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Competitive advantage of bacteriocinogenic strains within lactic acid bacteria consortium of raw milk cheese

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Summary

The presence of gene determinants for different bacteriocins has been already demonstrated in traditional Slovenian types of raw milk cheeses 'Tolminc' and 'Kraški'. These genes were present also in the cultivable microbiota. In this research the aim was to establish how the presence of gene determinants for bacteriocins in microbial consortia is reflected in its antimicrobial activity. In addition, one of the goals was to determine whether the strains that carry gene determinants for bacteriocins have any competitive growth advantage in microbial population. Microbial consortium of 'Tolminc' cheese was propagated in milk and examined at the end of propagation its antimicrobial activity and the presence of gene determinants for bacteriocins. Comparison of the results obtained before and after propagation leaded to the conclusion that most of the strains possessing gene determinants for bacteriocins were unable to persist during propagation. The strains which did persist during propagation carried gene determinants for enterocins P, L50B and cytolysin. Antimicrobial activity of consortium before and after propagation was not substantially different and cannot be attributed to any of detected bacteriocins.

Key words: traditional cheese, bacteriocin genes, bacteriocins, antimicrobial activity, competition

Introduction

Major role of lactic acid bacteria (LAB) in dairy production is to assure desired sensory properties of fermented products. LAB also have a significant role in assuring stability and safety of these products. Food safety is becoming increasingly important issue especially in traditional food production where artisanal cheeses represent an important group (Caplice and Fitzgerald, 1999; Šušković et al., 2010). Artisanal cheeses are traditionally made from raw milk which brings a diverse microbiota into these cheeses. The rich microbial composition of Slovene raw milk cheeses has been already confirmed by Čanžek Majhenič et al. (2005, 2007). They established the presence of numerous strains of *Lactobacillus* and *Enterococcus* that exerted antimicrobial activity against pathogenic and spoilage bacteria (Trmčić et al., 2010). Among several groups of metabolites responsible for antimicrobial activity, bacteriocins present one of the most specific and interesting one (Šušković et al., 2010). The term 'bacteriocins' represents a large and heterogeneous group of ribosomally synthesised proteins or peptides that are produced by bacteria to suppress growth of other competing bacteria in the environment (DeVuyst in Vandamme, 1994; Cleveland et al., 2001). Since microbiota of raw milk cheeses is both numerous and rich in composition (Habeš, 2002), the presence of LAB producing different bacteriocins might be expected as well. Trmčić et al.

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(2008) have already found the answer to this question by analysing metagenomic DNA of cheeses for the presence of genetic determinants of 19 well known LAB bacteriocins. In nine samples of traditional Slovene cheeses ('Tolminc' and 'Kraški ovčji sir') there were 11 bacteriocin determinants identified. The same determinants were also identified in microbial consortia isolated from these cheeses confirming that the genes for 11 bacteriocins examined belonged to the cultivable population of LAB.

Trmčić et al. (2008) identified in 'Tolminc' cheese genetic determinants for enterocins A, B, P, L50A, L50B, cytolysin, nisin, lacticin 481, acidocin B, helveticin J and plantaricin A. Some of these LAB bacteriocins have wide spectra of activity which is not so common since the majority of bacteriocins are active against related strains only. Bacteriocins are supposed to provide the producer bacteria with a competitive advantage over the other bacteria in the population (Caplici and Fitzgerald, 1999). A prerequisite for such a performance is that bacteriocin genes are expressed and bacteriocins released to the environment in their active form. In the present work the aim was to establish whether the LAB strains from microbial consortia of 'Tolminc' cheese carrying previously identified gene determinants for different bacteriocins really exert a competitive advantage *in situ* in the mixed community. This would also indirectly indicate the expression of bacteriocin genes.

Materials and methods

The microbial consortia analysed in this study were isolated from fully ripened 'Tolminc' cheese (T2) produced from raw cow's milk according to Šabec (1952) and Perko et al. (2010). The lactobacilli were isolated from cheese using Rogosa agar plates incubated at 37 °C, while mesophilic cocci and thermophilic cocci from M17 agar plates incubated at 30 °C and 42 °C. Although M17 agar is primarily intended for lactococci, it supported also the growth of enterococci which are well represented in 'Tolminc' cheese. Before used in the simulated cheese production experiment, all original microbial consortia were also subjected to antimicrobial activity assays and total DNA isolation according to the procedures described below.

