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DETECTION OF LACTIC ACID BACTERIA BACTERIOCINS' GENES AND THEIR EXPRESSION IN CHEESE

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DETECTION OF LACTIC ACID BACTERIA BACTERIOCINS' GENES AND THEIR EXPRESSION IN CHEESE

DOCTORAL DISSERTATION

ODKRIVANJE GENOV ZA BAKTERIOCINE MLEČNOKISLINSKIH BAKTERIJ IN NJIHOVO IZRAŽANJE V SIRU

DOKTORSKA DISERTACIJA

DETECTION ET ETUDE DE L'EXPRESSION DE GENES DE BACTERIOCINES DE BACTERIES LACTIQUES DANS LES FROMAGES

THÈSE DE DOCTORAT

Ljubljana, 2011

DETECTION ET ETUDE DE L'EXPRESSION DE GENES DE BACTERIOCINES DE BACTERIES LACTIQUES DANS LES FROMAGES

Nous avons étudié la présence de bactéries lactiques capables de produire des bactériocines dans des fromages traditionnels Slovènes. Dans cinq échantillons de Tolminc (fromage au lait de vache) et quatre échantillons de Kraški ovčji sir (fromage au lait de brebis), nous avons recherché la présence de 19 gènes de bactériocines par PCR. L'ADN a été extrait directement à partir des fromages ainsi qu'à partir de consortia microbiens de ces fromages isolés sur gélose CATC, Rogosa et M17. Nous avons utilisé des tests par diffusion sur gélose pour déterminer l'activité antimicrobienne des consortia sur différentes bactéries indicatrices. L'activité anti-staphylococcique a aussi été déterminée *in situ* dans du lait et du fromage. L'avantage compétitif des souches productrices de bactériocines a été évalué en effectuant des repiquages successifs des consortia dans du lait. Lors de ces essais, nous avons suivi la présence de différents déterminants génétiques de bactériocines ainsi que l'activité antimicrobienne des consortia. Ces mêmes propriétés ont aussi été déterminées pour des souches individuelles productrices de bactériocines, qui ont été isolées du consortium initial ou de consortia obtenus après propagation dans le lait. La mise en évidence de gènes de bactériocines par PCR dépendait de l'efficacité d'extraction de l'ADN. L'extraction de l'ADN total était plus efficace lorsque l'homogénéisation du fromage était réalisée avec une solution de citrate de sodium. Dans l'ADN issu des fromages et consortia microbiens, nous avons détecté plusieurs gènes de bactériocines. Leur nombre diminuait lors de la propagation dans le lait, ce qui indique un faible avantage compétitif des souches productrices de bactériocines. Les résultats n'ont montré un avantage compétitif que pour des souches comportant des déterminants génétiques de la cytolysine. Nous n'avons détecté une production de bactériocines dans les fromages que lorsque qu'une culture pure d'une souche productrice de nisine A a été ajoutée. La différence entre la détection de déterminants génétiques de bactériocines et l'activité antimicrobienne montre la complexité qui relie ces deux propriétés. En raison des limitations concernant les tests par diffusion sur gélose pour détecter l'expression des gènes *in situ*, nous avons mis en place une méthode moléculaire basée sur la reverse transcription et la PCR en temps réel. Cette méthode sera très utile pour de futures études concernant le rôle des bactériocines dans les fromages.

produits laitiers/fromages traditionnels/Slovénie/microbiologie/bactéries lactiques/bactériocines/génétique moléculaire/genes/expression de gènes

On the basis of the Statute of the University of Ljubljana and according to the decisions of the Senate of the Biotechnical Faculty and the University Senate dating 31st of May 2010, it has been confirmed that the candidate fulfils all the conditions for direct transition to Doctorate Postgraduate Study of Biological and Biotechnical Science and acquiring a PhD in Biotechnology. Sen. Res. Sci. Bojana Bogovič Matijašić, PhD has been appointed as his supervisor and Prof. Irena Rogelj, PhD as co-supervisor.

On the basis of the Agreement signed on 19th of June 2009 between the University of Ljubljana and AgroParisTech the research carried out by Aljosa Trmcic was guided by the appointed Thesis Directors: Sen. Res. Sci. Bojana Bogovič Matijašić, PhD and Prof. Irena Rogelj, PhD, representing the University of Ljubljana and Christophe Monnet, PhD, representing AgroParisTech. Furthermore, in accordance with the Agreement, representatives of the University of Ljubljana and of AgroParisTech were appointed to the Commission on doctoral dissertation assessment and defence. Copies of doctoral dissertation are deposited in University of Ljubljana and AgroParisTech according to their rules.

Commission on doctoral dissertation assessment and defence:

Chairman:	Prof. Peter RASPOR, PhD University of Ljubljana, Biotechnical faculty, Slovenia
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Doctoral dissertation is a result of personal research work.

Aljoša Trmčić

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne 31.5.2010 je bilo potrjeno, da kandidat izpolnjuje pogoje za neposreden prehod na doktorski Podiplomski študij bioloških in biotehniških znanosti ter za opravljanje doktorata znanosti s področja biotehnologije. Za mentorico je bila imenovana viš. znan. sod. dr. Bojana Bogovič Matijašić in za somentorico prof. dr. Irena Rogelj.

Na osnovi sporazuma, ki je bil dne 19.6.2009 sklenjen med Univerzo v Ljubljani in AgroParisTech, je bilo raziskovalno delo, ki ga je izvajal Aljoša Trmčić, vodeno s strani imenovanih svetovalcev: viš. znan. sod. dr. Bojana Bogovič Matijašić in prof. dr. Irena Rogelj, kot predstavnici Univerze v Ljubljani in dr. Christophe Monnet, kot predstavnik AgroParisTech. Prav tako skladno s sporazumom so v komisijo za oceno in zagovor bili imenovani predstavniki Univerze v Ljubljani in AgroParisTech. Izvodi doktorske disertacije so predloženi na Univerzi v Ljubljani in AgroParisTech v skladu z njihovimi pravili.

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AB We examined the presence of lactic acid bacteria (LAB) able to produce different bacteriocins in traditional Slovenian cheeses. In five samples of Tolminc cheese (from cows' milk) and four samples of Kraški ovčji sir (from ewes' milk) we looked for the presence of 19 LAB bacteriocins' gene determinants using PCR. The DNA analysed was extracted directly from cheese samples as well as from cultivable microbial consortia of cheese which were isolated on CATC, Rogosa and M17 agar media. We used diffusion tests to determine the antimicrobial activity of microbial consortia against different indicator bacteria. Anti-staphylococcal activity was also determined *in situ* in milk and cheese. Competitive advantage of bacteriocinogenic strains was examined by consecutive propagation of microbial consortia in milk. During this we followed the presence of different bacteriocin gene determinants and antimicrobial activity of microbial consortia. The same properties were also determined for individual bacteriocinogenic strains that were isolated from initial consortia and from consortia obtained after propagation in milk. The success of finding bacteriocin gene determinants with PCR depended greatly on the efficiency of DNA extraction. The extraction of total DNA was the most successful when sodium citrate was used for cheese homogenisation. In DNA of different cheeses and microbial consortia we detected a number of bacteriocin gene determinants. Their numbers were reduced during the process of propagation in milk, which indicates a poor competitive advantage of some bacteriocinogenic strains. The results demonstrated competitive advantage only for strains that carried gene determinants for cytolysin. We detected bacteriocin activity in cheese only when monoculture of nisin A producer was added. The discrepancy between detected bacteriocin gene determinants and antimicrobial activity shows the complexity that links these two properties. Since there are limitations in using bioassay methods for detection of *in situ* expression of bacteriocin genes in cheese we decided to introduce an alternative molecular method based on reverse transcription (RT) and real-time PCR. This method will be very useful in the future studies of the role of bacteriocins in cheese.

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AI V tradicionalnih slovenskih sirih smo iskali prisotnost sevov mlečnokislinskih bakterij (MKB), sposobnih proizvajati različne bakteriocine. V petih vzorcih sira Tolminc iz kravjega mleka in štirih vzorcih Kraškega ovčjega sira smo s pomočjo PCR ugotavljali prisotnost genskih determinant za 19 bakteriocinov MKB. Genske determinante smo iskali tako v skupni DNA izolirani neposredno iz sira, kot v DNA, ki smo jo izolirali iz osamljene mikrobne združbe. S trdnih gojišč CATC, Rogosa ter M17 smo osamili različne kultivabilne mikrobne združbe posameznih sirov. Z difuzijskimi testi *in vitro* smo ugotavljali njihovo protimikrobno aktivnost proti različnim mikroorganizmom. Aktivnost proti stafilokokom smo proučili tudi *in situ* v mleku in v siru. Kompetitivno prednost bakteriocinogenih sevov smo preverili z zaporednim precepljanjem mikrobne združbe enega od sirov v mleku, pri čemer smo spremljali prisotnost genskih determinant za bakteriocine in protimikrobno aktivnost mikrobne združbe. Iste lastnosti smo ugotavljali tudi pri posameznih bakteriocinogenih sevih, ki smo jih osamili iz prvotne mikrobne združbe in mikrobne združbe, pridobljene po zaporednem precepljanju v mleku. Uspešnost iskanja genskih determinant za bakteriocine s pomočjo PCR je bila v veliki meri odvisna od učinkovitosti izolacije DNA. K učinkoviti izolaciji DNA iz sira je prispevala uporaba natrijevega citrata za homogenizacijo vzorcev. V DNA različnih sirov in osamljenih mikrobnih združb smo našli številne genske determinante za bakteriocine. Tekom zaporednega precepljanja v mleku se je število teh determinant zmanjšalo, kar kaže na slabšo kompetitivno prednost nekaterih bakteriocinogenih sevov. Rezultati so pokazali kompetitivno prednost edino pri sevih s genskimi determinantami za citolizin. V siru smo zasledili bakteriocinsko aktivnost samo v primeru, ko je bila siru dodana monokultura seva, ki proizvaja nizin A. Diskrepanca med številom ugotovljenih genskih determinant in izkazano protimikrobno aktivnostjo kaže na kompleksnost, ki obe lastnosti povezuje. Ker obstajajo omejitve pri uporabi bioloških testov za določanje *in situ* izražanja bakteriocinskih genov v siru, smo se odločili vpeljati alternativno molekularno metodo na osnovi RT real-time PCR. Ta metoda bo zelo uporabna za nadaljnje študije vloge bakteriocinov v siru.

