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Characterization of bacteriocinogenic strains of lactic acid bacteria from traditional Slovenian cheese 'Tolminc'

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Summary

The purpose of this research was to examine traditional Slovenian 'Tolminc' cheese for the presence of lactic acid bacteria that produce several bacteriocins. The presence of gene determinants for different bacteriocins in this type of cheese and in the cultivable population of 'Tolminc' microbiota, have already been demonstrated, as well as its antimicrobial activity. Due to the difficulties in connecting the presence of gene determinants for bacteriocins with the observed antimicrobial activity it was decided to examine in this study the same features on the level of individual bacteriocinogenic strains. Like in previous results, enterococci and their bacteriocins prevailed in cheese microbial consortia. None of isolated strains inhibited growth of *Staphylococcus aureus*, while the other indicator strains were inhibited in a strain specific manner. Most of isolated strains carried gene determinants for cytolysin. On the basis of gene determinants for bacteriocins, antimicrobial activity, phenotyping by PhP (PhenePlate™) system and PCR identification, some similarities found were among *Enterococcus* isolates.

Key words: traditional cheese, bacteriocin genes, bacteriocins, enterocins, enterococci, antimicrobial activity

Introduction

Artisanal cheese and other dairy products are traditionally produced from raw milk. The chemical and microbiological properties of milk are besides technological procedure among the most important parameters that determine the final characteristics of a product. Majority of microbes present in the rich microbiota of raw milk are considered beneficial but we should also be aware of a possibility that certain microbes that came from milk to the final product can present a health risk for the consumer (Habeš, 2002). To minimize this risk, a number of different selected starter or protective cultures are being used (Šušković et al., 2010). The use of selected cultures is not desired in traditional cheese production since it can change the microbial composition and consequently the sensory properties of the product. One possible way towards solving this problem could be the use of cultures composed of the strains derived from particular artisanal product. The strains that produce different antimicrobial substances, such as bacteriocins are good candidates for the protective cultures. They may be selected by screening the natural microbiota of these cheeses (Radulović et al., 2010). Selection and combination of the autochtonous strains would enable the creation of "tailor made" starter cultures for particular types of traditional cheeses.

Čanžek Majhenič et al. (2005, 2007) who analysed natural microbiota of traditional Slovene cheeses 'Tolminc' (from cows' milk) and 'Kraški' (from ewes' milk), found that majority of the popu-

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lation consisted of the representatives of two genera, *Enterococcus* and *Lactobacillus*. They also determined the antimicrobial activity of some isolates. Their antimicrobial potential was confirmed in the metagenomic DNA study of nine cheeses for presence of gene determinants of different bacteriocins (Trmčić et al., 2008). The most promising results were obtained with 'Tolminc' cheese (T2) where they detected gene determinants for enterocins A, B, P, L50A, L50B, cytolysin, nisin, lacticin 481 and plantaricin A. Most of these determinants were also present in DNA extracted from the cultivable part of microbiota, i.e. microbial consortia, confirming the presence of viable bacteriocinogenic strains in the cheese microbiota.

In further studies they examined how the presence of gene determinants for different bacteriocins affects the antimicrobial activity of the microbiota and possibility of these bacteriocin genes being expressed and offering the producer strain a competitive advantage inside the microbial population of cheese. This was tested by consecutive transfers of microbial consortia in reconstituted milk for period of ten days and analysing the changes on the presence of bacteriocin gene determinants and antimicrobial activity. The main observation of the study was that the presence of bacteriocin gene determinants did not assure better survival although some of the strains carrying gene determinants for enterocin P, enterocin L50B and cytolysin did persist in the population. Bacteriocins of which gene determinants were detected could not be attributed to the observed antimicrobial activity and consequently not be directly attributed to the presence of bacteriocins' gene determinants in the particular consortia. Namely, although the presence of bacteriocin genes was reduced during consecutive propagation of consortia, their antimicrobial activity was not substantially changed. A similar result was observed in a recent study of Trmčić et al. (2010) where the antimicrobial activity was tested in the simulated conditions of real cheese production. Although the addition of viable cheese consortia did reduce the growth of *Staphylococcus aureus*, the inhibition could not be directly attributed to the activity of any bacteriocin.

In all of the studies by Trmčić et al. (2008, 2010) there were difficulties to link the antimicrobial activity of viable LAB consortia with the detected gene determinants for bacteriocins. We may speculate that this happened because of numerous interactions taking part inside a complex microbial population which affected antimicrobial activity. Therefore it was decided to examine in the present study the antimicrobial activity of previously studied LAB microbial consortia of traditional cheeses and the presence of bacteriocin genes on the level of single isolates.

