

In-situ inhibition of *Staphylococcus aureus* by lactic acid bacteria consortia from two traditional Slovenian raw milk cheeses

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Summary

Bacteriocin(s) producing lactic acid bacteria naturally present in traditional cheeses represent an inexhaustive pool of microbes with safeguarding potential. Some bacteriocins produced by cheese lactic acid bacteria were already described as successful anti-staphylococcal agents. The presence of genes for bacteriocins with potential anti-staphylococcal activity was also demonstrated in two Slovenian traditional raw milk cheeses, "Tolminc" and "Kraški ovčji sir". Same bacteriocin genes were also detected in viable lactic acid bacteria consortia's isolated from "bacteriocin positive cheeses" on Rogosa, M17 and CATC agar media. The aim of the research was to elucidate whether or not this particular cheese consortia, in which bacteriocin genes were detected, actually exhibit anti-staphylococcal activity in milk and/or cheese. For this purpose different cheese consortia were selected in relation to versatility of detected bacteriocin genes and used to perform challenge tests against *Staphylococcus aureus* in milk and cheese. In milk following the time/temperature regime of traditional cheese production all cheese consortia effectively inhibited growth of *Staphylococcus aureus* in the range of app. 2 to 3 log. In cheese the inhibition of staphylococci was less pronounced but still evident since inhibition of app. 1.5 log was detected. Sole inhibition by lactic acid production was ruled out while linking inhibition directly to bacteriocin production would take some additional work.

Key words: traditional cheeses, safety, LAB consortia, anti-staphylococcal activity

Introduction

Traditional raw milk cheeses are gaining interest of the research world in an attempt to characterize and preserve the traditional heritage (Samaržija et al., 2006). Some of the Slovenian traditional cheeses like "Tolminc" from cows milk and "Kraški ovčji sir" from ewes milk have already been well studied to identify the microbial community present (Čanžek Majhenič et al., 2005; Čanžek Majhenič, 2007). The results of these studies have shown that both cheeses are comparable with the other European raw milk cheeses and are a reach source of natural microbes, mainly lactobacilli and enterococci.

In addition to these studies an effort has been made to establish the safety of these cheeses in terms of antibiotic susceptibility and presence of virulence determinants. Since these cheeses are produced from thermally non treated milk there is another safety issue presented as a potential presence of different pathogenic and spoilage bacteria. The issue of cheese spoiler *Clostridium tyrobutyricum* in Slovenian cheeses has already been addressed by Bogovič Matijašič et al. (2007). This research begins addressing the safety issue, with a culture independent detection of bacteriocin genes in two cheeses "Tolminc" and "Kraški ovčji sir" (Trmčič et al., 2008).

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This study comprises of bacteriocin genes detection in metagenomic DNA of raw milk cheeses as well as in its microbial consortia isolated on three different selective culture media. The results of this study show the presence of different bacteriocin genes in both metagenomic DNA and DNA extracted from microbial cheese consortia which indicates the presence of live bacteriocinogenic bacteria. Of 19 different bacteriocin genes that were researched, the most commonly detected were genes for plantaricin A, enterocins A, B, P, L50A, L50B, cytolysin and nisin.

Bacteriocins like enterocin P (Cintas et al., 1997), L50A, L50B (Cintas et al., 1998) nisin (Rodríguez et al., 2000) and others are known to inhibit broad range of spoilage and pathogenic bacteria *in-vitro* as well as *in-situ* in cheese models. Among these pathogens there is also *Staphylococcus aureus* which has been recognized to present a potential risk in traditional Slovenian hard cheeses made from raw milk. Indigenous strains of LAB that are producing bacteriocins are very good candidates for tailor-made starter cultures that could be used to improve microbiological safety of raw milk cheeses.

In this study the aim was to examine if cheese consortia bearing the bacteriocin genes are able to express these genes and inhibit the growth of *S. aureus in-situ*, since this pathogen is known to be present in hard type cheeses and can present a potential risk for the consumer (Samaržija et al., 2007). Different cheese consortia were selected in

relation to versatility of detected bacteriocin genes (Trmčič et al., 2008) and used to perform challenge tests with *S. aureus* in milk and cheese.

Materials and methods

Three types of challenge tests were carried out. In the first one the aim was to examine the effect of the presence of different cheese consortia on the growth of *S. aureus* in milk. In the second experiment it was examined if the production of lactic acid had an effect on *S. aureus* growth in milk, and in the last one the aim was to examine if the presence of different cheese consortia had similar effect on *S. aureus* growth in model cheese as in milk.