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Priloga B: Protimikrobna aktivnost osamljenih mikrobnih združb sira T2 in njihovih koncentriranih supernatantov, dobljenih po kultivaciji v tekočih gojiščih (MRS, M17)

Annex C: Antimicrobial activity of isolated microbial consortia of cheese K3 and its concentrated supernatants, obtained after cultivation in liquid growth media (MRS, M17)
Priloga C: Protimikrobna aktivnost osamljenih mikrobnih združb sira K3 in njihovih koncentriranih supernatantov, dobljenih po kultivaciji v tekočih gojiščih (MRS, M17)

Annex D: Average counts of microorganisms in milk during propagation of T2 cheese consortia.
Priloga D: Povprečno število mikroorganizmov v mleku tekom zaporednega precepljanja mikrobne združbe sira T2.

Annex E: Antimicrobial activity of propagated mesophilic microbial consortia (ten transfers at 30°C) against *L. monocytogenes* N°10 S 4ab.
Priloga E: Protimikrobna aktivnost zaporedno precepljane mezofilne mikrobne združbe (deset precepljanj pri 30°C) proti *L. monocytogenes* N°10 S 4ab

Annex F: Antimicrobial activity of bacteriocinogenic strains *Lac. lactis* ssp. *lactis* M 78 and *E. faecium* ATCC 19434.
Priloga F: Protimikrobna aktivnost bakteriocinogenih sevov *Lac. lactis* ssp. *lactis* M 78 in *E. faecium* ATCC 19434.

Annex G: Antimicrobial activity of isolated strains against *Ec. faecium* ATCC 19434.
Priloga G: Protimikrobna aktivnost osamljenih sevov proti *Ec. faecium* ATCC 19434.

Annex H: Antimicrobial activity of isolate G0x42/9 against *L. monocytogenes* N°10 S 4ab.
Priloga H: Protimikrobna aktivnost izolata G0x42/9 proti *L. monocytogenes* N°10 S 4ab.

Annex I: Antimicrobial activity of isolate G0xR/1 against *E. durans* CCM 5612.
Priloga I: Protimikrobna aktivnost izolata G0xR/1 proti *E. durans* CCM 5612.

Annex J: Growth of isolated strains on solid CATC media.
Priloga J: Rast osamljenih sevov na trdnem gojišču CATC.

Annex K: *Lactococcus lactis* subsp. *lactis* M 78 nisin biosynthetic gene cluster, complete sequence
Priloga K: Skupek genov za biosintezo nizina pri sevu *Lactococcus lactis* subsp. *lactis* M 78, celotna sekvenca

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Priloga L: Celoten skupek genov nizina A pri sevu *Lactococcus lactis* M 78 (HM219853)-pridobitev nukleotidnega zaporedja in primerjava z ostalimi objavljenimi sekvencami nizina (Trmčić in sod., 2011c)

ABBREVIATIONS AND SYMBOLS
(OKRAJŠAVE IN SIMBOLI)

31	enterocin 31
A	enterocin A
A	adenine (adenin)
ABC	ATP binding cassette (ATP vezavna kaseta)
AcidA	acidocin A
AcidB	acidocin B
ANOVA	analysis of variance (analiza variance)
AS48	enterocin AS48
ATCC	American Type Culture Collection (Ameriška zbirka tipskih kultur)
ATP	adenosine 5'-triphosphate (adenozin 5'-trifosfat)
B	enterocin B
B.	*Bacillus*
BCCM	Belgian Co-ordinated Collections of Micro-organisms
BHI	brain heart broth (ime gojišča)
BLAST	Basic Local Alignment Search Tool
BMK	lactic acid bacteria (mlečnokislinske bakterije)
BP	Baird Parker (ime gojišča)
bp	base pair (bazni par)
C	cytosine (citozin)
C.	*Clostridium*
CATC	Citrate Azid Tween Carbonate (gojišče citrat azid tween karbonat)
CCM	Czech Collection of Microorganisms (Češka zbirka mikroorganizmov)
cDNA	complementary DNA (komplementarna DNA)
cfu	colony forming unit (kolonijska enota)
C_q	cycle of quantification (cikel kvantifikacije)
CurA	curvacin A (kurvacin A)
Cyl	cytolysin (citolizin)
DNA	deoxyribonucleic acid (deoksiribonukleinska kislina)
dNTP	deoxyribonucleoside-5'- triphosphate (deoksinukleozid-5'- trifosfat)
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen (Nemška zbirka mikroorganizmov in celičnih kultur)
E.	*Enterococcus*
E.	*Escherichia*
Ec.	*Enterococcus*
EDTA	ethylenediaminetetraacetic acid (etilendiaminotetraocetna kislina)
f	forward (naprej)
G	guanine (gvanin)

HelvJ	helveticin J
IM	Microbial Collection of Chair of Dairy Science, Biotechnical Faculty, University of Ljubljana (Mikrobna zbirka Katedre za mlekarstvo, Biotehniška fakulteta, Univerza v Ljubljani)
ISS	Microbial Collection of National Agricultural Research Foundation, Greece (Mikrobna zbirka National Agricultural Research Foundation, Grčija)
IU	international unit (mednarodna enota)
K	Kraški cheese (Kraški ovčji sir)
ke	colony forming unit (kolonijska enota)
L.	*Lactococcus*
L.	*Listeria*
L50A	enterocin L50A
L50B	enterocin L50B
LAB	lactic acid bacteria (mlečnokislinske bakterije)
Lac.	*Lactococcus*
Lac481	lacticin 481 (lakticin 481)
Lac705	lactocin 705 (laktocin 705)
Lb.	*Lactobacillus*
LccA	lactococcin A (laktokokcin A)
LMG	Bacteria collection of Belgian Co-ordinated Collections of Micro-organisms
MKB	lactic acid bacteria (mlečnokislinske bakterije)
mRNA	messenger RNA (infomacijska RNA)
MRS	De Man, Rogosa, Sharp (ime gojišča)
N	nucleotide (nukleotid)
NCBI	National Centre for Biotechnology Information
NCDO	National Collection of Dairy Organisms
Nis	nisin (nizin)
nisA	gene encoding peptide NisA (gen, ki kodira peptid NisA)
NisA	nisin A structural peptide (strukturni peptid nizina A)
nisB	gene encoding protein NisB (gen, ki kodira protein NisB)
NisB	dehydratase (dehidrataza)
nisC	gene encoding protein NisC (gen, ki kodira protein NisC)
NisC	cyclization enzyme (ciklizacijski encim)
nisE	gene encoding protein NisE (gen, ki kodira protein NisE)
NisE	immunity protein, ABC transporter (protein imunosti, ABC- prenašalec)
nisF	gene encoding protein NisF (gen, ki kodira protein NisF)
NisF	immunity protein, ABC transporter (protein imunosti, ABC-prenašalec)
nisG	gene encoding protein NisG (gen, ki kodira protein NisG)

NisG	immunity protein, ABC transporter (protein imunosti, ABC-prenašalec)
nisI	gene encoding protein NisI (gen, ki kodira protein NisI)
NisI	immunity protein (protein imunosti)
nisK	gene encoding protein NisK (gen, ki kodira protein NisK)
NisK	histidine kinase (histidin kinaza)
nisP	gene encoding protein NisP (gen, ki kodira protein NisP)
NisP	protease, activation protein (proteaza, aktivacijski protein)
nisR	gene encoding protein NisR (gen, ki kodira protein NisR)
NisR	response regulator protein (regulator odziva)
nisT	gene encoding protein NisT (gen, ki kodira protein NisT)
NisT	transport protein (transportni protein)
P	enterocin P
PCR	polymerase chain reaction (verižna reakcija s polimerazo)
PhP	PhenePlate^{TM} system (sistem PhenePlate^{TM})
PlaS	plantaricin S
PlnA	plantaricin A
qPCR	quantitative PCR (kvantitativna PCR)
r	reverse (nazaj)
RCM	reinforced clostridial medium (ime gojišča)
RIF	rimfapicin
RNA	ribonucleic acid (ribonukleinska kislina)
RPF	rabbit plasma fibrinogen (fibrinogen iz zajčje plazme)
rpm	rounds per minute (obratov na minuto)
rRNA	ribosomal RNA (ribosomska RNA)
RT	reverse transcription (obratna transkripcija)
RTD	Research and Technology Development
S.	*Staphylococcus*
SakP	sakacin P
ssp.	subspecies (podvrsta)
Staph.	*Staphylococcus*
Str.	*Streptococcus*
T	Tolminc cheese (Tolminc)
T	thymine (timin)
TRUEFOOD	Traditional United European Food
vol.	volume (volumen)
wt.	weight (masa)

1 GENERAL INTRODUCTION (SPLOŠNI UVOD)

1.1 GENERAL INTRODUCTION

Research results presented in this doctoral dissertation were partly obtained under the frame of a three-year applied research project 'Tailor-made starter cultures for production of traditional cheeses' and a four-year European project TRUEFOOD (Traditional United Europe Food), conducted under the 6th framework programme of the European Union. The joint goal of the TRUEFOOD project was to introduce suitable innovations into traditional European food production which would assure its persistence and competitiveness in the ever growing global European market. TRUEFOOD was directed towards small and medium enterprises which on one side represent the majority of the European food industry and on the other usually lack the access to good quality research and development work. One of the major challenges in traditional food production is the introduction of innovations that would meet the EU safety demands and consumer expectations for traditional food product. Balancing both is sometimes difficult. The product has to be microbiologically completely safe but still of high nutritional and sensory value with little or no processing and additives. This is even more difficult for small and medium enterprises because the financial and space limitations make it very difficult for them to establish a system of good microbiological and chemical monitoring (http://www.truefood.eu; 2010). The problem our group was working on inside the project was microbiological safety of traditional Slovenian hard cheeses, focusing on inhibition of bacterium *Staphylococcus* (*S.*) *aureus* in these cheeses. We tried to isolate the microbial consortia or single strains that would inhibit this pathogen and could be used as starter or protective cultures.