Materials and methods

Bacterial strains were isolated from fully ripened 'Tolminc' cheese (T2) produced from raw cows' milk according to Šabec (1952) and Perko et al. (2010). Different microbial consortia consisting manly of lactobacilli, mesophilic cocci and thermophilic cocci, were obtained on Rogosa and M17 agar plates (Trmčić et al., 2008). In addition to original consortia, consortia from milk were also isolated, during consecutive propagation of microbial consortia in milk.

For detection of antimicrobial activity the following indicator strains were used: *Lactobacillus sakei* ATCC 15521 (American Type Culture Collection, Manassas-Virginia, ZDA), *Enterococcus durans* CCM 5612 (Czech Collection of Microorganisms, Brno, Czech Republic), *Enterococcus faecalis* ATCC 19433, *Enterococcus faecalis* CCM 4647, *Listeria monocytogenes* N°10 S 4ab (National Agricultural Research Foundation, Greece), *Listeria innocua* ATCC 33090 in *Staphylococcus aureus* ISS 464 (National Agricultural Research Foundation, Greece). Strain *Enterococcus faecium* ATCC 19434 which produces enterocins A, B and P was used as a positive control for the presence of these bacteriocin gene determinants and for the antimicrobial activity assays.

M17 agar, MRS agar (pH 5.7), BHI agar and broth media were prepared according to manufacturer's instructions (Merck, Darmstadt, Germany). To avoid inhibitory action of organic acids (Zdolec et al., 2007) a modified MRS agar containing reduced concentration of glucose (2 g/L) which was prepared according to Jacobsen et al. (1999) was also used.

Individual bacteriocinogenic strains were obtained by plating proper dilutions of individual microbial consortia on different agar plates (modified MRS, 37 °C; M17, 30 °C; M17, 42 °C). After incubation the outgrown colonies were covered with

Table 1. Antimicrobial activity of individual strains from original microbial consortium of 'Tolminc' (T2) cheese and from propagated microbial consortia in milk

Tablica 1. Antimikrobna aktivnost pojedinih izolata iz originalne mikrobne zajednice sira 'Tolminc' (T2) i nakon uzgoja te mikrobne zajednice u mlijeku

Strain designation Oznaka izolata	Indicator strains/Indikatorski sojevi							
	Lb. sakei ATCC 15521	*L. monocytogenes* N°10 S 4ab	*L. innocua* ATCC 33090	*S. aureus* ISS 464	*Ec. durans* CCM 5612	*Ec. faecalis* ATCC 19433	*Ec. faecalis* CCM 4647	*Ec. faecium* ATCC 19434
G0x30/1	1 k p	1 k p	0	0	1 k p	1 k p	1 k p	1 k p
G0x30/3	1 k p	/	/	/	/	/	/	/
G0x30/4	2 k p	1 k p	0	0	1 k p	1 k p	1 k p	1 k p
G0x42/1	1 k p	1 k p	0	0	1 k p	2 k p	1 k p	1 k p
G0x42/2	1 k p	0	0	0	1 k p	1 k p	0	1 k p
G0x42/9	1 k p	1+4 k p*	6 k	0	1 k p	1 k p	1 k p	1 k p
G0xR/1	5 k p	0	0	0	4 k **	0	1 k p	4 k **
G0xR/2	5 k p	/	/	/	/	/	/	/
G10x30/4	2 k	1+6 k p*	7 k	0	1 k p	1 k p	1 k p	1 k p
G10x42/1	1 k p	/	/	/	/	/	/	/
G10x42/2	2 k p	/	/	/	/	/	/	/
G10x42/3	1 k p	1+4 k p*	6 k	0	1 k p	5 k p	1 k p	1 k p
G10x42/3.1	6 k p	5 k p	4 k p	0	1 k p	3 k p	3 k p	0
G10x42/5	2 k p	/	/	/	/	/	/	/
G10x42/6	1 k	/	/	/	/	/	/	/
G10x42/8	2 k p	/	/	/	/	/	/	/
G10x42/9	2 k p	1+4 k p*	6 k	0	1 k p	1 k p	1 k p	1 k p
G10x42/10	1 k p	/	/	/	/	/	/	/
G10x42L/1	/	2 k p	/	/	/	/	/	/
G10x42L/2	/	2 k p	/	/	/	/	/	/
G10x42L/3	/	2 k p	/	/	/	/	/	/
G10x42L/4	1 k p	1+4 k p*	6 k p	0	1 k p	1 k p	1 k p	1 k p
G10x42L/5	/	2 k p	/	/	/	/	/	/
Ec. faecium ATCC 19434	8 k p	6 k p	6 k p	0	6 k p	3 k p	3 k p	0

