* on udder health parameters. A total of 10 cows with subclinical mastitis were used in the study – 5 cows (19 udder quarters) in the experimental group and 5 other cows (19 udder quarters) in the control group. The experimental cows received intramammary infusions of LLG, given once per day, 3 times with the intervals of 48 h. Cows from the control group received infusions of sterile 0.15 M NaCl, given in the same way as the treated cows. The following parameters were measured: somatic cell count (SCC), the total number of blood leucocyte, differentiation between banded neutrophils, segmented neutrophils and lymphocytes, peripheral blood mononuclear cells and markers of cell activation. Besides bacteriological culturing was performed. Quarter milk and blood samples were taken several times from the 1st to the 21st day. During the treatment the number of lymphocytes and T helper cells significantly decreased in the blood of the treated group, the CD8+ cells did not change remarkably, the number of CD25+, CD38+, as well as CD69+ and CD95+ cells had diminished during the treatment. On the 21st day a rapid increase of IL-2 receptor bearing cells was detected. A significant elevation of SCC in the treated group was observed but pathogenic bacteria incidence decreased.

Key words: subclinical mastitis; immunomodulation; somatic cell count, lymphocyte subpopulations.

INTRODUCTION

Bovine subclinical mastitis is defined as an inflammatory condition of the mammary gland in response to infection, which is characterized by decreased milk production and altered milk quality but, in contrast to clinical mastitis, is without any visible changes in milk and udder (Harmon et al., 1994; Hillerton & Berry, 2005; Nogueira et al., 2012). The mammary host defence system has evolved into an extremely well-developed, complex, and highly effective barrier against pathogens, integrating

both the innate and adaptive immune systems. Modulation of the immune response may offer an alternative to antimicrobial therapy in the treatment of subclinical mastitis. In our chosen strategy of the modulation of host immune response we use a composition of well-known bactericidal protein lysozyme, lactic acid and glycopeptides isolated from *Lactobacillus spp.* (composition LLG). An effective systemic immune reaction is extremely important for a corresponding local response to ensure immune cells in peripheral blood directly involved in resolution of inflammation.

The treatment with immunomodulators has been intensively studied in the past three decades because the induction of systemic immune response could protect cattle from the development of clinical mastitis or lead to self-treating conditions. One of the promising immunomodulation approaches was treatment with cytokines: IL-2, GM-CSF, IL-8. Recombinant bovine IL-2 was used in intramammary treatment to promote T cell immune response and eventually to develop immunological memory (Daley et al., 1992; Erskine et al., 1998; Oviedo-Boyso et al., 2007; Zecconi et al., 2009). Another approach is to use herbal drugs with immunomodulating properties. V.D. Bhatt et al. (2014) obtained results that indicate significant decline of the total bacterial load after treatment with a topical herbal gel. However, the average differences in SCC after treatment were not significant. It has also been reported that intra-mammary administration of platelet concentrate may be useful for a quick resolution of the inflammatory response, which plays a role in tissue damage to the mammary gland and reduces the recurrence rates (Lange-Consiglio et al., 2014).

We have developed and studied the immunomodulation composition LLG which is unique because all the ingredients originate from milk. The aim of the study is to investigate the systemic immunomodulating effect and impact of composition LLG on udder health parameters.

MATERIAL AND METHODS

Study population and design

The clinical trial was conducted from May to June 2015 with lactating Holstein and Latvian Brown dairy cows located in one dairy farm in Latvia. The herd consisted of 320 dairy cows housed in tie-stall arrangement and milked twice a day. The sample of study was subclinically infected cows with somatic cell count (SCC) greater than or equal to 400,000 per mL in cow composite milk. All the cows included in this study had increased somatic cell count at least for two consecutive months. Cows which had received treatment with drugs for any reason within 30 days of the treatment start date were excluded from the study. The study sample consisted of 10 multiparous cows with 8,000 kg average 305-day yield during the previous lactation. Cows were in their 2nd or 3rd lactation, had been lactating for more than 60 days, and were less than 90 days from their expected dry-off dates. All the cows had no history of concurrent disease other than subclinical mastitis, and had insignificant physical examination findings. The study was performed as a simple randomized and placebo-controlled trial.