1.1.1 Introduction

Most of the traditional cheeses and milk products in general are made from raw milk. Its chemical and microbiological composition is, besides the technical procedure, the most important parameter in establishing characteristic features of a dairy product. Rich microbiota which comes from raw milk and cheese producing equipment gives the specific taste and aroma by which the traditional cheeses are well recognised and differentiated from the other cheeses. Besides beneficial microbiota, raw milk and the end products made from it also contain strains that can represent a potential risk for the consumer. Because of these, the main problem associated with these kind of products is how to offer a product with expected, specific sensory properties but still protect the consumer against potential infection or intoxication.

The use of protective starter cultures containing selected bacteriocinogenic strains of lactic acid bacteria (LAB) is already established in the cheese production. There are two problems concerning use of commercial starter or protective culture in traditional cheese production. The first one is that with starter culture you are adding strains that are not autochthonous to this cheese. The second problem is that the bacteriocinogenic activity of these strains may inhibit the naturally present beneficial microbiota and thus affect the microbial composition and physicochemical properties of the cheese. One of approaches to solve this problem is to search for the suitable bacteriocinogenic strains in the microbial population of different traditional cheeses. This way we could formulate starter cultures that are 'tailor made' for each traditional cheese. Using starter cultures like these would solve most of the problems associated with the use of traditional procedures (raw milk) and also preserve all of the specific properties of traditional cheeses including the sensory ones.

Analysing microbial population of traditional cheeses and screening for particular strains that produce different wide spectrum bacteriocins is a demanding procedure that takes a lot of time and material and the end result depends a lot on sensitive indicator strains and proteolytic enzymes being used. Our idea was to use the approach usually used in metagenomics. We extracted the total deoxyribonucleic acid (DNA) from individual traditional Slovenian cheeses and analysed it by polymerase chain reaction (PCR) for the presence of different bacteriocins' genes.

The presence of gene determinants for particular bacteriocin in total DNA of cheese however does not assures that these determinants come from cultivable or at least viable bacteria. Since only the cultivable strains are the ones that can be isolated and used as a component in starter culture, our next research challenge was to confirm the presence of cultivable bacteriocinogenic LAB strains in the cheese microbiota. Individual groups of LAB were isolated on selective agar plates and in total DNA extracted form these groups we again looked for the presence of different bacteriocin gene determinants.

The presence of particular bacteriocin gene determinants in DNA of cultivable LAB does not assure these determinants are also expressed in cheese. Only strains that actually produce bacteriocins in cheese are suitable for application as protective cultures with the potential to improve the safety of these products. That is why we tested *in vitro* as well as *in situ* the bacteriocinogenic activity of individual microbial consortia. *In vitro* testing of bacteriocin activity of microbial consortia, single strains and cell-free supernatants were performed by diffusion method on solid growth media. In addition, selected microbial consortia were applied *in situ* in cheese production and cheese extracts were tested for antimicrobial activity.

Demonstrating synthesis and activity of bacteriocins in cheese using bioassays has its limitations like high detection level, uneven distribution of bacteriocins in the cheese matrix, adsorption to cheese caseins or degradation by bacterial peptidases released from damaged cells. Besides these limitations we have to take into consideration unspecificity of bioassays, i.e. when more than one bacteriocin is present we can detect only the collective activity. In order to overcome these limitations, we decided to introduce an alternative molecular method for detection of *in situ* expression of bacteriocin genes in cheese, based on reverse transcription (RT) and real-time PCR. With this method the messenger ribonucleic acid (mRNA), produced at transcription of genes, is reverse transcribed to complementary DNA (cDNA) which can be further amplified by PCR. When combined with real-time PCR, the method offers not only qualitative but also quantitative analysis of gene expression.

1.1.2 Workflow

The starting point of our research work is described in the scientific paper Trmčić et al. (2008). Two traditional Slovenian cheeses, Tolminc (made of cows' milk) and Kraški ovčji sir (made of ewes' milk) were examined for the presence of bacteriocin gene determinants, using two procedures (Figure 1). With the first procedure we extracted total DNA directly from cheeses, using three different solutions for homogenisation of cheese and two different methods for extraction of DNA. With the second procedure we extracted the DNA from microbial cheese consortia of cultivable bacteria that consisted of three different groups of LAB: lactobacilli, enterococci and mesophilic cocci. We used PCR to look for presence of gene determinants of 19 different bacteriocins in DNA extracted directly from cheese and in DNA extracted from separate microbial consortia.

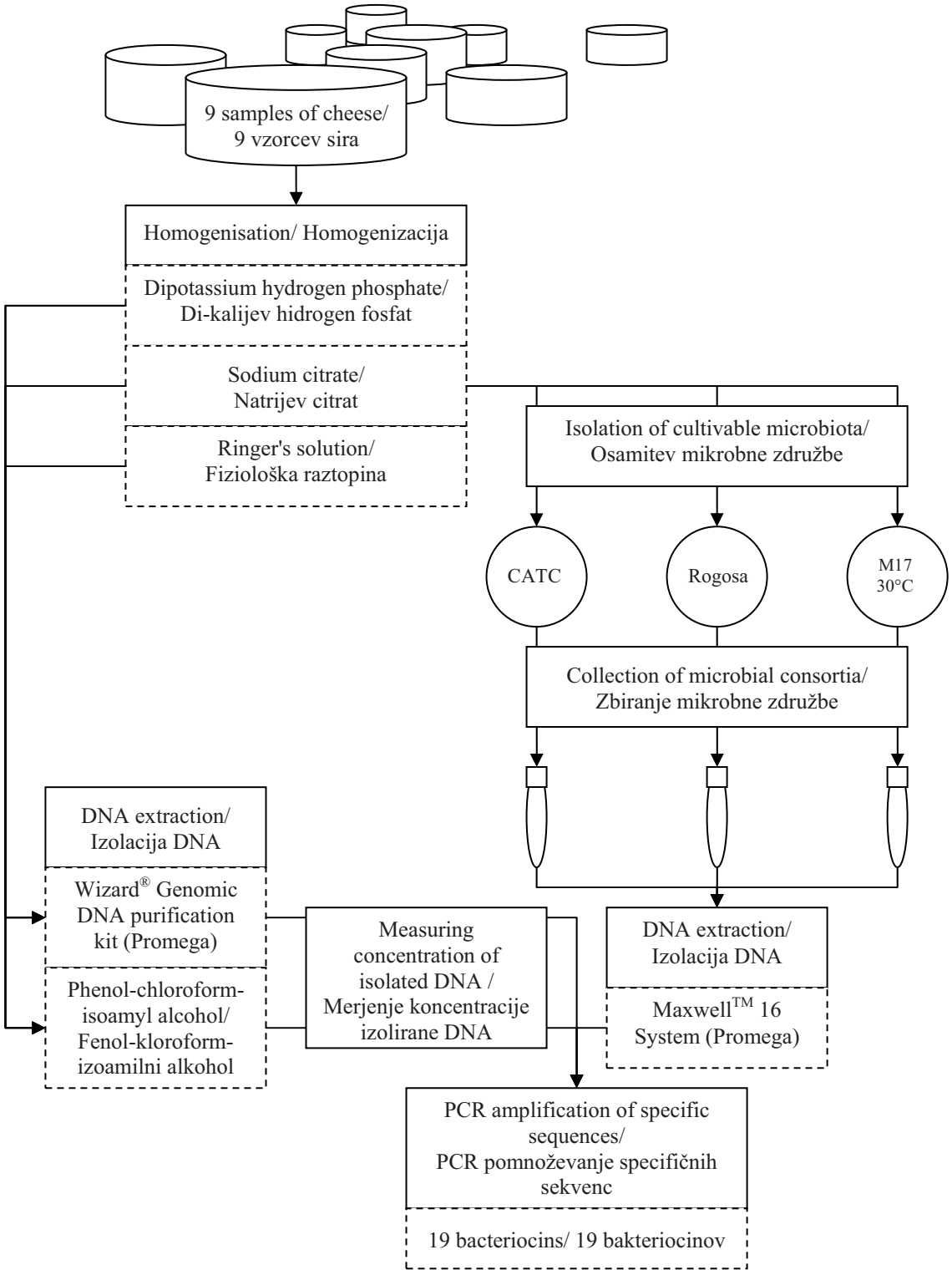

Figure 1: Schematic presentation of DNA isolation from cheese and its cultivable microbial consortia (Trmčić et al., 2008).