Legend:
- 0-8 … Size of inhibition zone measured from the edge of the spot (mm)/Veličina zone inhibicije od ruba rasta (mm)
- k … Proteinaceous nature of inhibition was confirmed with the use of chymotrypsin/Proteinska svojstva inhibicije bila su potvrđena upotrebom kimotripsina
- p … Proteinaceous nature of inhibition was confirmed with the use of proteinase K/Proteinska svojstva inhibicije bila su potvrđena upotrebom proteinaze K
- * … Double zone of inhibition, chymotrypsin effective in both zones while proteinase K effective only in smaller one/Dvojna zona inhibicije, kimotripsin djeluje na obje zone, proteinaza K djeluje samo na manju zonu
- ** … Weak effect of chymotrypsin on inhibition zone/Vrlo slab utjecaj kimotripsina na inhibicijsku zonu
- / … Spot-test was not performed/Spot-test nije bio izveden

soft overlay inoculated with indicator strains *L. monocytogenes* N°10 S 4ab, *S. aureus* ISS 464 or *Lb. sakei* ATCC 15521 (sensitive strain used as a positive control for antimicrobial activity). The colonies surrounded with clear inhibition halos were isolated from the agar and purified. Antimicrobial activity of each isolated strain was also tested against *L. innocua* ATCC 3090, *Ec. durans* CCM 5612, *Ec. faecalis* ATCC 19433, *Ec. faecalis* CCM 4647 and *Ec. faecium* ATCC 19434 .

The genomic DNA from selected isolates was extracted for further identification and analysis of the presence of gene determinants for different bacteriocins. Extraction of DNA was performed using commercial kit Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturers' instructions. Genomic DNA of each strain was searched for gene determinants of 10 LAB bacteriocins following the procedure described by Trmčić et al. (2008). Antimicrobial activity was tested according to Cogan et al. (1997). Chymotrypsin (10 mg/mL) and proteinase K (10 mg/mL) were used to determine the potential proteinaceous nature of inhibition. Proteolytic enzymes were applied as spots (3 μL) next to the spot of tested strain. Genus identification was performed by *Enterococcus* specific PCR using the oligonucleotide primers designed by Deasy et al. (2000). *Ec. faecalis* and *Ec. faecium* identification was performed in multiplex PCR reactions as described by Jackson et al. (2004).

Phenotyping was performed using PhP (PhenePlate™) which is based on measuring kinetics of biochemical reactions during growth in ready to use microtiter plates. The system for enterococci (PhP-FS) is composed of 23 different substrates and one control without substrate. The directions supplied by the manufacturer were followed. The results were evaluated by software PhPWIN which arranged analysed strains into groups based on the set coefficient of correlation 0.975.

The hemolytic properties of the strains were analysed by agar diffusion method. Cultures (18 h) of each strain were spotted (5 μL) on to surface of blood agar plate, prepared from blood agar base No. 2 (Merck, Darmstadt, Germany) and defibrinated bovine or sheep blood according to manufacturers' directions. After 24 h of incubation at either 30 °C or 37 °C we examined the plates for α, β or γ hemolysis.

Results and discussion

No colonies that would be inhibitory towards *S. aureus* were found by the screening of antimicrobial activity of different microbial consortia on agar plates. Nevertheless, from M17 agar plates incubated at both 30 °C and 42 °C, and also from MRS plates, strains that were effective against indicator strains *Lb. sakei* and *L. monocytogenes* (Table 1) were isolated. Although MRS agar was used to obtain the consortia rich with the representatives of *Lactobacillus*, the two isolates (G0xR/1, G0xR/2) with antimicrobial activity, turned out to be representatives of *Enterococcus* genera (Figure 1). These two strains were among the most effective isolates against indicator strain *Lb. sakei*. Limited selectivity of MRS and other growth media is well known (Menendez et al., 2001), however in this study the main purpose of collecting the viable consortia from different growth media incubated at different temperatures was to obtain as many as possible different representatives of cheese microbiota and to retain them during manipulation.

