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In Vitro Assessment for Antimicrobial Activity of *Lactobacillus Helveticus* and its Natural Glycopeptides Against Mastitis Causing Pathogens in Dairy Cattle

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Abstract: Probiotic lactic acid bacteria have a great potential to control bovine mastitis as well as they are favourable choice to treat many infectious diseases of human. These bacteria are well known as having many properties which make them beneficial to control pathogenic microorganisms. These include, the ability to adhere to cell, the reduction of pathogenic bacteria adherents, the co–aggregation, the production of organic acids, hydrogen peroxide, bacteriocin and etc., to be safe and non–pathogenic, which antagonize pathogenic microorganisms. However, each strain must be well identified and characterized *in vitro* before using for disease treatment. The aim of the present study was to screen three kind of test suspensions: TS1, TS2 and TS3, which contains probiotic lactic acid bacterium *Lactobacillus helveticus* or its natural glycopeptides, and other natural immunomodulators, in order to investigate which content were the most effective in inhibiting several mastitis causing bacteria in dairy cattle: coagulase–positive *Staphylococcus aureus*, coagulase–negative staphylococci *S. haemolyticus*, *S. saprophyticus*, *S. simulans*, *S. vitulinus*, and Gram–negative bacteria *Citrobacter freundii* and *Serratia liquefaciens*. Test suspensions TS1, TS2 and TS3 were adjusted by pH 6.3, then tested *in vitro* by well diffusion assay to determine their antimicrobial effect against bacteria. Furthermore haemolytic activity of applied test suspensions were determined. In results TS1 (9-13 mm) and TS2 (10-15 mm) showed the inhibition effect on four of eight tested bacterial strains, whereas TS3 did not displayed any antimicrobial effect. TS2 have a greatest antimicrobial activity as they resulted in the largest inhibition zones.

Keywords: Beta-glucans, Immunomodulators, Lactic acid bacteria, Lysozyme, Mastitis, Probiotics, Well diffusion assay

INTRODUCTION

Bovine mastitis (inflammation of the mammary gland) is an important disease in dairy industry, and the first cause of economic loss in milk production worldwide [1-3]. Although antibiotic therapy to control bovine mastitis is effective in most cases, it can be detrimental too, because of the emerging antibiotic resistance [4, 5] and occurrence of antibiotic residues in the milk and meat [6]. So an effective treatment by other substances than antibiotics becomes an urgent need [4].

Probiotic lactic acid bacteria with a variety of applications have a great potential to control bovine mastitis as well as they are favourable choice to treat many infectious diseases of human [4, 7-10]. These bacteria are well known as having many properties which make them beneficial to control pathogenic microorganisms. These include, the ability to adhere to cell, reduce pathogenic bacteria adherents, co–aggregate, produce organic acids, hydrogen peroxide, bacteriocin and etc., be safe and non–pathogenic, which antagonize pathogenic microorganisms [4].

Properties of synthetic and natural glycopeptides as well as their effects on immune system have been studied since sixties of the last century. Synthetic glycopeptides include MDP (Muramyl dipeptide) and GMDP (Glucosaminyl-muramyl dipeptide), GMDP is the main structural element of peptidoglycan of the cell wall of all Gram–positive bacteria, including almost all lactic acid bacteria. On it's basis two pharmaceutical preparations are produced in the world: Romurthide, an analogue of MDP is produced in Japan, and Lycopide, which is GMDP, is produced in Russia. Bulgarian scientists started the isolation of biologically active compounds from lyzozyme hydrolysate of cell walls of *Lactobacillus bulgaricus*. The isolated medicine was called Blastolysin and it consisted of glycopentapeptides. The increased interest about natural glycopeptides from lactic acid bacteria was due to their low toxicity, because of which food supplements containing them could be with lower degree of purification, accordingly their production would be cheaper [11]. GMDP is responsible for stimulating a specific immune response in host organism by activating macrophages, which in order activate T and B–lymphocytes, which are the major cellular components of the adaptive immune response [13]. It has been proven that glycopeptides are constantly delivered from gastrointestinal tract into the body environment and they are natural regulators of the immunity [13], as well as, the presence of glycopeptides was found in breast milk [14] and such products as yogurts [15], which are very healthy. There are no natural analogues of MDP and GMDP in the world at the moment.