Slika 1: Shematski prikaz izolacije DNA neposredno iz sira in iz njegove kultivabilne mikrobne združbe (Trmčić in sod., 2008).

Based on our research hypothesis we planned a circular flow of experiments as presented in Figure 2. From cheese with the highest number of detected bacteriocins' gene determinants we isolated cultivable microbial consortia (Trmčić et al., 2008). We used these microbial consortia in the next two experiments. First we tested the potential of microbial consortia to inhibit growth of *S. aureus in situ* in milk and cheese (Trmčić et al., 2010a). In the second experiment we propagated the microbial consortia in milk at two different temperatures (30°C and 42°C) and followed the presence of gene determinants for different bacteriocins (Trmčić et al., 2011a). During all phases of work we followed besides gene determinants for bacteriocins also the antimicrobial activity and tried to isolate individual bacteriocinogenic strains. Pure bacteriocinogenic strains isolated from original and propagated microbial consortia were the focus point of the scientific paper of Trmčić et al. (2010b).

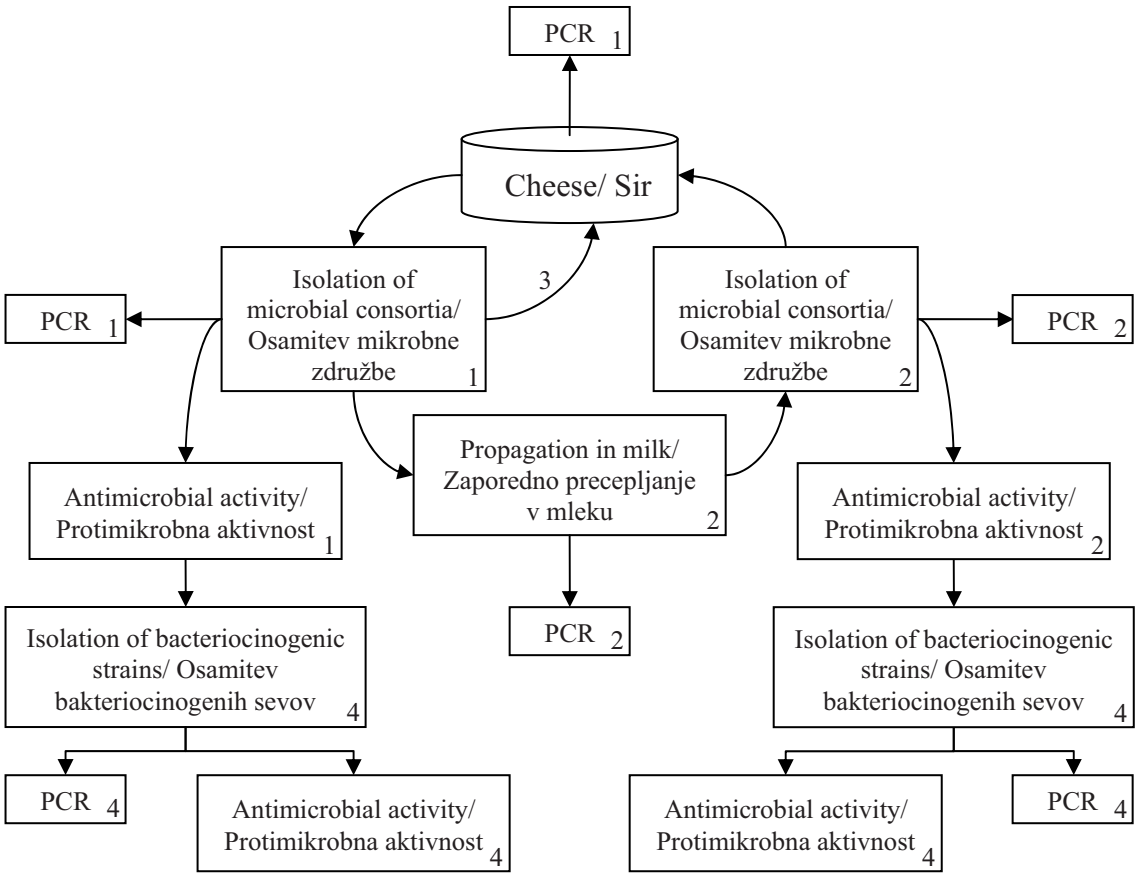

Figure 2: Overview of the study - detection of bacteriocin gene determinants and determination of antimicrobial activity. 1-Trmčić et al. (2008), 2-Trmčić et al. (2011a), 3- Trmčić et al. (2010a), 4-Trmčić et al. (2010b).

Slika 2: Pregled poteka raziskave - odkrivanja genskih determinant za bakteriocine in ugotavljanja protimikrobne aktivnosti. 1-Trmčić in sod. (2008), 2-Trmčić in sod. (2011a), 3-Trmčić in sod. (2010a), 4-Trmčić in sod. (2010b).

In the last part of our study we introduced a molecular method RT real time-PCR for the quantification of the expression of bacteriocin genes in cheese. In order to test the methods of mRNA extraction from cheese and the quantification of expression, we used a sterile cheese model inoculated with a monoculture of a strain that produces bacteriocin nisin A. Quantification of expression of 11 genes, involved in nisin A production and activation, during 24 h of fermentation is described in the scientific paper of Trmčić et al. (2011b).

1.1.3 Hypothesis

- Since traditional Slovenian cheeses are produced from raw milk there is a strong possibility that their rich microbiota includes also LAB able to produce different bacteriocins with the potential to inhibit outgrowth of undesired or pathogenic bacteria.
- The use of an approach based on molecular methods could enable faster detection of strains that produce different bacteriocins.
- Due to their competitive advantage, bacteriocinogenic strains preserve the cultivability and bacteriocin activity in cheese.
- The use of an approach based on molecular methods could enable quantitative determination of bacteriocin gene expression in cheese.

1.2 SPLOŠNI UVOD

V doktorski disertaciji so prikazani rezultati raziskav, ki so delno potekale v okviru triletnega aplikativnega raziskovalnega projekta 'Po meri narejene starterske kulture za izdelavo tradicionalnih sirov' in štiriletnega evropskega projekta šestega okvirnega programa Evropske skupnosti TRUEFOOD (Traditional United Europe Food). Skupni cilj TRUEFOOD projekta je bil vpeljati primerne inovacije na področju proizvodnje tradicionalnih živil, ki bi zagotovila ohranitev in povečanje njihove kompetitivnosti na vse bolj globalnem evropskem tržišču. TRUEFOOD je bil usmerjen k manjšim in srednje velikim podjetjem, ki predstavljajo večinski delež evropske živilske industrije, obenem pa običajno nimajo dostopa do kvalitetnega raziskovalno-razvojnega dela. Eden od glavnih izzivov v tradicionalni proizvodnji hrane je vpeljava inovacij, ki so v skladu s priporočili in zakoni Evropske unije ter zagotavljajo varnost tradicionalnih živil, obenem pa zadostijo splošnim pričakovanjem in zahtevam potrošnikov za te izdelke. Usklajevanje obeh aspektov je včasih težavno. Izdelek mora biti na primer po eni strani mikrobiološko neoporečen, po drugi strani pa izdelan s čim manj obdelave in dodatkov ter imeti visoko prehransko in senzorično kakovost. Slednje je še posebno zahtevno za manjša in srednje velika podjetja, ki zaradi finančnih in prostorskih omejitev ne morejo vedno vzpostaviti pravega sistema za zagotavljanje mikrobiološke in kemijske neoporečnosti živil (http://www.truefood.eu; 2010). Problem, s katerim se je znotraj projekta ukvarjala naša skupina, je bil mikrobiološka varnost tradicionalnih slovenskih trdih sirov, s poudarkom na zaviranju bakterijske vrste *Staphylococcus* (*S.*) *aureus* v teh sirih. Poskušali smo osamiti mikrobne združbe in posamezne seve, ki bi zavirali to pogosto patogeno bakterijsko vrsto in bi jih lahko uporabili v starterskih ali zaščitnih kulturah.

1.2.1 Uvod

Večina tradicionalnih sirov in mlečnih izdelkov na splošno je izdelanih iz surovega mleka, ki je s svojo kemijsko in mikrobiološko sestavo poleg tehnološkega postopka izdelave glavni parameter, ki določa karakteristične lastnosti izdelka. Pestra mikrobna združba, ki pride v sir s surovim mlekom in sirarsko opremo, oblikuje značilen okus in aromo, po katerih so tradicionalni siri razpoznavni in se ločijo od množice ostalih sirov. Poleg zaželjene mikrobne združbe se v surovem mleku zelo pogosto pojavljajo tudi sevi, ki lahko v končnem izdelku predstavljajo potencialno nevarnost za potrošnika. Zaradi tega je ključni problem, povezan z izdelki te vrste, kako ponuditi potrošniku izdelek z željenimi specifičnimi senzoričnimi lastnostmi, obenem pa ga zaščititi pred potencialno infekcijo ali intoksikacijo.