Immunomodulating composition

For the treatment, a composition of lactic acid, lysozyme and other component from *Lactobacillus spp.* bacteria in 10 mL 0.15 M NaCl solution was applied (composition LLG). The composition was developed in Riga Stradins University, Institute of

Microbiology and Virology (PCT application LV 2015/000005). The sterility of the preparations was checked by inoculation of 0.1 mL from each batch on blood agar, followed by incubation at 37 °C for 48 h, and observation of visible colony forming.

Treatment procedure

The treatment regimen consisted of intramammary infusion in 19 udder quarters of 5 cows of 10 mL LLG, given after milking once per day, 3 times at the intervals of 48 h (day 1, day 3 and day 5). The negative (placebo) control was a 10 mL infusion of sterile 0.15 M NaCl, given in the same way – once per day, 3 times at the intervals of 48 h, administered in 19 quarters of other 5 cows.

Following milking, the teat end was disinfected by scrubbing with a single-use cotton pad saturated with 70% isopropanol. The LLG was then infused through the teat canal in teat cistern using the individual syringe fitted with a sterile J-12 cannula (Jorgensen Laboratories, USA). Following infusion, the mammary gland was massaged upwards briefly to distribute the treatment into the gland. Rectal temperature and milk yield were measured every day to evaluate the effects of treatment on the physiological homeostasis of the animals and on any side effects.

Sampling

Udder quarter milk samples were collected by the project veterinarian (first author) in accordance with Bradley et al. (2012) aseptic technique, from 38 udder quarters five times during the study: day 1 (before treatment), day 3, day 5, day 7 and day 14 after first treatment. Sterile laboratory plastic vials of 40 mL capacity were used for detecting the milk somatic cell count, but sterile no additive tubes of 8 mL capacity (Vacutest Kima, Italy) were used for bacteriological analyses. After collection, milk samples were immediately refrigerated, placed on ice and transported to the laboratory at 4–6 °C. In the laboratory, samples were stored at 4–5 °C while analyses were completed within 12 hours of the milk sample collection on the farm.

Peripheral blood samples were taken by the farm veterinarian from the tail vein of 10 cows four times during the study: day 1 (before treatment), day 3, day 7 and day 21 after the first treatment. Blood samples were collected in tubes containing EDTA as an anti-coagulant (Vacutest Kima, Italy) and immediately transported to the laboratory at 4–6 °C. In the laboratory, analyses were completed within 12 hours of the milk sample collection on the farm.

Evaluation methods

The total number of blood leucocytes was determined with blood cell with 5-part differential haematology analyser (ABX Pentra 60, HORIBA ABX SAS, Japan) according to the standard protocol and differentiation between banded neutrophils, segmented neutrophils and lymphocytes was performed by light microscopy. Peripheral blood mononuclear cells and markers of cell activation were determined using fluorescence-activated cell analysis. CD4+, CD8+, CD14+, CD16+, CD18+,CD19+, CD25+, CD38+, CD69+ and CD95+ cells were detected by a flow cytometer (FACS Calibur, Becton Dickinson, USA) using corresponding mouse antibodies (for example, IgG1 isotope murine monoclonal antibodies, specific for CD4+ and CD8+) according to the manufacturer′s protocol.

Milk SCCs were determined by fluoro-opto-electronic cell counting using a Fossomatic FC (Foss A/S, Denmark) somatic cell counter in accordance with the standard LVS EN ISO 13366-2:2007 'Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-opto-electronic counters'.

SCC data about days 22, 50 and 83 were obtained from the state institution 'Agricultural Data Centre' and they characterise a cow's composite milk.