Bacterial cultures and media

For all of the experiments, *S. aureus* (Č.Sa.4.2), strain that was isolated from "Tolminc" cheese was used. The strain was cultivated in BHI (Merck) broth and quantified by plating on BP (Biolife) agar supplemented with RPF (Biolife). Microbial cheese consortia were isolated from two cheeses ("Tolminc" and "Kraški ovčji sir") on three selective media Rogosa (Merck), CATC (Merck) and M17 (Merck). Complete cheese consortium was obtained by joining consortia from isolation on all three media. Genes for individual bacteriocins that were detected in sample cheeses and cheese consortia in previous studies (Trmčič et al., 2008) are listed in Table 1. 10 % reconstituted skim milk (Merck) was prepared

Table 1. Different bacteriocin genes detected in total DNA extracted directly from "Tolminc" cheese (T2) and cheese "Kraški ovčji sir" (K3) and from their microbial consortia (Trmčič et al., 2008)

Tablica 1: Različiti geni za bakteriocine detektirani u ukupnoj DNA izoliranoj iz sireva "Tolminc" (T2) i "Kraški ovčji sir" (K3) i u DNA mikroflore izolirane iz tih sireva (Trmčič i sur., 2008.)

Cheese/Sir	PCR amplification of bacteriocin genes										
	DNA from cheese/DNA from cheese consortia										
	PCR umnažanje bakteriocinskih gena										
	DNA sira/DNA mikroflore sira										
	Target bacteriocin ^a /Ciljni bakteriocin ^a										
	Enterocins/Enterocini										
	A	B	P	L50A	L50B	Cyt	Nis	Lac481	AcidB	HelvJ	PlnA
T2	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/-	-/+	-/-	+/+
K3	-/-	-/-	-/+	-/-	-/-	-/-	-/+	-/-	-/-	-/+	+/+

cytolysin/citolizin (Cyt), nisin/nizin (Nis), lacticin 481/lakticin 481 (Lac481), acidocin B/acidocin B (AcidB), helveticin J/helveticin J (HelvJ), plantaricin A/plantaricin A (PlnA)

for challenge tests in milk, while preheated cows milk (65 °C, 15 min) was used for challenge tests in cheese.

Challenge tests

In the milk challenge tests, the milk was inoculated with 18 hour culture of *S. aureus* (app. 10^2 cfu/mL beginning concentration in milk), divided into three aliquots and refrigerated at 4 °C for 13,5 hours to simulate storage of contaminated milk before cheese production. One aliquot was used as a control, the second aliquot was additionally inoculated (1 % vol/vol) with lactobacilli cheese consortium and the third aliquot with complete cheese consortium (1 % vol/vol). Cheese consortia were cultivated separately in appropriate growth media for 18 hours prior to addition in milk experiment. All three inoculated milks were incubated following the time/temperature regime of "Tolminc" cheese production, excluding the ripening period. During the procedure, pH level of milk, concentration of consortia constitutes and *S. aureus* were also monitored. Challenge tests were done in two separate experiments using consortia isolated from each of two traditional Slovenian cheeses.

Challenge test in cheese was done similarly, with the difference that the procedure followed the actual "Tolminc" cheese production including the 15 day ripening period (Šabec, 1952; Perko et al., 2010). Challenge test in cheese was done once, with cheese consortia isolated from "Tolminc" cheese.

The second experiment where the effect of lactic acid production on growth of *S. aureus* in milk was evaluated, included four samples of inoculated milk. Two samples were inoculated with approximately 10^2 cfu/mL and two with 10^3 cfu/mL of *S. aureus*. From the two samples with the same *S. aureus* inoculation level, one was adjusted with lactic acid (Kemika) to pH level of 5.0, and the other was not adjusted (pH level was approximately 6.5). The procedure and sampling was done identically as in milk challenge test described above.

Bacteriocin detection

During challenge tests, milk and cheese samples were taken for determination of bacteriocin activity. Bacteriocin activity was examined in extracts

obtained from 10 g of cheese or milk as described by Bogovič Matijašič et al. (2007). Additionally, peaces of cheese (app. 10x10x5 mm) were overlaid with lawn of indicator strain as described by Foulquié Moreno et al. (2003). In all bacteriocin detection assays *Lactobacillus sakei* NCDO 2714 as the indicator strain was used. Confirmation of proteinaceous nature of inhibition would be performed by abolishing inhibitory activity with proteinase K or chimotripsine treatment of extracts.