Uporaba zaščitnih starterskih kultur, ki vsebujejo izbrane bakteriocinogene mlečnokislinske bakterije (MKB), se je v proizvodnji sira že uveljavila. Pri izdelavi tradicionalnih izdelkov pa je uporaba zaščitnih ali starterskih kultur problematična iz dveh vidikov. Prvi je, da s startersko kulturo dodajamo seve, ki niso avtohtoni za posamezen sir, drugi pa je, da lahko ti sevi s svojo bakteriocinsko aktivnostjo zavirajo tudi naravno prisotno koristno mikrobno združbo in s tem še dodatno spreminjajo mikrobiološko sestavo in posledično tudi fizikalno-kemijske lastnosti sira. Eden od pristopov k reševanju opisanega problema je iskanje primernih bakteriocinogenih sevov v naravno prisotni mikrobni združbi posameznih tradicionalnih sirov. S takšnimi sevi bi lahko sestavili starterske kulture, ki so 'po meri' posameznega tradicionalnega sira. Uporaba takšnih straterskih kultur bi preprečila večino težav, ki spremljajo tradicionalno tehnologijo (surovo mleko) in obenem ohranila vse specifične lastnosti tradicionalnega sira, vključno s senzoričnimi.

Analiza mikrobne združbe, ki jo vsebujejo tradicionalni siri, in iskanje posameznih sevov, sposobnih sinteze širokospektralnih bakteriocinov, je dokaj zahteven in dolgotrajen postopek, ki je povezan tudi s porabo velike količine materiala, poleg tega pa je njegova uspešnost zelo odvisna od izbire indikatorskih sevov in proteolitičnih encimov. Zato je bila naša ideja, da uporabimo pristop, ki ga sicer uporabljajo v metagenomiki. Iz posameznih tradicionalnih sirov smo izolirali celokupno bakterijsko deoksiribonukleinsko kislino (DNA), ter s pomočjo verižne reakcije s polimerazo (PCR) in specifičnih začetnih oligonukleotidov iskali v njej prisotnost genskih determinant za posamezne bakteriocine.

Prisotnost genskih determinant za posamezne bakteriocine v celokupni DNA sirov pa še ne zagotavlja, da ta DNA izhaja iz kultivabilnih ali vsaj živih celic MKB. Le kultivabilni bakterijski sevi pa so tisti, ki jih lahko osamimo in nadalje uporabimo kot komponento v starterski kulturi. Zato je bil naš naslednji raziskovalni izziv potrditi prisotnost kultivabilnih bakteriocinogenih MKB v sirih. Posamezne skupine MKB smo osamili na selektivnih gojiščih, nakar smo raziskali, ali skupna DNA, pridobljena iz mešanice kolonij s posameznih gojišč, vsebuje različne bakteriocinske determinante.

Prisotnost genskih determinant za posamezne bakteriocine v DNA kultivabilnih MKB pa še ne zagotavlja, da se bakteriocinski geni tudi izražajo. Le sevi, ki dejansko proizvajajo bakteriocine v siru, so lahko učinkoviti v smislu povečanja varnosti izdelka za potrošnika. Zato smo *in vitro* kot tudi *in situ* raziskali tudi bakteriocinsko aktivnost posameznih mikrobnih združb. *In vitro* testiranja bakteriocinske aktivnosti mikrobnih združb, posameznih sevov ter brezceličnih pripravkov kultur le-teh so potekala z difuzijsko metodo na trdnem gojišču. Izbrane mikrobne združbe pa smo tudi dodajali pri proizvodnji sira ter

tako preverili protimikrobno aktivnost tudi *in situ,* pri čemer smo preizkusili tudi bakteriocinsko aktivnost ekstraktov sirov.

Dokazovanje sinteze in aktivnosti bakteriocinov v siru z uporabo bioloških testov ima nekatere omejitve, kot so visoka meja detekcije, neenakomerna razporeditev bakteriocinov v siru, adsorbcija na kazeine sira ali razgradnja bakteriocinov z ekstracelularnimi ali intracelularnimi bakterijskimi peptidazami, ki se sprostijo iz poškodovanih celic. Poleg omenjenih omejitev je pomembno tudi upoštevati, da kadar je v vzorcu več različnih bakteriocinov, ne moremo zaznati aktivnosti posameznih bakteriocinov, pač pa le celokupno aktivnost. Naš zadnji raziskovalni izziv je bil zato vpeljati alternativno molekularno metodo za ugotavljanje produkcije bakteriocinov *in situ* v siru, ki bi temeljila na merjenju izražanja bakteriocinskih genov s pomočjo obratne transkripcije (RT) in verižne reakcije s polimerazo v realnem času (real-time PCR). Pri tej metodi informacijsko ribonukleinsko kislino (mRNA), ki nastane s prepisovanjem genov, prepišemo z reverzno transkripcijo v komplementarno DNA (cDNA), ki jo je mogoče pomnoževati s PCR. V povezavi s PCR v realnem času metoda omogoča ne samo kvalitativno ampak tudi kvantitativno analizo izražanja posameznih genov.

1.2.2 Potek dela

Izhodišče našega raziskovalnega dela je opisano v članku Trmčić in sod. (2008), kjer smo kot začetni raziskovalni material uporabili vzorce dveh tradicionalnih slovenskih sirov, Tolminc (iz kravjega mleka) in Kraški ovčji sir. Vse vzorce sirov smo vzorčili za dva postopka ugotavljanja prisotnosti bakteriocinskih determinant, ki so shematsko predstavljeni na sliki 1. Pri prvem postopku smo izolirali skupno DNA neposredno iz sira, pri čemer smo kombinirali tri različne raztopine za homogeniziranje sira in dva različna postopka izolacije DNA. Pri drugem postopku ugotavljanja prisotnosti bakteriocinskih determinant smo izolirali DNA iz mikrobnih združb, ki so vsebovale tri različne skupine MKB: laktobacile, enterokoke in mezofilne koke. Tako v DNA, pridobljeni neposredno iz sira, kot v tisti iz posameznih združb smo s pomočjo PCR iskali prisotnost strukturnih genov za 19 različnih bakteriocinov.

Skladno s postavljenimi hipotezami smo načrtovali krožni potek poskusov, ki so predstavljeni na sliki 2. Iz sira z največjo raznovrstnostjo genskih determinant za bakteriocine smo osamili tri različne mikrobne združbe in jih združili v eno samo (Trmčić in sod., 2008). Tako sestavljeno mikrobno združbo smo uporabili v dveh nadaljnjih poskusih. V prvem poskusu smo testirali zmožnost mikrobne združbe, da omeji rast *S. aureus in situ* v mleku in siru (Trmčić in sod., 2010a). V drugem poskusu smo isto mikrobno združbo zaporedno precepljali v mleku pri dveh temperaturah (30°C in 42°C) in

spremljali prisotnost genskih determinant za različne bakteriocine (Trmčić in sod., 2011a). V vseh fazah dela smo poleg genskih determinant za bakteriocine spremljali tudi protimikrobno aktivnost, obenem pa poskušali osamiti posamezne bakteriocinogene seve. Posamezni bakteriocinogeni sevi, osamljeni iz prvotne mikrobne združbe in mikrobne združbe po zaporednem precepljanju v mleku, so bili predmet raziskave v članku Trmčić in sod. (2010b).

V zadnjem delu raziskave smo vpeljali molekularno metodo RT real time-PCR za kvantifikacijo izražanja bakteriocinskih genov v siru. Z namenom preveriti uspešnost izolacije mRNA iz sira ter kvantifikacije izražanja smo uporabili sterilni model sira, inokuliranega z monokulturo seva, ki proizvaja bakteriocin nizin A. Merjenje transkripcije 11 genov, ki so vključeni v proizvodnjo in aktivacijo nizina A, med 24-urno fermentacijo modelnega sira je predmet raziskave, predstavljene v članku Trmčić in sod. (2011b).

1.2.3 Hipoteze

- Ker so tradicionalni slovenski siri izdelani iz surovega mleka, je zelo verjetno, da so v pestri mikrobni združbi prisotni tudi sevi MKB, ki proizvajajo različne bakteriocine in lahko zavirajo potencialni razrast neželjenih oziroma patogenih bakterij.
- Uporaba primerne molekularne metode bi lahko omogočila hitrejše odkrivanje sevov, ki proizvajajo različne bakteriocine.
- Zaradi kompetitivne prednosti ohranijo bakteriocinogeni sevi kultivabilnost in bakteriocinsko aktivnost tudi v siru.
- Uporaba primerne molekularne metode, bi lahko omogočila kvantitativno ugotavljanje izražanja genov za bakteriocine v siru.

2 SCIENTIFIC PAPERS (ZNANSTVENI ČLANKI)

2.1 SHORT COMMUNICATION: CULTURE-INDEPENDENT DETECTION OF LACTIC ACID BACTERIA BACTERIOCIN GENES IN TWO TRADITIONAL SLOVENIAN RAW MILK CHEESES AND THEIR MICROBIAL CONSORTIA

Kratki znanstveni prispevek: Od gojenja neodvisno odkrivanje genov za bakteriocine mlečnokislinskih bakterij v dveh tradicionalnih slovenskih sirih iz surovega mleka in njihovi mikrobni združbi

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Journal of Dairy Science 91, 4535-4541 (2008)

S pomočjo PCR analize smo raziskali prisotnost genov za 19 bakteriocinov mlečnokislinskih bakterij v dveh tradicionalnih slovenskih sirih iz surovega mleka, v Tolmincu (iz kravjega mleka) in Kraškem ovčjem siru. Analizirali smo tako skupno DNA, osamljeno neposredno iz 9-ih sirov, kakor tudi DNA iz mikrobnih združb sevov, predhodno osamljenih iz teh sirov. Enajst genov za bakteriocine smo zasledili v vsaj enem siru, v pripadajoči združbi ali v obeh. Različni siri ali združbe so vsebovali determinante za 3 do 9 bakteriocinov. Genske determinante za plantaricin A smo odkrili v vzorcih DNA iz vseh sirov in združb. Geni za enterocine A, B, P, L50A in L50B ter bakteriocin citolizin so bili pogosto prisotni, kakor tudi geni za nizin. Rezultati kažejo na to, da so bakteriocinogeni sevi iz rodov *Lactobacillus*, *Enterococcus* in *Lactococcus* običajni predstavniki naravne mikrobiote sirov iz surovega mleka, ki tako predstavlja dober vir novih zaščitnih sevov.