Milk samples for bacteriological culturing were serially diluted 3-fold in buffered peptone water (Oxoid, England). Baird Parker agar with egg yolk supplement (Biolife, Italia) for the enumeration of staphylococci was used in accordance with the standard LVS EN ISO 6888-1: 1999/A1:2003 'Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*S. aureus* and other species) – Part 1: Technique using Baird-Parker agar medium – Amendment 1: Inclusion of precision data'. The enumeration of bacteria of the family *Enterobacteriaceae* was carried out on pour plate technique on Violet Red Bile Glucose agar (VRBG, Biolife, Italia) in accordance with the standard LVS ISO 21528-2:2007 'Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Enterobacteriaceae* – Part 2: Colony-count method'. *E. coli* detection was carried out in accordance with previously described technique (Leininger, Roberson, Elvinger, 2001) but blood agar was used for isolating *Streptococcus* and *Enterococcus spp*. following UK Standards for Microbiology Investigations (UK Standards…, 2014).

S. aureus and *E.coli* was considered as major pathogen while other isolated microorganisms (including coagulase positive and negative staphylococci, environmental streptococci, enterococci, other bacteria of the family *Enterobacteriaceae* instead of coliforms) were defined as minor pathogens in accordance with Reyher et al. (2012).

Statistical analysis

Data were analysed using the GraphPad Prism computer program (Prism 7.0 for Mac, La Jolla, California, USA). Dynamic changes of measured parameters and differences among groups at were assessed on the cow level with ordinary two-way ANOVA (taking into account two main sources of variation – time and treatment) followed by two-stage step-up method of Benjamin, Krieger and Yekutieli or Fisher's LSD test for between-groups comparisons or RM two-way ANOVA followed by Holm-Sidak's multiple comparison test for intra-group comparisons to assess the time factor. Results are expressed as median, more objective measure of central tendency for given data sets, and ± IQR (interquartile range) as dispersion characteristics. Statistical significance was set at $p < 0.05$ for all statistical analyses.

RESULTS AND DISCUSSION

Peripheral blood cell analysis

The infected mammary gland (MG) tissue and milk contain mainly neutrophils, recruated to the site of infection and act as phagocytes (Werfel et al., 1997; Mehrzad et al., 2003). Nonspecific or innate responses are mediated by macrophages, neutrophils, natural killer (NK) cells, cytokines (CK), and complement. Neutrophils are essential cells for innate host defence; the rapid influx of neutrophils with high antimicrobial

activity to the foci of infection is the main process that leads to elimination of infection (Mehrzad et al., 2005). If the invading bacteria survive, neutrophil infiltration is replaced by T and B lymphocytes and monocytes (Raihard & Riollet, 2003). Another important cell involved in antimicrobial defence is the monocyte/macrophage expressed CD14 receptor – high-affinity protein for the complex of bacterial LPS and LPS-LBP protein (Nemchinov et al., 2006). Not only does the number of polymorph nuclear neutrophilic leukocytes (PMNL) increase enormously but their defensive responses (e.g., phagocytic activity) increase as well (Targowski, 1983).

No remarkable changes in the total number of leucocytes, neutrophils and CD14+ monocytes in peripheral blood were detected in the treated and the control groups during the observation (3rd to 21st day) so it may be concluded that phagocytic activity does not differ meaningfully between both groups after the treatment with LLG (data not shown). However, the number of lymphocytes was changed significantly in the treated group: during the treatment (on day 3) and two days after the end of the intramammary administration of the immunomodulator (on day 7), the number of lymphocytes had decreased in comparison with the pre-treatment amount ($p = 0.0317$ and $p = 0.013$). Sixteen days after the last treatment (on day 21) the number of lymphocytes increased and was close to the pre-treatment amount (Fig. 1).