Original consortia of lactobacilli, mesophilic cocci and thermophilic cocci were subcultivated in MRS (37 °C), M17 (30 °C) and M17 (42 °C) broths, respectively. Equal parts of all cultivated consortia were combined together into a mixed consortium and 500 μL of it was used for inoculation of two 50 mL aliquots of 10 % reconstituted milk. The first aliquot was incubated at 30 °C and another at 42 °C. With these two temperatures of cultivation milk coagulation and scalding temperatures were simulated, respectively. After 24 h of fermentation, both cultures were transferred (1 %) to fresh reconstituted milk, and this procedure was repeated every day for ten days. After the fifth and the tenth transfer the fermented milk were sampled for isolation of different microbial consortia and for the extraction of total DNA. The isolation of microbial consortia was performed identically as above described isolation of original consortia from cheese, with one modification. Besides using Rogosa agar (37 °C) and M17 agar (30 °C, 42 °C) this time also CATC agar (37 °C) was used which is selective for *Enterococcus* genus. Determination of antimicrobial activity was performed for each isolated microbial consortia separately. Total DNA was extracted from combined microbial consortia which consisted of consortia from all selective growth media and both fermentation temperatures (30 °C, 42 °C). The DNA was also extracted from the original microbial consortia of cheese used to initiate the fermentation experiment.

Extraction of total DNA directly from fermented milk (10 mL) or from isolated microbial consortia (1 mL) was performed using Maxwell™ 16 System (Promega) together with reagent kit Maxwell™ 16 Cell DNA Purification Kit (Promega). Bacteria were collected by centrifugation (6000 x g, 10 min, 4 °C) and the pellet transferred to lysis solution (50 mM EDTA, 10 mg/mL lysozyme, 25 U/mL mutanolysin) for 1 h pre-treatment at 37 °C. The PCR amplifications of parts of 10 known bacteriocins' genes (Table 1) were performed using specific oligonucleotide primers and procedures described by Trmčić et al. (2008).

For determination of antimicrobial activity spectra of original microbial consortia from cheese 36 different indicator strains were used: 20 *Staphylococcus aureus*, 7 *Lactobacillus* sp., 3 *Enterococcus* sp., 2 *Clostridium* sp., 2 *Listeria* sp. and one *Escherichia coli* and one *Bacillus cereus*. The origin of this indicator strains is of human, animal, milk and

cheese source. For testing of antimicrobial activity of consortia obtained after the consecutive propagation in milk, 10 indicator strains were used selected on the basis of testing of original microbial consortia. Antimicrobial activity was tested by conventional spot-test assay using cultivated microbial consortia, culture supernatants, neutralised culture supernatants and lyophilised supernatants (25-fold concentrates). The spot-test assay with microbiota cultures was performed according to Cogan et al. (1997). The base agar layer was adapted to each microbial consortia type (M17, MRS agar) and a modified MRS agar which contained 10 % of usual D-glucose concentration was also used (Jacobsen et al., 1999). The soft cover layer of agar was adapted to indicator strain used, MRS agar for lactobacilli and M17 agar for all other indicator strains. In the spot-tests with supernatants, the supernatants were applied (3 times 5 μL) onto the overlay of soft agar inoculated with the indicator strain. For determination of proteinaceous nature of inhibitor, the proteolytic enzyme proteinase K (10 mg/mL) was applied (3 μL) next to the spot of microbial consortia or supernatant.