Ključne besede: Slovenski siri iz surovega mleka, geni za bakteriocine, verižna reakcija s polimerazo

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Short Communication: Culture-Independent Detection of Lactic Acid Bacteria Bacteriocin Genes in Two Traditional Slovenian Raw Milk Cheeses and Their Microbial Consortia

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ABSTRACT

Two Slovenian traditional raw milk cheeses, Tolminc (from cows' milk) and Kraški (from ewes' milk), were examined for the presence of 19 lactic acid bacteria bacteriocin genes by PCR analysis of total DNA extracts from 9 cheeses and from consortia of strains isolated from these cheeses. Eleven bacteriocin genes were detected in at least one cheese or consortium, or from both. Different cheeses or consortia contained 3 to 9 bacteriocin determinants. Plantaricin A gene determinants were found in all cheese and consortia DNA extracts. Genes for enterocins A, B, P, L50A, and L50B, and the bacteriocin cytolysin were commonly detected, as were genes for nisin. These results indicate that bacteriocinogenic strains of *Lactobacillus*, *Enterococcus*, and *Lactococcus* genera with protective potential are common members of indigenous microbiota of raw milk cheeses, which can be a good source of new protective strains.
Key words: Slovenian raw milk cheese, bacteriocin gene, polymerase chain reaction

There is increasing interest in preserving the production of traditional cheeses. However, because such cheeses are typically produced from raw milk (as thermal treatment would damage the natural microbiota), food safety is of concern. A possible alternative for thermal treatment is the introduction of selected microbial communities that are able to inhibit pathogenic or spoilage microorganisms, generally referred to as natural protective cultures. However, these introduced cultures must not unduly influence the desired microflora and typical organoleptic properties of the cheese.

Certain bacteriocins (i.e., naturally occurring inhibitory peptides) inhibit food pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridia* species, and thus can be considered as natural preservative agents (Deegan et al., 2006; Gálvez et al., 2007). The potential of enterococcal and lactococcal bacteriocins to increase food protection has been extensively studied in vitro and in model food fermentations, including raw milk cheeses, and several applications have been developed (Rodríguez et al., 2001; De Vuyst et al., 2003; Guinane et al., 2005; Foulquié Moreno et al., 2006). Bacteriocin-producing lactic acid bacteria (**LAB**) that occur naturally in traditional cheeses represent an extensive pool of microbes with inhibitory potential (Martínez et al., 1995; De Vuyst et al., 2003; Majhenič et al., 2007).

The presence and diversity of bacteriocinogenic strains in the natural microbiota of raw milk have been studied previously. In the study of Rodríguez et al. (2000), DNA extracted from individual isolates served as a target for PCR amplification of different bacteriocin genes. The presence of 8 known structural genes for enterocins in enterococci isolates from different sources, including 28 isolates from cheeses, was examined by De Vuyst et al. (2003). In the present study, we examined the presence of 19 known bacteriocin genes typically produced by representatives of *Lactobacillus*, *Enterococcus*, and *Lactococcus* genera, directly in Tolminc cows' milk cheeses and Kraški ewes' milk cheeses produced in small-scale cheese plants in Slovenia. In addition to analysis of the total DNA extracted from cheeses, the DNA from bacterial consortia from the cheeses, grown on different selective agar media, was examined to establish the viability of bacteriocin producers whose bacteriocin genes were detected in the cheese samples. The aim of this study was to obtain insight into the prevalence of bacteriocinogenic strains in the natural microbiota of traditional cheeses and to evaluate the importance of these products as a source of interesting strains with protective potential.

Five Tolminc cheeses (T1 to T5), made from raw cows' milk, and 4 Kraški cheeses (K1 to K4), made from raw ewes' milk, were obtained from different producers. The mature 2- to 3-mo-ripened cheeses were sampled. Samples (10 g) were homogenized (Bagmixer R400, Interscience, St. Nom, France) in 3 buffers (90 g):

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2% (wt/vol) dipotassium hydrogen phosphate (Merck, Darmstadt, Germany), 2% (wt/vol) trisodium citrate dihydrate solution (Kemika, Zagreb, Croatia) prepared according to the International Dairy Federation (1992), or quarter-strength Ringer's solution (Merck). Each of two 10-mL aliquots of each cheese homogenate was centrifuged (6,000 × *g*, 10 min, 4°C), the fat layer was removed, and the pellet was resuspended in 1 mL of sterile filtered water.

Extraction of DNA from cheese was carried out using 2 methods. One of the two 1-mL aliquots of cell suspension in water was transferred to a 2-mL tube containing 0.3 to 0.5 g of 0.1-mm zirconi/silica beads (BioSpec Products, Bartlesville, OK), and DNA was extracted from the cell pellet using phenol-chloroform-isoamylalcohol as described by Cocolin et al. (2001). The second 1-mL aliquot was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). The cell pellet was first resuspended in 600 μL of 50 m*M* EDTA containing 10 mg/mL lysozyme and 25 U/mL mutanolysin (Sigma-Aldrich, Steinheim, Germany) and incubated at 37°C for 1 h. Subsequent DNA purification steps were performed following the kit manufacturer's instructions. The DNA was finally resuspended in 100 μL of water. To determine the efficiency of the different DNA extraction protocols, the DNA concentration in extracts from cheeses was measured using the QUBIT Quantitation Platform fluorometer (Invitrogen, Paisley, United Kingdom). The effect of different extraction protocols on the yield of DNA in the cheese extracts was statistically evaluated using one-way ANOVA (Excel, Microsoft Corp., Redmond, WA). In addition, Student's *t*-test ($P < 0.05$) was applied when the effect of the treatment was found to be significant.

Cheese samples homogenized in quarter-strength Ringer's solution as described above were appropriately diluted and plated for viable count determination. Citrate azide Tween carbonate (**CATC**) and Rogosa (Merck) agar plates used for enterococci and lactobacilli, respectively, were incubated at 37°C for 48 h. Rogosa agar plates were incubated in anaerobic conditions (GENbox anaer, Bio-Mérieux, Marcy l'Etoile, France). M17 agar plates (Merck), incubated at 30°C for 48 h, were used to grow mesophilic LAB, including lactococci. For DNA extraction from consortia of strains, approximately 300 colonies were rinsed from the surface of CATC, Rogosa, and M17 agar plates with 2 mL of quarter-strength Ringer's solution. Then, DNA was isolated from 1 mL of each suspension using the Maxwell 16 Cell DNA Purification Kit and Maxwell 16 System (Promega), and finally resuspended in 300 μL of elution buffer.

Polymerase chain reaction amplification of structural genes for LAB bacteriocins was performed as described previously in the publications listed in Table 1, except for helveticin J and lactocin 705. Helveticin J primers were constructed based on the gene sequence of helveticin J stored in GenBank (accession number M59360). The primers anneal to the structural gene (ORF 3) of helveticin J and enable the amplification of 200-bp PCR products (Joerger and Klaenhammer, 1990). The lactocin 705 forward and reverse primers were derived from the gene sequences (GenBank accession number AF200347) of the α and β complementary subunits of lactocin 705, respectively (Cuozzo et al., 2000). The PCR amplification of lactocin 705 structural genes results in 298-bp products. Amplification was carried out as follows: 95°C/3 min; 35 cycles: 95°C/30 s, 55°C/30 s, 72°C/ 30 s; 72°C/5 min. Oligonucleotide primers were purchased from Invitrogen. Amplifications were carried out in a thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). GoTaq DNA Polymerase with appropriate buffer, $MgCl_2$, and deoxyribonucleotide triphosphate solutions were purchased from Promega. All PCR amplifications were performed in a final volume of 20 μL. The reaction mixtures contained DNA (0.5 μL of cheese extract or 1.5 μL of consortia extract), GoTaq buffer (including 1.5 μ*M* $MgCl_2$), GoTaq DNA Polymerase (0.5 U), dNTP (100 μ*M*), and 1 μ*M* of appropriate primers, except for plantaricin A (Maldonado et al., 2004), for which an appropriate quantity of 25 m*M* $MgCl_2$ was added to reach a final concentration of 2.5 m*M*. Amplified fragments were resolved by electrophoresis on 1.8% (wt/vol) agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen).

To confirm the specificity and identity of the PCR amplicons, the corresponding bands were excised from agarose gel, purified by using the Wizard SV Gel and PCR Clean-Up System (Promega), and subjected to sequencing analysis (Microsynth AG, Balgach, Switzerland). Homologies of the sequences were searched in the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A reference bacteriocinogenic *Enterococcus faecium* LMG 11423, a producer of enterocins A, B, and P was purchased from BCCM/LMG (Gent, Belgium). Helveticin J producer strain *Lactobacillus helveticus* 481NCK228 Hlv+ was kindly provided by Todd R. Klaenhammer (Department of Food Science and Microbiology, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh). *Lactobacillus helveticus* and *E. faecium* were grown at 37°C in de Man, Rogosa, and Sharpe broth and M17 broth (Merck), respectively, and applied as positive controls in PCR reactions. The PCR mixture without DNA served as a negative control for PCR reactions.