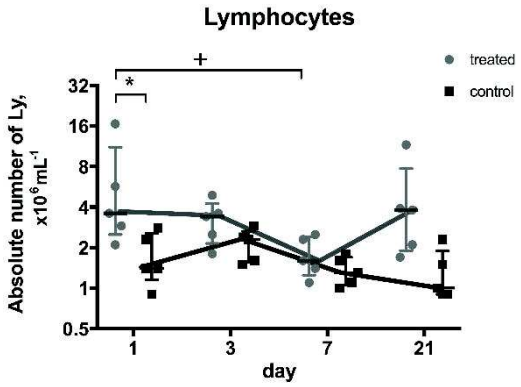

Figure 1. Number of lymphocytes (median ± IQR) in treated (n = 5) and control (n = 5) cows' peripheral blood before the treatment (day 1), during the treatment (day 3) and after the treatment from 7th to 21st day. + P value vs day 1; * P value vs control.

Activation of specific immune defences results in the selective elimination of mastitis-causing pathogens (Paape, 2003). Recognition of pathogenic factors is mediated by several lymphocyte (ly) populations (CD4+, CD8+, CD19+ cells), macrophages, and antibodies (Sordillo, 2009).

CD4+ T helper cells may be involved in protective immunity against the challenge of intra-mammary infection as the main inductor of adaptive immune response. This is supported by the observation that CD4+ cells are increased in the milk of the cattle during mastitis (Banos et al., 2013). CD4+ cells have the ability to secrete certain cytokines, therefore they play an important role in the activation of CD19+ B ly, CD8+ T cytotoxic ly, and macrophages (Sordillo et al., 1997). In mastitis CD4+ ly is predominantly activated by molecular complex recognition, formed between MHC II molecules or by antigen presenting cells, B lymphocytes and macrophages (Ohtsuka et

al., 2004; Park et al., 2004). Cytotoxic CD8+ T lymphocytes recognize and eliminate host cells expressing foreign antigens in association with MHC class I molecules. Suppressor CD8+ T ly controls or modulates the immune response during bacterial infection (Oviedo-Boyso et al., 2007).

The number of T helper cells (CD8+) had diminished significantly in the treated group on days 3 (median 850 cells mm^{-3}) and 7 (median 863 cells mm^{-3}) during the study in comparison with day 1 (median 1,176 cells mm^{-3}, $p = 0.0180$ and $p = 0.0066$ respectively); in contrast – CD8+ cells did not change remarkably during the observation (Fig. 2). In the control group an opposite tendency was observed – increasing number of T cells on day 3 followed by decreasing of both subpopulations until day 7 and 21.

The decreased number of CD4+ T lymphocytes in the peripheral blood during the treatment that we observed in our study might be explained by possible faster migration to the local site, induced by composition LLG. On the 21st day the number of CD4+ and CD8+ T lymphocytes was very similar to the pre-treatment number in both groups (Fig. 2).