Results and discussion

In all milk challenge tests (Figure 1, 2 and 3) the concentration of *S. aureus* was not increased during the 13.5 hours storage at 4 °C. This confirms the role of good manufacture practice in limiting the contamination and on spread of potential illnesses. Nevertheless, under the time/temperature regime simulating cheese production, *S. aureus* was able to grow by more than three log units if present alone in milk. The results of challenge tests in milk showed that the presence of any cheese consortium could limit the growth of *S. aureus* during cheese production. The lactobacilli consortia from both cheeses had a similar effect (Figure 1 and 2). In the beginning phase, heating to 32 °C (30 min) and maintaining this temperature (2 h), resulted in *S. aureus* growth limitation by cheese consortia, and after 30 minutes scalding step at 45 °C they even reduced the concentration of *S. aureus*. On the other hand the growth of *S. aureus* in control samples seemed unaffected by scalding time/temperature regime. In case of complete cheese consortia, the one isolated from "Kraški ovčji sir" showed only a limited effect on the growth but there was still 2 log difference in final concentration in comparison with growth in control samples (Figure 2).

Complete consortia from cheese "Tolminc" showed the most promising results. After initial growth of *S. aureus* its concentration decreased rapidly to undetectable levels of less than 10 cfu/mL (Figure 1).

Although the consortia from "Tolminc" cheese were more effective in inhibition of *S. aureus* in milk it is impossible to assign this inhibition to any of bacteriocins for which genes have been detected in the total DNA isolated from the consortia. Extracts obtained from milk and cheese sam-

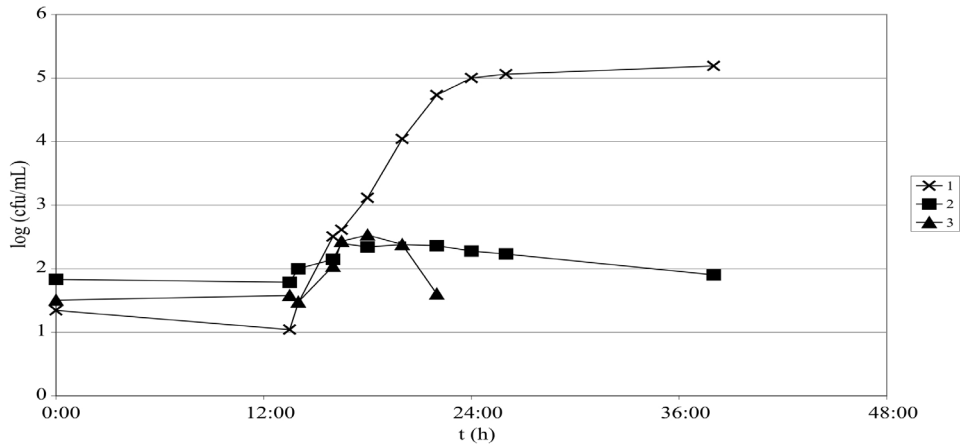


Figure 1. Growth of *Staphylococcus aureus* in milk with or without the presence of "Tolminc" (T2) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

Slika 1. Rast *Staphylococcus aureus* u mlijeku sa ili bez prisutnosti mikroflore "Tolminc" (T2) sira: bez prisutnosti mikroflore (1), prisutnost mješovite kulture laktobacila (2), prisutnost cjelokupne mikroflore sira (3)