Because many researchers have highlighted the importance of choosing a reliable extraction method for

Table 1. Oligonucleotide primers for PCR and target bacteriocins

Primer sequence[1] (5′ to 3′)	Target bacteriocin	Abbreviated name	Reference
f: 5′-ggt acc act cat agt gga aa-3′ r: 5′-ccc tgg aat tgc tcc acc taa-3′	Enterocin A	A	De Vuyst et al. (2003)
f: 5′-caa aat gta aaa gaa tta agt acg-3′ r: 5′-aga gta tac att tgc taa ccc-3′	Enterocin B	B	De Vuyst et al. (2003)
f: 5′-gct acg cgt tca tat ggt aat-3′ r: 5′-tcc tgc aat att ctc ttt agc-3′	Enterocin P	P	De Vuyst et al. (2003)
f: 5′-cct acg tat tac gga aat ggt-3′ r: 5′-gcc atg ttg tac cca acc att-3′	Enterocin31	31	De Vuyst et al. (2003)
f: 5′-gag gag tat cat ggt taa aga-3′ r: 5′-ata ttg tta aat tac caa-3′	Enterocin AS 48	AS48	De Vuyst et al. (2003)
f: 5′-atg gga gca atc gca aaa tta-3′ r: 5′-ttt gtt aat tgc cca tcc ttc-3′	Enterocin L50A	L50A	De Vuyst et al. (2003)
f: 5′-atg gga gca atc gca aaa tta-3′ r: 5′-tag cca ttt ttc aat ttg atc-3′	Enterocin L50B	L50B	De Vuyst et al. (2003)
f: 5′- ggc ggt att ttt act gga gtn-3′ r: 5′- cct act cct aag cct atg gta-3′	Cytolysin	Cyl	De Vuyst et al. (2003)
f: 5′-aag aat ctc tca tga gt-3′ r: 5′-cca tgt ctg aac taa ca-3′	Nisin	Nis	Rodríguez et al. (2000)
f: 5′-tct gca ctc act tca tta gtt a-3′ r: 5′-aag gta att aca cct ctt tta-t3′	Lacticin 481	Lac481	Rodríguez et al. (2000)
f: 5′-caa tca gta gag tta tta aca ttt g-3′ r: 5′-gat tta aaa aga cat tcg ata att at-3′	Lactococcin A	LccA	Rodríguez et al. (2000)
f: 5′-tgg tgt gca ttg tac t-3′ r: 5′-ttg atc ggc aac gat t-3′	Acidocin A	AcidA	Majhenič et al. (2003)
f: 5′-aga tgc agt ggc ttc t-3′ r: 5′-cca tgc agg taa tgt c-3′	Acidocin B	AcidB	Majhenič et al. (2003)
f: 5′-tga aaa ttc aaa tta aag gta tga agc-3′ r: 5′-tta cca tcc cca ttt ttt aaa cag ttt c-3′	Plantaricin A	PlnA	Maldonado et al. (2004)
f: 5′-aay aar yti gci tay aay atg-3′ r: 5′-gcy tty aar rai ccy tci cc-3	Plantaricin S	PlaS	Remiger et al. (1996)
f: 5′-atg gaa aag ttt att gaa tta-3′ r: 5′-tta ttt att cca gcc agc gtt-3′	Sakacin P	SakP	Remiger et al. (1996)
f: 5′- gta aaa gaa tta agt atg aca-3′ r: 5′-tta cat tcc agc taa acc act-3′	Curvacin A	CurA	Remiger et al. (1996)
f: 5′-aga cat ggg aat ttg ctg gt-3′ r: 5′- ggc gcg att caa gta gga ta –3′	Helveticin J	HelvJ	This study
f: 5′- tcc aag acg tcc ctt ttt gt –3′ r: 5′- tta cag gtg gtg gat ttt gg-3′	Lactocin 705	Lact705	This study

[1]f = forward; r = reverse.

DNA when investigating microbial communities in complex media, we first evaluated the suitability of 2 DNA extraction protocols and 3 diluents for cheese homogenization. There was no significant difference in the total cheese DNA concentrations given by the 2 extraction methods (i.e., phenol-chloroform-isoamylalcohol extraction and Wizard Genomic DNA Purification kit). However, for all 18 extracts (9 cheeses, 2 DNA extraction procedures), we gained significantly ($P \leq 0.05$) greater concentrations of DNA using Na citrate buffer to obtain cheese homogenates than when K_2HPO_4 or quarter-strength Ringer's solution were used (Table 2).

The PCR reactions with DNA extracts from cheese were run for each of the bacteriocins in 6 parallel series because 6 samples were obtained from each cheese (3 homogenization solutions and 2 extraction protocols). We considered that a cheese contained genes for individual bacteriocin when a PCR product of expected size was amplified in at least 1 of the 6 DNA extracts. In general, the presence of individual bacteriocin genes was detected in all 6 DNA extracts from the same cheese. However, negative results in some of the extracts from the same cheese were also observed in some cases (results not shown). Although there were some significant differences in the yield of DNA among the buffers and protocols used (Table 2), the success of reactions was not directly correlated to the concentration of DNA or restricted to any particular combination of protocol and homogenization solution.

The screening of total DNA extracts from 9 cheeses for the presence of genes encoding 19 known LAB bacteriocins revealed the presence of between 1 (in K2 and K3 cheeses) and 9 genes (in T2 and K4 cheeses; Table 3). Enterocin genes except those for enterocin 31 and enterocin AS-48 (which were not detected in any sample) were commonly detected. Of the 3 bacteriocins typically produced by *Lactococcus lactis* species,

Table 2. Concentration of DNA (μg/mL) in extracts from cheeses obtained by different extraction protocols and buffers[1]

	Diluent		
Extraction method	Na citrate	K_2HPO_4	1/4 Ringer's solution
Phenol-chloroform-isoamylalcohol	22.16 ± 24.46[a]	2.82 ± 4.17[b]	7.76 ± 10.06[ab]
Wizard Genomic DNA Purification kit[2]	34.85 ± 23.23[a]	7.89 ± 16.14[b]	20.00 ± 20.43[ab]
Both extraction methods	28.51 ± 24.04	5.35 ± 11.73	13.88 ± 16.84

[a,b]Means with different superscripts in the same row differ significantly, $P < 0.05$.

[1]The concentrations were measured by QUBIT fluorimeter (Invitrogen, Paisley, United Kingdom). The presented values are means of 9 values ± SD or 18 values (combination method). The values presented in the first and second rows are mean values ± SD of DNA concentration of 9 different cheese samples. In the third row, the average values were obtained from all 18 measurements of DNA concentration; that is, 9 cheeses, each undergoing 2 extraction methods.

[2]Promega (Madison, WI).

nisin determinants were detected in 6 of the cheeses and lacticin 481 was detected in 2, whereas lactococcin A-specific sequences were not found. Plantaricin A determinants were present in all cheese samples and acidocin B genes in only 1 cheese sample, whereas another 6 bacteriocins usually produced by lactobacilli (i.e., helveticin J, lactocin 705, acidocin A, plantaricin S, sakacin P, and curvacin A) were not found in the cheeses.

The viable counts of lactobacilli, enterococci, and mesophilic LAB (predominantly lactococci) from cheeses are presented in Table 4. Simultaneously, the total DNA samples isolated from the consortia of viable bacterial population grown on Rogosa, CATC, and M17 plates were analyzed by PCR. Positive results for consortia in Table 3 signify that specific products (i.e., bacteriocin genes) were amplified with at least one consortium of an individual cheese sample. In general, enterocin genes were detected in consortia obtained from CATC and M17 agar plates, indicating that bacteriocinogenic enterococci were grown on these 2 media. Genes for plantaricin A, helveticin J, and acidocin B (i.e., bacteriocins commonly produced by lactobacilli) were detected in consortia from Rogosa and M17 agar, whereas nisin and lacticin 481 determinants were detected in M17 consortia only (results not shown). With the exception of lacticin 481 genes that were found only in Tolminc cheese and consortia samples,

Table 3. Detection of different bacteriocin genes in total DNA extracted directly from 5 Tolmine (T1 to T5) and 4 Kraški (K1 to K4) cheeses and from consortia of viable strains, by PCR amplification[1]

	Target bacteriocin[2]										
	Enterocins										
Cheese/consortia sample	A	B	P	L50A	L50B	Cyl	Nis	Lac481	Acid B	HelvJ	PlnA
T1	+/+	+/+	+/+	−/+[4]	+/+	+[3]/+	+/+	−/−	−/−	−/−	+[3]/+
T2	+/+	+/+	+/+	+/+	+[3]/+	+/+	−/+[4]	+[3]/+	−/+[4]	−/−	+/+
T3	+/+	+[3]/+	−/+[4]	−/+[4]	+/+	−/+[4]	−/+[4]	−/+[4]	−/−	−/−	+/+
T4	+[3]/+	+/+	+/+	−/+[4]	+/+	+/+	−/+[4]	−/+[4]	−/−	−/+[4]	+/+
T5	+/+	−/−	+/+	−/−	−/+[4]	−/−	+[3]/+	−/−	+/+	−/−	+/+
K1	+/+	−/+[4]	+[3]/+	−/+[4]	+/+	−/−	−/−	−/−	−/−	−/−	+/+
K2	−/−	−/−	−/−	−/−	−/−	−/−	−/+[4]	−/−	−/−	−/+[3,4]	+/+
K3	−/−	−/−	−/+[4]	−/−	−/−	−/−	−/+[4]	−/−	−/−	−/+[4]	+/+
K4	+/+	+/+	+/+	+[3]/+	+/+	+/+	+/+	−/−	+[3]/+	−/−	+/+
Positive samples (cheese/consortia)	78/78	67/56	67/89	22/67	78/67	44/56	67/56	22/22	22/33	0/33	100/100

[1]+ = PCR product of expected size was amplified in at least 1 of 6 DNA extracts obtained from each cheese or in DNA extract from at least 1 consortium of 2 (Rogosa, citrate azide Tween carbonate, M17) obtained from each cheese; − = PCR product of expected size was not amplified in any of 6 DNA extracts obtained from each cheese or in any of 3 consortia (Rogosa, citrate azide Tween carbonate, M17) from each cheese.