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In our study we used glycopeptides with Beta Glucan (TS3), preparation outworked in Riga Stradins University, containing glycopeptides from *Lactobacillus helveticus* and β-glucans from mushrooms - shiitake (*Lentinula edodes*) and chanterelle (*Cantharellus cibarius*). It had been shown that 1-3 β–glucan and 1-6 β–glucan are polysaccharides producing immunomodulative effects with a significant role in infectious and antitumoral immunity as well as in prevention of toxic damage of bone marrow [12].

Due to a promising medical properties of glycopeptides, there is a potential to use them also in treatment of mastitis in dairy cows. However, each strain must be well identified and characterized *in vitro* before using for disease treatment. Therefore, in the present study, we aimed to investigate *in vitro* the ability of probiotic lactic acid bacteria *Lactobacillus helveticus* and their natural glycopeptides to exert antagonistic activity against mastitis causing bacteria.

MATERIALS AND METHODS

Bacterial Strains and Culturing Conditions

A total of 564 raw milk samples obtained from cow composite milk were examined for their microbiological content as it is described previously [16]. The bacterial strains used in the study were isolated from raw milk of cows with subclinical mastitis, including such strains: *Staphylococcus aureus* (typical strain), *S. aureus* (small colony variant), coagulase–negative staphylococci *S. haemolyticus, S. saprophyticus, S. simulans, S. vitulinus*, and Gram–negative bacteria *Citrobacter freundii* and *Serratia liquefaciens*. Bacterial strains were identified using a MALDI Biotyper (Bruker, Germany), and an identification system "BBL Crystal Gram-positive and Enteric/Nonfermenter ID" (Becton, Dickinson and Company, USA). Strains were maintained at -20 °C in freezing medium of Brain Heart Infusion broth (Oxoid, England) supplemented with 30% glycerol, prior to use. After unfreezing, cultures were transferred to sterile BHI broth, and incubated at 37 °C overnight. Plates of nutrient agar (Biolife Italiana, Italia) were inoculated from cultures and incubated for 24 h at 37 °C.

Applied Test Suspensions of *L. Helveticus* and Glycopeptides

Natural glycopeptides that derived from the *L. helveticus* with lysozyme (TS1), inactivated *L. helveticus* in titre of 10^{10} with lysozyme (TS2), and milk polypeptides with β–glucans (TS3) were diluted in sterile sodium chloride 0.9% (0.75 g of TS in 10 mL^{-1} of saline) and adjusted to pH 6.3 using a sodium hydrogen carbonate, then well diffusion assay was performed for determination of the antimicrobial activity of the test suspensions against mastitis causing bacteria. Aminoacid analyses were performed by amino acid analyzer (Biotronic) in peptide laboratory of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS. The composition of amino acids from *L. helveticus* includes Ala, Glu, Lys, and Asp. β–glucans originated from shiitake (*Lentinula edodes*) and chanterelle (*Cantharellus cibarius*) mushrooms.

Antimicrobial Activity by a Well Diffusion Assay

Inhibitory activity of the test suspensions was investigated by a well diffusion method [4]. From the nutrient agar plates, colonies of *S. aureus* (2 strains), *S. haemolyticus, S. saprophyticus, S. simulans, S. vitulinus, C. freundii* and *S. liquefaciens* were transferred in 5 mL buffered peptone water (Oxoid, England) and adjusted using 0.5 McFarland's standard, then mixed using a vortex. Subsequently 100 µl of bacterial cultures was inoculated on Muller Hinton agar (Oxoid, England), by streaking the swab over the entire MH agar surface. Wells sized 6 mm were cut with a sterile metal cylinder into the agar plate. On the each MH agar plate 4 wells were cut. Then, 60 µl of each test suspension was placed into each well. The plates were incubated for 24 h at 37 °C and inhibition was examined by growth–free inhibition zones surrounding each well. Inhibition zones were measured in millimetres (mm) by the diameter of the wells. As the controls, sterile peptone water were used. This experiment was carried out in duplicate.

Haemolytic Activity

The four test suspensions which were used in testing of antimicrobial activity, were inoculated as a 60 µL spot on blood agar plates containing 5% of sheep blood (Oxoid, England) for the haemolytic activity tests. The plates were incubated at 37 °C for 48 h. According to P.A. Maragkoudakis et al. [17] and S. Tejero-Sarinena et al. [18], strains that produce green-hued zones around the spots (α–haemolysis) or do not produce any effect on the blood plates (γ–haemolysis) are considered non–haemolytic. Strains displaying blood lysis zones around the spots were classified as haemolytic (β–haemolysis). Absence of haemolytic activity indicates that a test suspensions are non–virulent [19].