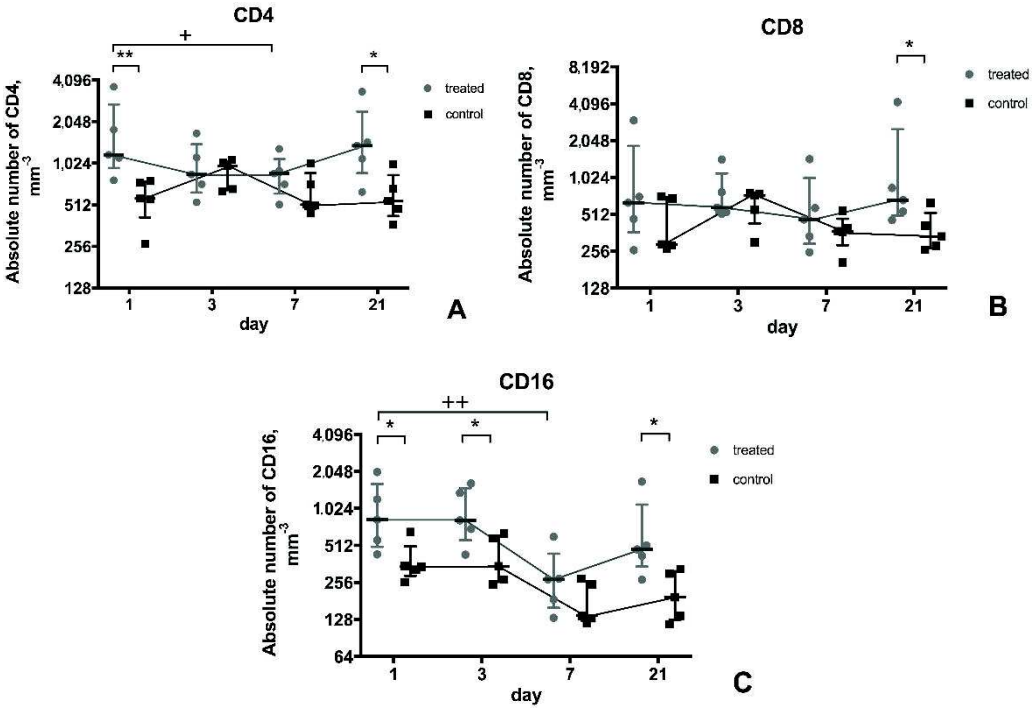

Figure 2. Number of CD4+ cells (A), CD8+ cells (B) and CD16+ cells (C) (median ± IQR) in treated (n = 5) and control (n = 5) cows' peripheral blood before the treatment (day 1), during the treatment (day 3) and after treatment from 7th to 21st day. + P value vs day 1; * P value vs control.

Various surface markers are expressed during the activation of immune cells; some are involved in cell proliferation and signal transduction, some are indicators of cell maturation, some reflect capacity of functional activity. CD69 expression is induced by the activation of T lymphocytes and some NK cells. CD69 is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes (Werfel et

al., 1997). CD25 is the alpha chain of a type I transmembrane protein present in activated T cells, some thymocytes, that can act as a high-affinity receptor for IL-2, glycoprotein that stimulates the growth of T cells and provides other biochemical signalling to the immune system (http://www.kingfisherbiotech.com). CD95 (Fas-R) is a cell surface receptor that, when engaged by Fas ligand or specific agonistic antibodies, triggers apoptosis. Fas-R is expressed in blood neutrophils and lymphocytes rendering the cells highly sensitive to apoptosis (Hu et al., 2001).

We observed that NK cells represented by an innate immune response react slower with a decreasing of absolute count in peripheral blood after the last treatment. During the treatment, a stable number of NK cells was detected, but after the end of the treatment on day 7 (median 246 cells mm^{-3}) the amount of NK cells decreased significantly in comparison with the amount of day 1 (median 566 cells mm^{-3}, $p = 0.0048$) (Fig. 3c). No differences were detected in CD16+ in the control group (Fig. 2c).