13 isolates were found effective against *L. monocytogenes* N°10 S 4ab, five of which exerted double inhibition halos of which the larger one was sensitive to action of chymotrypsin alone and the smaller halo to action of both chymotrypsin and proteinase K (Table 1). Double inhibition halos were most probably a result of activity of two different bacteriocins of which one has better diffusion properties in agar. In general these five strains had a similar profile of inhibition which was in case of *L. innocua* ATCC 33090 effected only by chymotrypsin. Similar double inhibition halos were also observed in previous study where mesophilic part of cheese population was tested after ten transfers in reconstituted milk. This observation confirms the results of previous study where some bacteriocinogenic strains did persist in the population and meaning that this feature offers a potential growth advantage.

Nine of eleven strains analysed by species specific PCR were identified as *Ec. faecalis* and two as *Ec. faecium* (Figure 1). The same two groups were identified using PhP system (Figure 2). Within *Ec.*

Table 2. Presence of different bacteriocin gene determinants in individual strains of isolated microbial consortia

Tablica 2. Prisutnost različitih genskih determinanta za bakteriocine kod pojedinih sojeva mikrobne zajednice

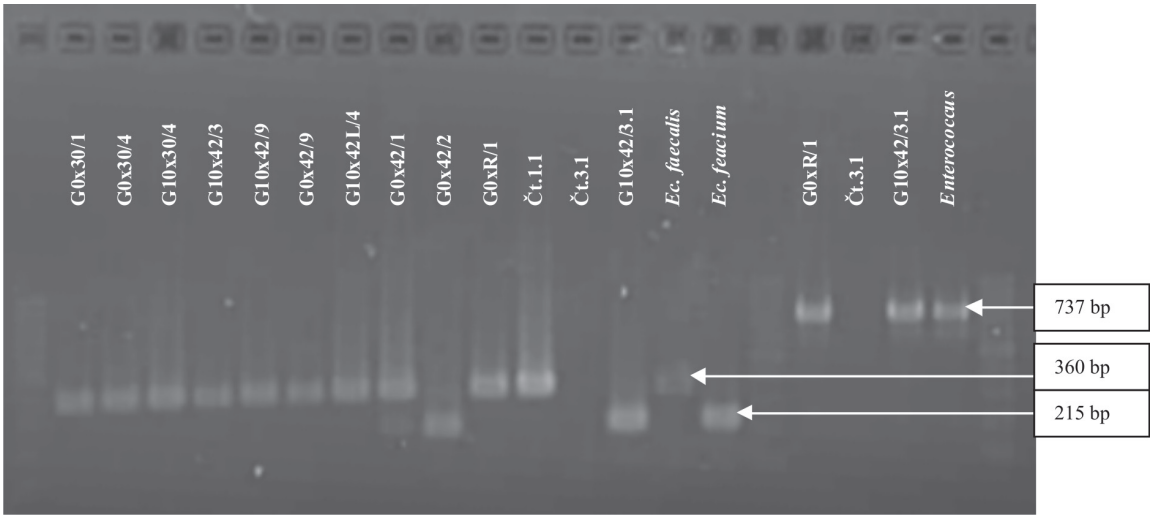
Isolate designation Oznaka izolata	Presence of gene determinant for bacteriocin Prisutnost genske determinante za bakteriocin									
	Enterocin						Nizin/Nisin	Lakticin 481 Lacticin 481	Acidocin B	Plantaricin A
	A	B	P	L50A	L50B	Cytolysin Citolizin				
G0x30/1	-	-	-	-	+	+	-	-	-	-
G0x30/4	-	-	-	-	-	+	-	-	-	-
G0x42/1	+	+	+	+	+	+	-	-	-	-
G0x42/2	+	-	+	+	+	+	-	-	-	-
G0x42/9	-	-	-	-	-	+	-	-	-	-
G0xR/1	-	-	-	-	-	+	-	-	-	-
G10x30/4	-	-	-	-	-	+	-	-	-	-
G10x42/3	-	-	-	-	+	+	-	-	-	-
G10x42/3.1	+	+	+	-	-	-	-	-	-	-
G10x42/9	-	-	-	-	-	+	-	-	-	-
G10x42L/4	-	-	-	-	-	+	-	-	-	-

Legend: + … PCR product of corresponding length is present/PCR produkt prave dužine je prisutan
- … PCR product of corresponding length is not present/PCR produkt prave dužine nije prisutan

faecalis group there were three smaller groups identified, while the two *Ec. faecium* strains together with *Ec. faecium* ATCC 19434 seemed less related.