Statistical Analysis

Statistical parameters were calculated with the Microsoft Excel 2013 Software (Microsoft Corp., Redmond, US); data about growth–inhibition zones (diameter, mm) were expressed as mean ± standard deviation.

RESULTS

Antimicrobial Activity

The inhibitory activity exerted by *L. helveticus* and glycopeptides against pathogens is presented in Table **1**.

TS1 (9-13 mm) and TS2 (10-15 mm) showed the inhibition effect on four of eight tested bacterial strains, whereas TS3 did not displayed any antimicrobial effect (Table **1**). TS2 have a greatest antimicrobial activity as they resulted in the largest inhibition zones. Even when TS1 and TS2 did not inhibited bacterial growth, around the wells we observed large zones of a yellow colouration, presumably due to some kind of antagonistic effect [18]. However, TS3 did not created such zones. The bacterial growth inhibition and zones of the yellow colouration are displayed in Fig. (**1**). The standard deviations of zones diameter measurements were ± 0 in all cases. The controls showed no inhibitory activity.

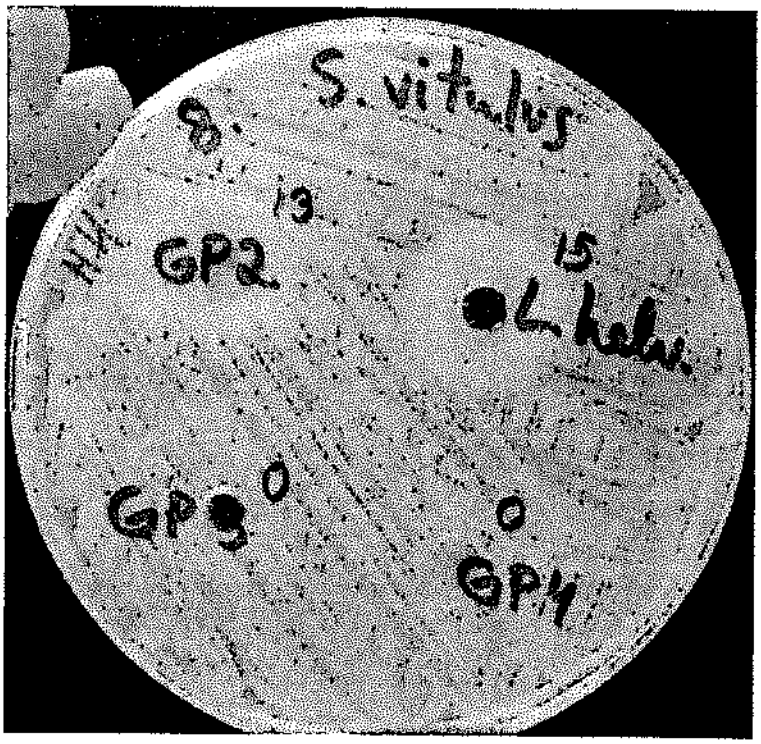

Fig. (1). Well diffusion assay on Muller Hinton agar medium.
MH – Muller Hinton agar medium, *S. vitulus* – *S. vitulinus* culture; GP2 – TS1 (creates 13 mm of bacterial clearance); L. helv. – TS2 (creates 15 mm of bacterial clearance); GP3 and GP4 – TS3 (do not create zone of bacterial clearance).

Table 1. Inhibitory effects of test solutions against a mastitis causing bacteria.

Bacterial strains	TS1 glycopeptides with lysozyme	TS2 inactivated *L. helveticus* with lysozyme	TS3 milk polypeptides with β–glucans
	Growth–inhibition zone, mm		
S. aureus 1 (typical strain)	9 ± 0	10 ± 0	0 ± 0
S. aureus 2 (small colony variant)	0 ± 0	0 ± 0	0 ± 0
S. haemolyticus	8 ± 0	10 ± 0	0 ± 0
S. saprophyticus	0 ± 0	0 ± 0	0 ± 0
S. simulans	12 ± 0	13 ± 0	0 ± 0
S. vitulinus	13 ± 0	15 ± 0	0 ± 0
Citrobacter freundii	0 ± 0	0 ± 0	0 ± 0
Serratia liquefaciens	0 ± 0	0 ± 0	0 ± 0

Haemolytic Activity

This experiment evidenced that TS1 and TS2 was not found to be haemolytic (γ–haemolysis), whereas TS3 created β–haemolysis on the blood agar plate.