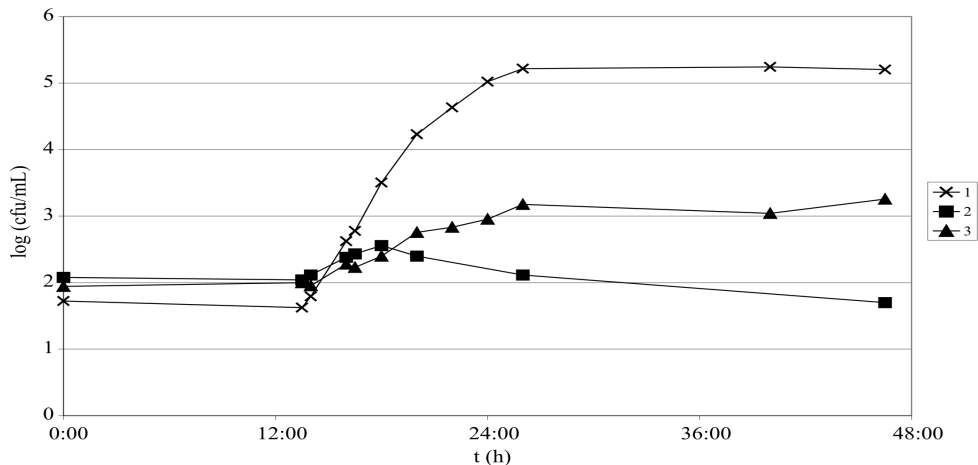


Figure 2. Growth of *Staphylococcus aureus* in milk with or without the presence of "Kraški ovčji sir" (K3) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

Slika 2. Rast *Staphylococcus aureus* u mlijeku sa ili bez prisutnosti mikroflore "Kraškog ovčjeg sira" (K3): bez prisutnosti mikroflore (1), prisutnost mješovite kulture laktobacila (2), prisutnost cjelokupne mikroflore sira (3)

ples showed no presence of inhibitory substances. A more detailed production of hydrogen peroxide or any other antimicrobial metabolite was not determined, but at least the most probable antimicrobial factor was ruled out, the organic acids production and decreased pH. In all cases the pH level of control treatment remained at a level of 6.5, while

treatments with lactobacilli consortia reduced initial pH level to 6.3 and remained at that level during entire fermentation. In treatments with complete cheese consortia the pH level was falling constantly and reached pH level of 6.1 after 36 hours and 5.9 after 48 hours of the experiment. Poorer acid formation by lactobacilli consortia is consistent with the

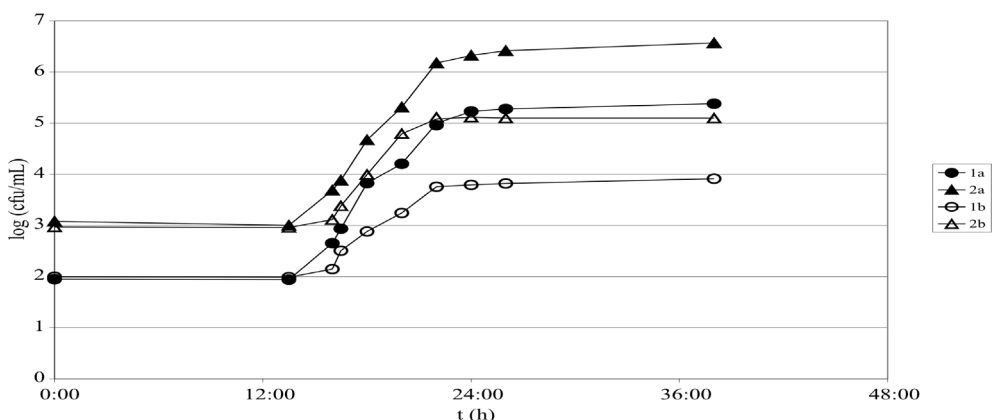


Figure 3. Growth of *Staphylococcus aureus* in milk in the presence of lactic acid: starting inoculation 10^2 cfu/mL non-adjusted pH level (1a), starting inoculation 10^3 cfu/mL non-adjusted pH level (2a), starting inoculation 10^2 cfu/mL pH level adjusted to 5.0 (1b), starting inoculation 10^3 cfu/mL pH level adjusted to 5.0 (2b)

Slika 3. Rast *Staphylococcus aureus* u mlijeku uz prisutnost mliječne kiseline: početna inokulacija 10^2 cfu/mL bez podešavanja pH (1a), početna inokulacija 10^3 cfu/mL bez podešavanja pH (2a), početna inokulacija 10^2 cfu/mL s podešavanjem pH na 5,0 (1b), početna inokulacija 10^3 cfu/mL s podešavanjem pH na 5,0 (2b)