Results

Examination of antimicrobial activity of original three microbial consortia isolated from 'Tolminc' cheese (T2), using Rogosa agar (37 °C) or M17 agar (30 °C or 42 °C) against 36 indicator strains did not reveal an inhibition caused by bacteriocin activity. The strongest antimicrobial activity was detected with microbial consortium represented by lactobacilli (from Rogosa agar). The inhibition halos observed on MRS agar did not have a well defined sharp edge typical of inhibition caused by bacteriocins. Since inhibition halos were not detected when cultures were tested on modified MRS agar or when neutralised supernatants were applied, the inhibition was likely caused by organic acids produced by LAB during the growth. Moderate inhibition of *Ec. durans* CCM 5612 and *Ec. faecium* CCM 4647 by mesophilic cocci consortium (from M17 agar at 30 °C) was observed. There was also a slight reduction of these inhibitions when proteinase K was applied, which indicates a possible involvement of antimicrobial substance of proteinaceous nature. The concentration of antimicrobial substance in the supernatant (untreated or treated) however was not sufficient to be observed by the antimicrobial assay.

The main goal of this work was to examine whether the strains that carry bacteriocins' genes have growth advantage in mixed population at given conditions and which bacteriocins could be eventually involved. Results of the total DNA analysis during consecutive transfers of microbial consortia in milk are presented in Table 1. Consecutive subculturing at 30 °C resulted in the reduction of the number of gene determinants detected in milk. Namely,

Table 1. Comparison of the presence of different bacteriocin gene determinants during propagation of microbial consortia in milk

	Propagation temperature	Number of transfers in propagation	Presence of gene determinant for bacteriocin									
			Enterocin						Nisin	Lacticin 481	Acidocin B	Plantaricin A
			A	B	P	L50A	L50B	Cytolysin				
FM	30 °C	5x	-	-	+	-	-	+	-	-	-	-
		10x	-	-	-	-	-	-	-	-	-	-
	42 °C	5x	-	-	+	-	-	-	-	-	-	-
		10x	-	-	+	-	-	+	-	-	-	-
MC	X	0x	+	+	+	+	+	+	-	+	-	+
	30 °C + 42 °C	5x	-	-	+	-	+	+	-	-	-	-
		10x	-	-	+	-	+	+	-	-	-	-

FM - DNA extracted directly from fermented milk during propagation of microbial consortia; MC - DNA extracted from isolated microbial consortia of cheese and fermented milk during its propagation; X - Isolated microbial consortia before propagation; + - PCR product of corresponding length is present; - – PCR product of corresponding length is not present

after five passages only two bacteriocins (enterocin P and cytolysin) of ten tested were detected, and after ten passages none of them. Gene determinants for the same two bacteriocins, enterocin P and cytolysin were however still detected after ten subculturing at 42 °C. When DNA was isolated from the microbial cells collected after plating fermented milk on three different agars (Rogosa, M17, CATC) to obtain four different consortia, gene determinants for enterocin L50B were detected after fifth as well as after tenth passage, in addition to enterocin P and cytolysin genes detected also directly in milk. Analysis of the DNA extracted from original microbial consortia isolated from cheese 'Tolminc' revealed positive results for eight out of ten tested bacteriocins which confirm results obtained previously by Trmčić et al. (2008).

After each subculturing of fermented milk, it was plated onto Rogosa, CATC or M17 agar, the latter incubated at 30 °C and 42 °C in order to obtain four microbial consortia which were tested for antimicrobial activity against ten indicator strains. The results of antimicrobial assays are presented in Table 2. After ten consecutive transfers of fermented milk incubated at 42 °C, it was not possible to detect any enterococci (CATC) or other mesophilic cocci (M17, 30 °C), while lactobacilli and thermophilic cocci were found at all samplings. All lactobacilli

Table 2. Comparison of antimicrobial activities of isolated microbial consortia during propagation in milk