DISCUSSION

Immunomodulators from the natural sources, like a lactoferrin, lysozyme, and β–glucans etc., nowadays play important role in treatment of different diseases as they increase the host's natural resistance to pathogens [13].

Bovine mastitis produces a wide variety of problems in the dairy farm. The treatment of this disease is based on the use of antibiotics which are not always effective, and usage of them is questionable, particularly in cases where infections are caused by minor pathogens like a coagulase–negative staphylococci. This is because of antibiotics are able to cure these kind of mastitis only temporarily, but cannot entirely eradicates an infections of opportunistic pathogens from the herd. According to J. Duval, antibiotics have not reduced the incidence of mastitis; the incidence of contagious mastitis (like induced by *S. aureus* or *S.*

agalactiae) has diminished through the use of antibiotics, but this has been paralleled by an increase in the level of environmental mastitis [20]. Moreover, antibiotics are responsible for the presence of residues in the milk and the increase of antibiotic-resistant strains [9]. Natural immunomodulators and probiotic products may become as a valid alternative to antibiotic therapies, so the problems that are associated with antibiotics usage can to a great extent be solved.

The overall aim of our researches is to test an efficacy of the *L. helveticus* and their glycopeptides in the treatment and prevention of subclinical mastitis in dairy cows during their lactation. The objective of the current investigations was to screen three test suspensions: natural glycopeptides that derived from the *L. helveticus* with lysozyme (TS1), inactivated *L. helveticus* with lysozyme (TS2), and milk polypeptides with β–glucans (TS3), in order to investigate which content were the most effective in inhibiting several mastitis causing bacteria in dairy cattle.

In a composition of used test suspensions are not only *L. helveticus* or their glycopeptides, but also other natural substances with immunomudulatory properties: lysozyme, β–glucans of mushrooms, and peptides derived from milk proteins. Milk polypeptides have an ability to affect biological functions of an organism. These effects can be antimicrobial and probiotic, i.e., prevent the growth and proliferation of undesirable and pathogenic organisms, or they may promote the growth of desirable bacteria in the digestive tract of humans and animals. They may also influence the immune system and treat or mitigate the effects of diseases [21]. Lysozyme is an enzyme present in the milk of some species (cow, goat, human etc.) that causes bacterial cell wall lysis, increases IgA production, and contributes to macrophage activation performing an immunomodulatory effects in host organism. In comparison with a human breast milk, lysozyme activity are limited in cow milk, but it increases due to mastitis and by high somatic cell counts in milk [22]. Acting alone, lysozyme lyses and kills a number of Gram–positive bacteria by damaging their surface exposed peptidoglycan [22]; for this reason it is possible to observe antibacterial action of lysozyme *in vitro*. In a host organism lysozyme possesses antibacterial activity usually functions in association with lactoferrin or immunoglobulin A. As well as, lysozyme can limit the migration of neutrophils into damaged tissue and might function as an anti-inflammatory agent [23, 24].

The β-glucans belong to a group of natural, physiologically active compounds, generally called biological response modifiers; together with chitin, the β-glucans are components of cell walls in yeasts and filamentous fungi. Glucans are well known biologic response modifiers that function as immunostimulants against infectious diseases and cancer [25, 26]. A survey of P. Persson Waller *et al.* [27] with the aim to investigate if intramammary infusion with the β–glucan at drying off can make the udder more resistant to experimental intra mammary *S. aureus* infection, indicated a slight, but not statistically significant, positive effect of β–glucan on the antibacterial response to *S. aureus* infection. Furthermore, in the same survey results demonstrated no therapeutic effect of β–glucan treatment of lactating udder quarters with chronic subclinical *S. aureus* mastitis [27]. As well as, test suspension TS3 that contained β–glucan, displays no inhibitory effect on the bacterial growth in our study. So, the health benefit of β–glucan in the treatment of bovine mastitis are still questionable.

The efficacy of the lactic acid bacteria against pathogenic bacteria are based on the action of bacteriocins and a combination of antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriophages [32]. In the literature are available much information about antimicrobial activity of probiotic lactobacilli, and several species are found to be antagonistic to pathogenic bacteria [9, 18, 28, 32]. But there are no publications available which describe usage of natural glycopeptides, derived from the probiotic bacteria.