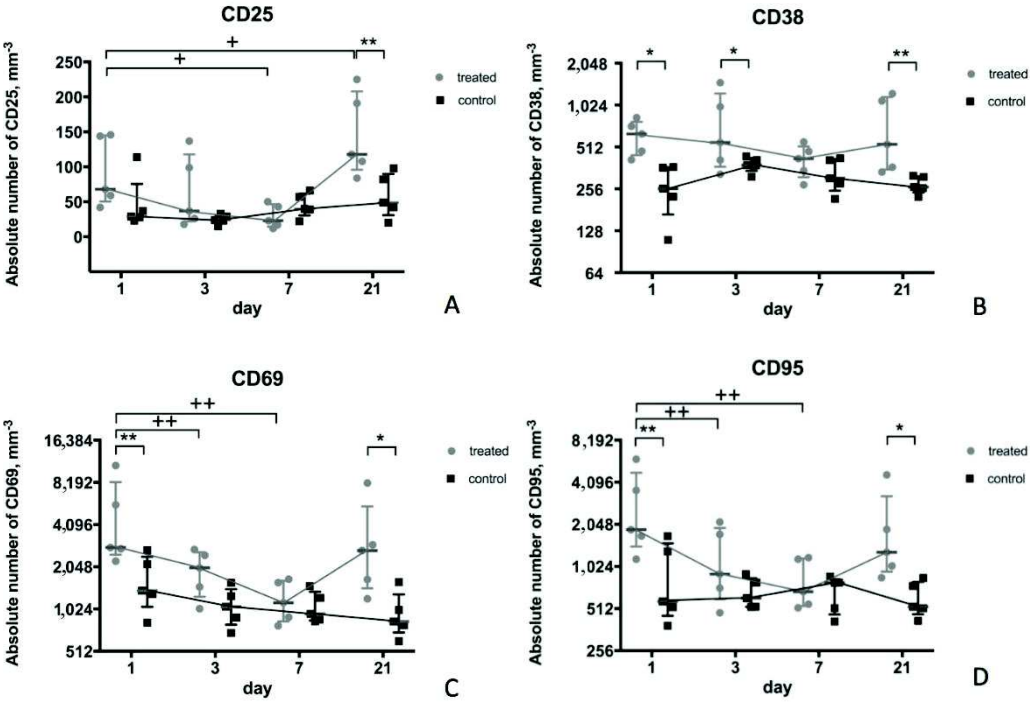

Figure 3. Number of CD25+ cells (A), CD38+ cells (B), CD69+ cells (C) and CD95+ cells (D) (median ± IQR) in treated (n = 5) and control (n = 5) cows' peripheral blood before the treatment (day 1), during the treatment (day 3) and after the treatment from 7th to 21st day. + P value vs day 1; * P value vs control.

It was reported that NK cells can modulate adaptive immune responses via early production of T helper type 1 associated cytokines or interactions with antigen presenting cells (Banos et al., 2013). We can speculate that the NK attempts to boost the function of the T arm of the adaptive immune system in peripheral blood in case of CD4+ ly early migration to the tissue.

The tendency of quantitative changes of CD19+ B lymphocytes was similar to that of NK cells (but not significant) in both groups during the observation (data not shown).

The number of lymphocyte expressed markers of activation belonging to T cells CD25 and CD69 decreased significantly two days after the last treatment (Fig. 3a, c). The common lymphocyte activation marker CD38 bearing cells remained relatively stable, possibly due to the constant number of CD8+ cells during the follow-up period (Fig. 3b). The number of CD25+, CD69+ and CD95+ cells was diminished during the treatment (Fig. 3 a, c, b). On day 21, a rapid increase of IL-2 receptor bearing cells was detected as compared to day 7 (median 118 cells mm³ and 23 cells mm³ respectively, $p < 0.0001$) (Fig. 3a). The number of cells expressed CD38, CD69 and CD95 molecules did not differ significantly from the pre-treatment count. No remarkable changes in the number of cells expressed markers of activation was noticed in the control group (Fig. 3b, c).

Milk somatic cell count analysis and bacteriological culturing

We assessed the local effect due to the treatment using quarter milk SCC analysis and bacteriological culturing which are traditional and well-established methods of mastitis diagnosis.

Mastitis is an inflammation predominantly caused by pathogenic bacteria and it is therefore not surprising that mastitis will result in an acute upregulation of the immune system (Sordillo & Streicher, 2002). As a result, a rapid transient increase in immune components in milk can be observed including elevated SCC. The SCC measures all types of cells in milk, including epithelial cells, lymphocytes, macrophages, and polymorph nuclear neutrophilic leukocytes (Kehrli & Shuster, 1994).

We observed no differences in the SCC between the treated and the control groups at the beginning (prior the treatment) of the observation ($p = 0.222$). During the intra-mammary administration of composition LLG a significant increase of SCC was detected in treated group (Fig. 4). Similarly, the infusion of recombinant bovine sCD14 lead to an increase in SCC, due to more rapid recruitment of neutrophils that was accompanied by a faster clearance of bacteria (Erskine et al., 2004).