Isolated microbial consortia	Propagation at 30 °C										Propagation at 42 °C									
	5 transfers					10 transfers					5 transfers					10 transfers				
	Ec.	*Lb.*		MK	TK	*Ec.*	*Lb.*		MK	TK	*Ec.*	*Lb.*		MK	TK	*Ec.*	*Lb.*		MK	TK
		M	m				M	m				M	m				M	m		
L. sakei ATCC 15521	0	0	0	0	0	0	0	0	1p	0	0	0	0	0	0	/	/	1	/	9*
L. monocytogenes N°10 S 4ab	0	10	3	0	0	6	15	2	1p	0	0	7	2	0	0	/	5	3	/	9
L. innocua ATCC 33090	0	6	0	0	0	3	15	2	9	0	3	2	0	0	0	/	7	3	/	1
E. faecalis ATCC 19433	0	/	0	0	0	0	/	0	0	0	0	/	0	0	0	/	/	0	/	0
E. faecium CCM 4647	0	/	2	0	0	1*	/	0	1*	0	0	/	0	0	0	/	/	0	/	1
E. durans CCM 5612	0	/	0	0	0	4*	/	0	2*	0	0	/	0	0	0	/	/	0	/	0
S. aureus ISS 465	0	6	0	0	0	0	15	0	0	0	0	11	2*	0	0	/	2	0	/	0
S. aureus ISS 464	0	4	0	0	0	0	10	0	0	0	0	12	1*	0	0	/	5	0	/	7*
S. aureus ISS 23	0	3	0	0	0	0	2	0	0	0	0	3	1*	0	0	/	1	0	/	0
S. aureus Č.Sa.4.2	0	2	0	0	0	0	2	0	0	0	0	4	0	0	0	/	3	1*	/	0

M - Spot-test performed on regular MRS; m - Spot-test performed on modified MRS; *Ec.* - Enterococci; *Lb.* - Lactobacilli; MK - Mesophilic cocci; TK - Termophilic cocci; 0-15 - Inhibition halo size measured from outside edge of spot to outside edge of inhibition (mm); * - Clear halo with well defined edge; p - Double zone of inhibition (5+1 mm), proteinase K effective only in smaller one; / - Test was not performed

consortia examined exerted inhibition on MRS plates, but no inhibition or weak inhibition was observed on a modified MRS medium. In some cases, inhibition on modified MRS was not observed when the test was repeated. Most of the clear and sharp inhibition halos were observed with the samples obtained after ten passages, but they were not reduced by proteinases. The only microbial consortium that showed an antimicrobial activity sensitive to the activity of proteolytic enzymes was the mesophilic cocci consortium (M17, 30 °C) after ten transfers at 30 °C. The activity of the latter was also present against *L. monocytogenes* where two inhibition halos were observed; the larger one was 5 mm wide and was not affected by the proteinase K while the clear small 1 mm halo with sharp edge was reduced in the presence of this enzyme. In the assay with *Lb. sakei* indicator strain only the small 1mm inhibition halo was present which was affected by proteinase K.

Discussion

The number of genetic determinants for bacteriocins in the total DNA from microbial consortia was reduced from initial nine to three during consecutive transfers in milk. This indicates that the strains carrying majority of bacteriocin determinants were not able to persist in the microbiota or that their ratio was significantly decreased. On the other side the strains that carried genes for enterocin P, L50B and cytolysin were able to persist during all transfer steps. Enterocin L50B is a bacteriocin that is produced exclusively by *Ec. faecium* while enterocin P can be produced not only by *Ec. faecium* but also by *Ec. durans*. Although there are some reports that some *Ec. faecium* representatives can produce cytolysin, its production is more characteristic for *Ec. faecalis* species (De Vuyst et al., 2003). Besides bacteriolytic activity cytolysin has also hemolytic properties which are the reason that this bacteriocin is considered a virulence factor and cytolysin-producing strains undesired in food products. The reason for the predominance of enterococci in the cheese microbiota examined in this study is the high initial concentration of this genus in the original microbial consortium from 'Tolminc' cheese used. In addition, it is well known from the studies of microbial dynamic in cheese that concentration of enterococci is elevated during the initial stages of cheese production. Later during the ripening period the enterococci are overtaken by lactobacilli. With each subsequent transfer in milk the ratio of enterococci in the fermented milk microbiota increased and the number of lactobacilli decreased. This may be the reason why in the fermented milk there was an absence of genes for plantaricin A. Genetic determinants for plantaricin A, a bacteriocin produced by *Lb. plantarum*, were otherwise the most frequently detected bacteriocin genes in 'Tolminc' cheese (Trmčić et al., 2008). Besides observed differences in the presence of bacteriocin genes, the antimicrobial activity of microbial consortia was also changes during the consecutive propagation in milk. Although a possible bacteriocin activity against *Enterococcus* was detected, indicator strains were not able to confirm the same in propagated consortia. The only inhibition that was reduced by proteinase-K action was against indicator strains *Lb. sakei* and *L. monocytogenes*. This inhibition may hardly be attributed to enterocins, since the inhibition was not observed with enterococci consortium obtained from the CATC selective medium. However it should also be considered the observation of Reuter (1992) who reported a poor growth of *Ec. faecium* strains on CATC medium.