As in our previously studies about mammary quarters's microflora it is revealed, some of the most distributed pathogens which cause subclinical mastitis in dairy cows are *Staphylococcus aureus*, several coagulase negative staphylococci and variety of Gram–negative bacilli from genus *Enterobacteriaceae* [29]; therefore cultures of mentioned pathogens, isolated from mastitic milk, were included in our investigations about glycopeptides' antimicrobial activity. In this study we included also two strains of *S. aureus* – one typical strain, and one small colony variant, which are a slow–growing subpopulation of *S. aureus* species. Phenotypically, small colony variants have a slow growth rate, atypical colony morphology and unusual biochemical characteristics. Clinically, small colony variants of *S. aureus* are able to persist viable inside host cells and modulate host defences, they are less susceptible to antibiotics than typical strains, and cause latent or recurrent infections [30, 35, 36].

In the current study we obtained results that TS1 and TS2 display an antimicrobial activity against pathogenic bacterial strains only partially, whereas TS3 did not displayed any antimicrobial effect. The applied test suspensions demonstrated no antagonistic effect on the growth of small colony variant *S. aureus*, *S. saprophyticus* and Gram–negative bacteria. A possible reason for the observed resistance of SCV *S. aureus* strain are the previously mentioned its' natural resistance factors. Regarding to resistance of *S. saprophyticus* strain, should be mentioned that, unlike most other coagulase-negative staphylococci, *S. saprophyticus* is rarely resistant to most antibiotics which are active against most of Gram–positive pathogens, apparently these species are difficult to treat with natural antibiotics, too [31]. It is known that most Gram–negative microorganisms are resistant to destroying by the proteins such as lysozyme, because Gram–negatives possess an outer membrane that shields the peptidoglycan murein sacculus that is not easily penetrated by the enzyme [22]. So the outer membrane of Gram–negative bacteria is a permeability barrier to lysozyme. In contrast, lysozyme lyses and kills Gram–positive bacteria by damaging their surface exposed peptidoglycan.

In our study TS2 which contains inactivated *L. helveticus* and lysozyme, showed constantly better results in the inhibiting of pathogen growth unlike in TS1 which consist of

glycopeptides and lysozyme, however, these study are conducted *in vitro*, but only *in vivo* conditions activity of glycopeptides may include the reactivity of the host immune system. Whereas *in vitro* settings the ability of the probiotic bacteria to produce organic acids have the greatest role performing antimicrobial activity on plates. The significance of pH, performing *in vitro* assessment for antimicrobial activity, is supported by E.T. Lima who observed that usage of the modified culture medium supplemented with only 0.05% glucose and adjusted to pH 6 causes the decrease in the production of organic acids of the *Lactobacillus* bacteria, and it results in a lesser inhibition capacity of the growth of pathogens [32]. Also several other researchers have found out that growth–inhibiting activity are generally attributed to the fact that lactic acid bacteria lower pH and/or produce organic acids [18, 28, 33, 34,], for example, S. Tejero-Sarinena et al. [18] studied the relationship between pH and inhibition of different strains of lactic acid bacteria against bacterial pathogens, and the results showed a significant correlation - the lower the pH results in the higher the bacterial growth inhibition.

We observed an interesting phenomenon around the wells on the plates - large zones of a yellow colouration, even if the test suspension did not inhibited bacterial growth. The colouration zones were present for the test suspensions of TS1 and TS2, but did not appear in TS3; the same observation with action of several lactobacilli are made previously by other studies, and Tejero-Sarinena *et al.* [18] explained it as presumably due to some kind of an antagonistic effect of lactic acid bacteria.

CONCLUSIONS

1. The applied test suspension TS1 which contains natural glycopeptides of *L. helveticus* and lysozyme with bacterial clearance zone between 9 mm and 13 mm, and TS2 which contains inactivated *L. helveticus* bacteria in titre of 10^{10} and lysozyme with clearance zone between 10 mm and 15 mm, displayed antimicrobial activity against *S. aureus* (typical strain), *S. haemolyticus, S. simulans*, and *S. vitulinus*.
2. The applied test suspension TS3 which contains natural glycopeptides of *L. helveticus* with milk polypeptides and β-glucans did not showed any antimicrobial effect against tested bacterial strains.
3. Test suspensions TS1 and TS2 displayed no haemolysis (γ–haemolysis) on sheep blood agar indicating that it is non–pathogenic, whereas TS3 created β–haemolysis.

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