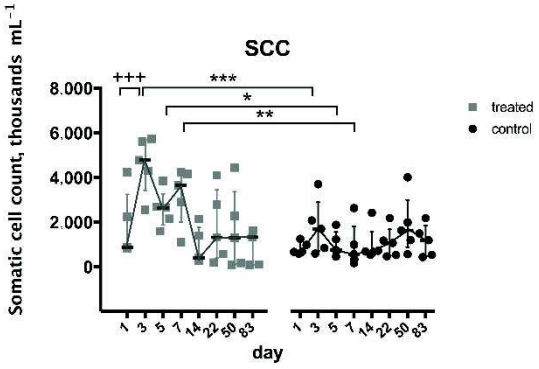

Figure 4. Variation of quarter milk somatic cell count (median ± IQR) in treated (n = 19) and control (n = 19) group before treatment (day 1), during the treatment (day 3 and day 5) and after the treatment from 7th to 14th day in conjunction with cow composite milk somatic cell count in post-trial period (day 22, day 50 and day 83). + P value vs day 1; * P value vs control.

It has also been reported that immunomodulators could improve the capacity of the adaptive immune system to respond to pathogenic challenge, with subsequently lower incidence of clinical and subclinical mastitis (Banos et al., 2013). If the innate immune response is unable to contain the infection, then the adaptive immune system will come into play via T and B lymphocyte responses.

The antibacterial activity of milk lysozyme and lactic acid as part of the unspecific innate defence mechanism is well established fact (Brul & Coote, 1999; Benkerroum, 2008; Ella et al., 2011; Espeche et al., 2012). In this study we found that the composition of LLG reduces the prevalence of pathogenic bacteria in milk, but the incidence of minor mastitis pathogens did not change significantly.

Before treatment, 16% of treated quarter milk samples and 53% of control quarter milk samples were bacteriologically negative; coagulase negative staphylococci were isolated from 47% of treated quarters and 32% from that of control quarters; *S. aureus* (16%) and bacteria from the *Enterobacteriaceae* family were detected only in the treated group (10%); mixed culture were isolated from 10% of treated quarter milk samples and 16% from the control group.

The authors report that major mastitis pathogens *S. aureus* and coliforms are usually considered more virulent and damaging to the udder than minor mastitis pathogens such as coagulase-negative staphylococci (CNS) (Reyher et al., 2012). As we isolated major pathogens only from the experimental group quarter milk samples, several analysed parameters (for example, the number of lymphocytes and CD molecules) significantly differed on day 1 (before treatment) between the control and the experimental group.

The prevalence of minor pathogens (such as CNS) changed insignificantly both in the treated and the control group. Besides we observed a significant decrease of major pathogens in the treated group milk two days post-treatment (day 7) – *S. aureus* were detected in one sample (5%) but we did not isolate bacteria from the *Enterobacteriaceae* family. Thereby prevalence of major pathogens had reduced by 75% in the experimental group milk after the application of LLG.

In total on day 7 53% of the experimental and 63% of the control group udder milk samples were bacteriologically negative. It means that 37% in the experimental group and 11% in the control group udder quarters were bacteriological cured after the application of LLG.

None of the cows in the treated and the control groups showed any abnormal clinical symptoms or any visible local reactions were observed in the areas injected with the control or LLG solution. Feed intake and milk yield remained unchanged during the observation in the treated group, whilst 1 cow from the control group developed acute clinical mastitis 20 days after the beginning of the study.

CONCLUSIONS

Our data show that the composition LLG modulates the T arm of the adaptive immune system and the expression of markers of lymphocyte activation. The proven immunomodulation composition reduced prevalence of pathogen bacteria (*S. aureus* and *Enterobacteriaceae* family) in milk by 75%. Our results suggest that the composition LLG demonstrates immunomodulatory and antibacterial activity in subclinical mastitis and could be used as alternative treatment of subclinical udder infection in cows.

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