Therefore the *Ec. faecium* produced bacteriocins could also be involved in the inhibition observed in this study even though their genetic determinants were not detected in isolated enterococci consortia. Analysis of DNA extracted directly from fermented milks indicates that enterococci preferred temperature 42 °C over 30 °C which is probably related to thermodynamic properties of their metabolic processes, since some species of this genera can grow at both of this temperatures. This fact was confirmed by the results of antimicrobial activity testing. If inhibition observed in the spot-test assays was a result of the activity of enterococci then it can be concluded that on M17 growth media enterococci prefer temperature of 30 °C which is the opposite as observed in milk fermentations. The results lead to the conclusion that the observed antimicrobial activity of mesophilic microbial consortium (M17, 30 °C) was predominantly a result of the activity of bacteriocins not targeted in this study.

Conclusions

The results of this study may lead to the conclusion that some LAB derived from 'Tolminc' cheese which are carrying gene determinants for bacteriocins can persist in the mixed microbial cheese consortia however the possession of bacteriocins' genes does not assure them a significant competitive advantage and consequently a better survival. In this study it was established that *Enterococcus* bacteriocinogenic strains were able to persist in the population which most certainly has to do with the nature of these bacteria and the experimental conditions selected for this study. It was however not possible to attribute the antimicrobial activity observed in spot-test assays, to enterococci that were able to maintain their presence in the microbiota during consecutive propagation in milk. There is a possibility that inhibition observed was caused by the strains that carry gene determinants for bacteriocins that were not examined in the study, or even by the cytolysin producers which are for the safety reasons not acceptable for the use in food production.

Kompetitivna prednost bakteriocinogenih sojeva konzorcija bakterija mliječne kiseline izoliranih iz sira proizvedenog od sirovog mlijeka

Sažetak

Prisutnost genskih determinanti za stvaranje različitih vrsta bakteriocina već je ranijim istraživanjima utvrđena u slovenskim ovčjim sirevima "Tolminac" i "Kraški sir" koji se proizvode na tradicionalan način. Isti geni za izoliranu mikrobnu populaciju (konzorcij) također su potvrđeni i u kultiviranim uvjetima. U ovom radu pokušalo se utvrditi kako se prisutnost tih genskih determinanti u mikrobnom konzorciju odražava na njegovu antimikrobnu aktivnost. Osim toga, utvrđivao se i utjecaj bakterijskih sojeva koji nose gene za stvaranje bakteriocina na kompetitivnost rasta u mješovitoj populaciji. Mikrobni konzorcij izoliran iz sira "Tolminac" propagiran je u mlijeku 10 dana, nakon čega je utvrđena antimikrobna aktivnost kulture i prisutnost genskih determinanti za stvaranje bakteriocina. Usporedbom rezultata za sposobnost stvaranja bakteriocina prije i nakon precjepljivanja u mlijeku, utvrđen je gubitak te sposobnosti za većinu izoliranih sojeva. Sojevi koji su izdržali ponovljena precjepljivanja u mlijeku nosili su genske determinante za bakteriocine: enterocin P, enterocin L50B i citolizin. Antimikrobna aktivnost konzorcija prije i nakon precjepljivanja nije se značajno razlikovala te se ne može pripisati niti jednom od u ovom pokusu potvrđenih bakteriocina.

Ključne riječi: tradicionalni sir, bakteriocinski geni, bakteriocini, antimikrobna aktivnost, kompeticija

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