Selected representatives of each PhP group were also analysed for the presence of specific gene determinants for bacteriocins already identified in the original microbial population of T2 cheese (Trmčić et al., 2008). All tested strains, except one, carried the gene determinants for cytolysin which is unusual for cheese enterococci especially for isolates belonging to *Ec. faecium* species (Mohar Lorbeg, 2008; Katana Burja, 2006; Čanžek Majhenič et al., 2005; Čanžek Majhenič, 2006). De Vuyst et al. (2003) identified among 37 isolates only one *Ec. faecium* that carried gene determinants for cytolysin and even this one was a clinical isolate. It is also notable that in three strains of *Ec. faecalis* gene determinants for enterocin L50B were identified and in one strain also determinants for enterocins A, B, P and L50A which are characteristic of *Ec. faecium* species. A possible explanation for this is that isolates contained more than one strain which was the case for isolate G0x42/1 (Figure 1). Namely, sometimes it is practically impossible to separate and purify particular strains because they grow in the symbiosis.

All of isolates were similarly effective against indicator strains from genera *Enterococcus*. The only exception was isolate G0xR/1 which was the most effective against *Ec. durans* CCM 5612, but the inhibition halos were not affected by any proteolytic enzyme (Table 1). The most effective against both *Listeria* indicator strains was the isolate G10x30/4, while the isolate G10x42/3.1 turned out to be the most effective in general, since it inhibited most of the indicator strains used. This strain identified as *Ec. faecium* (Figure 1), was, according to detected gene determinants for bacteriocins and antimicrobial activity, very similar to strain *Ec. faecium* ATCC 19434 (Tables 1 and 2), but the results of PhP analysis displayed an obvious difference (Figure 2). From the fact that strain G10x42/3.1 was not effective against *Ec. faecium* ATCC 19434 it was concluded



Legend: JCM5804 … *Ec. faecium* ATCC 19434

Figure 1. PCR products from isolates using genera (*Enterococcus*) and species (*Ec. faecium* and *Ec. faecalis*) specific primers

Slika 1. PCR produkti izolata sa specifičnim početnicama za rod (*Enterococcus*) i vrste (*Ec. faecium* i *Ec. faecalis*)

that its antimicrobial activity was based on secretion of one or more enterocines A, B, P. Enterocin P has a wide antimicrobial spectrum including also representatives of *S. aureus*. None of two strains possessing enterocin P genes inhibited *S. aureus* ISS 464 indicator strain (Tables 1 and 2), but this does not necessary mean they would not be effective against some other strains of this species.

Since the aim was to determine whether the antimicrobial activity is a result of enterocins A, B or P activity, isolate G10x42/3.1 and additional ten isolates were tested against *Ec. faecium* ATCC 19434 indicator strain (Table 1). It turned out that all tested strains except G10x42/3.1 were effective against *Ec. faecium* ATCC 19434. The inhibition halos were 1 mm wide and of proteinaceous nature, similar to the inhibition halos observed against *Ec. durans* CCM 5612 (Table 1). Based on these results and the fact that all of the isolates, except G10x42/3.1, carried the gene determinants for cytolysin it was speculated that this bacteriocin could be responsible for the observed inhibition. Since cytolysin has also hemolytic properties it was decided to test this possibility *in-vitro* using blood agar growth media. None of tested isolates exerted hemolytic activity (results not shown). In study conducted by De Vuyst et al. (2003) there were eleven enterococci strains identified carrying gene determinants for cytolysin of which eight proved to be also positive for β hemolysis. Based on these results it can be concluded that cytolysin was not responsible for the observed inhibition. There is a possibility that inhibition was not directly correlated to cytolysin secretion but is rather a result of genotypic/phenotypic feature which is linked to the presence of cytolysin genes.

The G0xR/1 isolate was the only one that exerted a stronger antimicrobial activity against *Ec. faecium* ATCC 19434 and also against *Ec. durans* CCM 5612 (Table 1). On the other side it was not possible to confirm reliably the proteinaceous nature of these inhibitions by using proteolytic enzymes. Even though the proteolytic enzymes were not effective, the appearance of the inhibition halos (clear with well defined edges) indicated that this was the result of bacteriocin activity. Based on given results it could not be concluded which bacteriocin was responsible for this inhibition since only the cytolysin gene determinants were identified. Isolate G0xR/1 belongs to *Ec. faecalis*, and representatives of this species are known to produce also several other bacteriocins (Galvez et al., 1998; Eguchi et al., 2001; Franz et al., 2002; Martin-Platero et al., 2006) which were not looked for in this study.