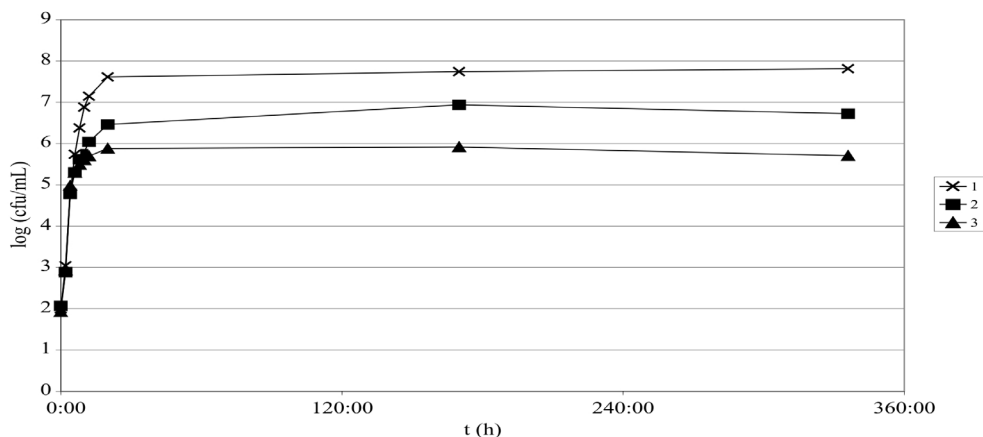


Figure 4. Growth of *Staphylococcus aureus* in cheese with or without the presence of “Tolminc” (T2) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

Slika 4. Rast *Staphylococcus aureus* u siru sa ili bez prisutnosti mikroflore “Tolminc” (T2) sira: bez prisutnosti mikroflore (1), prisutnost mješovite kulture laktobacila (2), prisutnost cjelokupne mikroflore sira (3)

fact that lactobacilli are auxotrophic for some amino acids and are stimulated by the presence of microbes like streptococci that produce formic acid and other metabolites. However, the differences in pH levels are not consistent with different growth patterns of *S. aureus*, indicating that acid production was only a minor factor in inhibition of *S. aureus*. This observa-

tion was confirmed with additional experiment results of which are shown in Figure 3. By addition of lactic acid the pH level of milk was lowered to 5.0, which is much lower pH level from the one detected in challenge tests. Even at this lower pH level the *S. aureus* was inhibited almost for 1.5 log unit difference in final concentration. Charlier et al. (2008)

reported the similar results of *S. aureus* growth in the presence of different concentrations of lactic acid. From their results it is evident that pH level of 5.0 is a limit under which a substantial inhibition by acids takes place.

Since the results in milk were promising, it was decided to perform challenge tests in the model cheese also. Microbial consortia from "Tolminc" cheese were used. In general the growth of *S. aureus* in cheese seemed to be higher than in milk. This was probably due to cheese making procedure where microflora was concentrated in the cheese curd during syneresis. Another difference between milk and cheese challenge tests was the milk. For cheese production fresh thermally treated cow's milk was used and not reconstituted milk like for milk challenge tests. Again the complete cheese consortium was the most effective in inhibiting *S. aureus*. It managed to lower the final concentration by two log units, while lactobacilli consortium lowered the final concentration by one log unit (Figure 4). The experiment was finished after two weeks when in both cheeses treated with cheese consortia, *S. aureus* concentration was in a slight decline while the concentration in control treatment was still slightly rising. Once again the difference between treatments in the growth of *S. aureus* occurred after the scalding step. This can indicate that thermophilic microflora which was stimulated by scalding step was responsible for *S. aureus* inhibition. In our previous studies on bacteriocin gene presence in cheese the appropriate PCR primers for *Str. thermophilus* bacteriocins were not found and therefore data about the bacteriocinogenic potential of this large part of thermophilic microflora are absent.

There was no difference between the pH level of control samples and samples treated with the lactobacilli consortium. In both cases it was lowered to only 6.4 while the treatment with complete cheese consortium resulted into reduction of pH to the level of 5.5 in one day and continued to drop to 5.2 until the end of experiment. Once again, poor fermentative characteristics of consortial lactobacilli was demonstrated, confirming the presumption that the inhibition of *S. aureus* seems to involve some other mechanisms besides acid production. Charlier et al. (2008) came to similar conclusions that inhibition of *S. aureus* in cheese could occur regardless of acidification. It still remains to be determined

what are the possible factors involved in *S. aureus* inhibition. It is assumed that it is nutrient-related phenomenon. Delbes et al. (2006) reported that pH level had little effect in the first 6 hours of cheese production. In this period, the pH level however should be lowered below 5.8 since the subsequent growth and enterotoxin production between 6 and 24 hours of fermentation is greatly influenced by it. In this study none of added consortia were able to lower the pH to appropriate level in cheese or milk. In all three treatments, concentration of *S. aureus* reached the levels above 10^6 cfu/mL, which is the limit for cheese in order to be considered as safe. At high *S. aureus* concentrations there is a risk of enterotoxin accumulation (Lindqvist et al., 2002). According to some studies, 10^3 cfu/mL can be enough for sufficient toxin accumulation (Charlier et al., 2008). It seems that the consortia should be refined by selecting the strains that enable appropriate acidification and inhibition of *S. aureus*. The next goal would be to determine if bacteriocins are responsible for the inhibition observed in this study. In addition, the following experiments that will be conducted in cheese should include the monitoring during entire 60 day of ripening period since major changes in *S. aureus* presence may happen during the second month of ripening (Rodríguez et al., 2000).

Conclusions

This study was performed in milk and cheese model. In both cases the conditions of the real cheese making process typical for "Tolminc" cheese production were simulated. The study revealed that all tested cheese consortia had an obvious effect on the growth of pathogenic bacteria *S. aureus* and consequently possibly also on the enterotoxin production *in-situ*. Although acid formation is known to be an important factor for survival of pathogens (Zdolec et al., 2007), in this study it was found to be only a minor factor in *S. aureus* inhibition. These results were consistent with the results of some other studies on inhibition of *S. aureus* in cheese and milk (Delbes et al., 2006; Charlier et al., 2008). In cheese consortia from "Tolminc" cheese a number of bacteriocin genes that, if expressed, could play an important role in inhibition (Trmčić et al., 2008) was previously detected. The consortia from "Tolminc" cheese exhibit more pronounced

inhibition of *S. aureus* than consortia from "Kraški ovčji sir". Inhibition of *S. aureus* could be a matter of one single bacteriocin, like enterocin P whose genes were detected in both cheese consortia. Additionally the results raised a question of possible non-acid and non-bacteriocin type of inhibition for which there are only a few documentations (Šušković et al., 2010). Most probably among the mechanisms involved in inhibition, a nutrient-related phenomenon, like competition or limitation of nutrients which in fact can trigger other mechanisms, can be considered. Further studies are needed to determine the exact mechanisms of inhibition and the role of different factors including bacteriocins. In the following studies it would be a great advantage if classical fermentation and cheese production experiments were supported by the modern molecular methods.

In-situ inhibicija *Staphylococcus aureus*
bakterijama mliječne kiseline iz dva
tradicionalna slovenska sira
proizvedena od sirovog mlijeka

Sažetak

Bakterije mliječne kiseline (BMK), prirodno prisutne u tradicionalnim sirevima, predstavljaju neograničen izvor mikroba sa zaštitnim svojstvima. Za neke bakteriocine, koje proizvode BMK iz sira, antistafilokokna svojstva već su poznata. Prisutnost gena za bakteriocine s antistafilokoknim svojstvima potvrđena je u dva tradicionalna slovenska sira iz sirovog mlijeka; to su sir "Tolminc" i "Kraški ovčji sir". Prisutnost istih bakteriocina potvrđena je i kod bakterija mliječne kiseline, koji su izolirali iz tih sireva na agar podlogama Rogosa, M17 i CATC. Cilj je bio provjeriti da li izolirani sojevi bakterija mliječne kiseline, koji nose gene za bakteriocine, zaista pokazuju antistafilokokna svojstva u mlijeku i/ili siru. Na temelju prisutnih gena za bakteriocine ispitana je antimikrobna aktivnost združene kulture bakterija mliječne kiseline prema bakteriji *Staphylococcus aureus* u mlijeku i siru. U mlijeku kojeg smo podvrgnuli vremensko-temperaturnom režimu proizvodnje tradicionalnih sireva, združena kultura bakterija mliječne kiseline inhibirala je rast bakterije *Staphylococcus aureus* u rasponu od 2 do 3 logaritamske jedinice. U siru je inhibicija stafilokoka bila nešto manje izražena, ali još uvijek očita sa 1,5 logaritamskih jedinica

slabijim rastom *S. aureus* nego u kontrolnom siru. Učinak inhibicije izazvane mliječnom kiselinom bio je isključen, ali bi bilo potrebno izvesti još dodatna ispitivanja, da se utvrdi izravno inhibicijsko djelovanje bakteriocina na stafilokoke.

Ključne riječi: tradicionalni sirevi, bakterije mliječne kiseline (BMK), antistafilokokna aktivnost

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