In individual isolates from microbial population of 'Tolminc' (T2) cheese only two out of nine gene determinants for bacteriocins that were originally

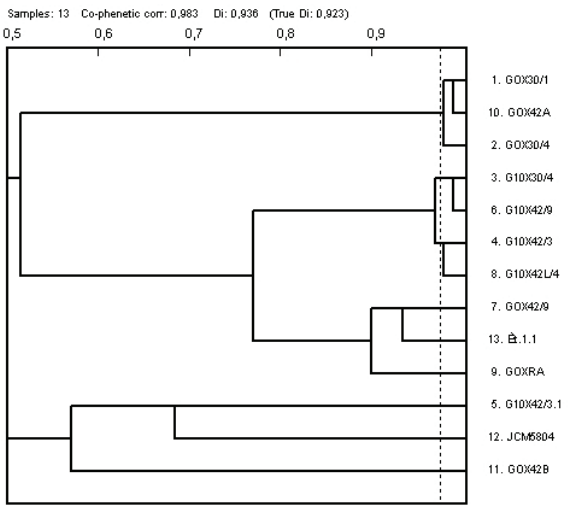


Figure 2. Dendrogram of PhP classification of enterococci
Slika 2. Dendrogram enterokoka svrstanih u PhP grupe

identified in this cheese were identified (Trmčić et al., 2008). To find the strains that carried other seven bacteriocins' genes more strains from the examined microbial consortia of this cheese have to be screened. Even then some of the determinants might not be found since some strains can lose their cultivability. Also there is a possibility that some bacteriocins are not expressed, synthesized or secreted properly which is a prerequisite for their detection.

Conclusions

With this study of 'Tolminc' cheese microbiota we concluded the complete analysis of its antimicrobial potential, going from entire cheese to strain level and from genotypic to phenotypic level of bacteriocinogenic potential. In previous closely related studies (Trmčić et al., 2008, 2010) similar conclusion were given, that the presence of gene determinants of different bacteriocins itself does not assure consistent antimicrobial activity. In addition, even when antimicrobial activity of proteinaceous nature is detected, it is very difficult to attribute it to any of the detected bacteriocin gene determinants, especially since the majority of bacteriocins are not looked for or have not been yet identified. Obviously there is a gap between the analysed gene determinants and *in vitro*, *in vivo* or *in situ* observed inhibition. In order to resolve these gaps observed during these studies it would be necessary to incorporate studies at a transcriptomic and proteomic level. On one side the gene determinants detected could be analysed for transcription which is a major part of expression. Expression analysis could help us understand the dynamics of bacteriocin production in complex media like cheese where conventional biological methods are not enough sensitive and specific. On the other side the proteomic tools could help to identify the bacteriocins secreted by bacteriocinogenic strains. This way it can also be analysed and characterized new bacteriocins and bacteriocinogenic strains that are with certainty present in diverse microbial community of such a rich medium like raw milk cheeses.

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Karakterizacija bakteriocinogenih sojeva bakterija mliječne kiseline iz tradicionalnog slovenskog sira 'Tolminc'

Sažetak

Cilj ovog rada bio je provjeriti prisutnost sojeva bakterija mliječne kiseline koji proizvode različite bakteriocine u tradicionalnom slovenskom siru 'Tolminc'. Prisutnost genskih determinanti za pojedine bakteriocine u ovoj vrsti sira i izoliranoj populaciji mikrobiote sira 'Tolminc' već je bila prikazana, a također i njihova antimikrobna aktivnost. Zbog poteškoća pri povezivanju detektiranih genskih determinanti za bakteriocine i antimikrobne aktivnosti, odlučeno je u ovom radu analizirati ista svojstva i kod pojedinačnih bakteriocinogenih sojeva. Slično prethodnim istraživanjima, enterokoki i njihovi bakteriocini najbolje su bili zastupljeni. Nijedan od izoliranih sojeva nije inhibirao bakteriju *Staphylococcus aureus*, dok su ostale indikatorske mikroorganizme inhibirali različito. Većina sojeva nosila je genske determinante za bakteriocin citolizin. Na temelju genskih determinanti za bakteriocine, antimikrobne aktivnosti, fenotipizacije s PhP (PhenePlate™) sistemom i identifikacije roda i vrste sojeva, mogu se naći neke sličnosti između *Enterococcus* sojeva.

Ključne riječi: tradicionalni sirevi, bakteriocinski geni, bakteriocini, enterocini, enterokoki, antimikrobna aktivnost

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