[2]Cyl = cytolysin; Nis = nisin; Lac481 = lacticin 481; Acid B = acidocin B; HelvJ = helveticin J; PlnA = plantaricin A. The following bacteriocins were not detected in any sample and are therefore not presented in the table: enterocin 31, enterocin AS 48, lactococcin A, acidocin A, lactocin 705, plantaricin S, sakacin P, and curvacin A.

[3]PCR products were sequenced to confirm the positive result.

[4]Results obtained on DNA extracts from cheese and from consortia were not identical.

Table 4. Viable counts of mesophilic lactic acid bacteria (M17 agar, 30°C), enterococci (citrate azide Tween carbonate agar, CATC, 37°C), and lactobacilli (Rogosa agar, 37°C, anaerobic) in 5 Tolminc (T1 to T5) and 4 Kraški (K1 to K4) cheeses

Cheese	Viable count (cfu/g)		
	M17 (30°C)	CATC (37°C)	Rogosa (37°C)
T1	1.0×10^8	8.3×10^6	1.0×10^8
T2	1.1×10^7	8.5×10^5	1.1×10^8
T3	5.3×10^6	2.7×10^6	2.5×10^7
T4	4.2×10^7	2.0×10^6	5.5×10^7
T5	5.2×10^7	3.2×10^5	4.1×10^8
K1	2.0×10^8	5.7×10^6	5.1×10^8
K2	3.8×10^6	6.0×10^6	1.8×10^7
K3	7.1×10^6	7.4×10^7	6.2×10^7
K4	6.8×10^7	6.7×10^6	3.9×10^7

the presence of individual bacteriocin determinants was not restricted to a particular cheese type. Most of the results (Table 3) obtained with the total cheese DNA extracts were identical to those obtained with the consortia DNA extracts, indicating that bacteriocinogenic strains are members of viable bacterial populations of Tolminc and Kraški cheeses. The finding of bacteriocin genes detected in DNA extracts from cheese but not in those from consortia (6 cases) can be explained by the amplification of DNA derived from nonviable cells present in cheeses. In contrast, in 14 cases, bacteriocin genes were detected in consortia but not in DNA extracts from cheese. For instance, helveticin J was not detected in any of cheese extracts, but was found in 3 consortia from Rogosa agar plates (Table 4). This observation indicates that the proportion of cells that possess helveticin J genes was probably low.

All consortia were also tested previously in vitro by agar diffusion assay (Bogovič-Matijašić et al., 1998) for antimicrobial activity against 25 indicator strains belonging to different genera: *Bacillus*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Listeria*, and *Staphylococcus* (results not shown). Although the inhibition of several strains was observed by different consortia, only one of them (enterococci consortium from K1 cheese) was confirmed to produce proteinase-K–sensitive antimicrobial metabolites, which is an indication of the bacteriocin nature of the substances involved. Bacteriocin-like inhibition was observed against *Enterococcus faecalis*, *E. faecium*, *Enterococcus durans*, *L. monocytogenes*, *Lactobacillus paracasei*, *Clostridium perfringens*, and *Clostridium tyrobutyricum* strains. Because genes for 4 enterocins were detected in the total DNA of this consortium (enterococci, K1 cheese), and enterocins are known to be active against bacteria belonging to different genera, it may be possible that enterocins were involved in the inhibition observed (Foulquié Moreno et al., 2006). The failure to demonstrate active bacteriocin production in vitro by the other consortia that also contained bacteriocin genes could be explained by nonsusceptibility of the indicator strains chosen, inappropriate agar media or incubation conditions for the production of particular bacteriocins, or the presence of silent genes, as previously reported for enterocin A (O'Keeffe et al., 1999). In addition, it should be considered that in vitro antimicrobial tests do not accurately reflect the interactions among bacteria in cheese in situ. For instance, in agar diffusion assays, different strains are in closer contact in consortia than in cheese and may inhibit growth and bacteriocin production and diffusion among each other.

Except for enterocins A, B, P (De Vuyst et al., 2003), and helveticin J (Joerger and Klaenhammer, 1986), positive control strains containing appropriate bacteriocin genes were not available, but we established the sequence of randomly chosen PCR amplification products of expected size. The nucleotide sequences of amplified fragments of cytolysin, nisin, lacticin 481, plantaricin A, acidocin B, helveticin J, and 5 enterocin (L50A, L50B, A, B, and P) genes were found to be identical with the sequences for these bacteriocins available in GenBank.

The advantages of using PCR analysis with bacteriocin-specific primers instead of the common approach of bacteriocin identification by biochemical techniques have been emphasized previously (Remiger et al., 1996; Moschetti et al., 2001; Majhenič et al., 2003). However, this approach has usually been used at the strain level, after isolation of DNA from pure cultures. There have been some exceptions to this. For instance, Moschetti et al. (2001) carried out amplification of nisin genes in DNA extracted directly from milk and cheese, and Matijašič et al. (2007) demonstrated the presence of the bacteriocinogenic strain *Lactobacillus gasseri* K7 (Rif^r) in cheese throughout ripening using PCR amplification of specific bacteriocin genes.

Most of the enterococcal and lactococcal bacteriocins whose genes were detected in Tolminc and Kraški cheeses (i.e., enterocins, nisin, and lacticin 481) have the ability to inhibit not only related LAB species but also members of other genera, among them certain spoilage or pathogenic bacteria (foodborne pathogens) such as *L. monocytogenes*, *Staph. aureus*, *Escherichia coli*, and *B. cereus* (Piard et al., 1990; Delves-Broughton et al., 1996; Foulquié Moreno et al., 2006). In contrast, helveticin J produced by *Lb. helveticus* (which is commonly associated with a cheese environment) has a narrow spectrum of inhibition including closely related *Lactobacillus* species (e.g., *bulgaricus*, *lactis*, and *helveticus* species; Joerger and Klaenhammer, 1986). Acidocin B, another typical lactobacilli bacteriocin found in Kraški and Tolminc cheeses, is more interesting from the ap-

plication point of view, as it was found to be active against *L. monocytogenes*, *Clostridium sporogenes*, and *Brochothrix thermosphacta*, but affects only a few *Lactobacillus* strains (van der Vossen et al., 1994). Plantaricin A, found in all cheeses in this study, is known to be bacteriocidal toward some species of LAB belonging to *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* genera, but not against other gram-positive or gram-negative bacteria or yeasts (Daeschel et al., 1990). Therefore, enterocins, nisin, and acidocin B producers in Kraški and Tolminc cheese may play a protective role against potentially pathogenic microorganisms in cheeses.

The idea of applying bacteriocinogenic strains to inhibit technologically undesirable bacteria or those that present a health risk is not new. For instance, nisin-producing cultures have been widely used in different foods to increase safety (Delves-Broughton et al., 1996; Deegan et al., 2006). Bacteriocinogenic *Enterococcus* cultures have been studied under pilot-scale conditions in a variety of fermented foods including cheese (Foulquié Moreno et al., 2006). For example, bacteriocin AS-48–producing *E. faecalis* strain was found to be effective at controlling *Staph. aureus*, *B. cereus*, and *L. monocytogenes* in milk and cheeses (Rodríguez et al., 2001; Muñoz et al., 2004, 2007).

However, in traditional cheeses, nonindigenous bacteriocinogenic strains cannot simply be applied as adjunct cultures because they may negatively influence the typical microflora needed to obtain the characteristic organoleptic properties and diversity of such products. For this reason, bacteriocinogenic strains suitable for application as protective cultures in cheeses should be found in the indigenous microbiota.

The prevalence of plantaricin A genes in cheeses and in viable lactobacilli populations, the less frequent detection of helveticin J and acidocin B, and the absence of some other lactobacilli bacteriocins observed in this study is in accordance with the observations of Majhenič et al. (2007), who reported that *Lactobacillus plantarum* was found to be the most representative species of Kraški ewes' milk cheese, whereas *Lb. helveticus* and *Lactobacillus acidophilus* were not. Enterocin P is usually produced by *E. faecium* and *E. durans* strains, cytolysin by *E. faecium* and *E. faecalis*, and enterocins A, B, L50A, and L50B exclusively by *E. faecium* strains (Foulquié Moreno et al., 2006). Interestingly, in this study, enterocins typically produced by *E. faecium* prevailed over those typical for *E. faecalis* in cheese and consortia samples, although *E. faecalis* was previously recognized as the predominant enterococcal species of the viable microflora of the Tolminc cheese at the end of ripening (Majhenič et al., 2005).

In conclusion, the presence of 6 different *Enterococcus* bacteriocin genes, 2 *Lactococcus* bacteriocin genes, and 3 *Lactobacillus* bacteriocin genes was established in traditional Tolminc cows' milk cheese and Kraški ewes' milk cheese, or in different consortia (i.e., viable populations of lactobacilli, enterococci, or lactococci derived from the cheeses), or in cheeses and consortia. These results indicate that bacteriocinogenic strains of the 3 genera are common members of the indigenous microbiota of these traditional cheeses. In future studies we will focus on the isolation of particular bacteriocin producers from cheese, especially those with wider spectra of activity such as enterocins, nisin, and acidocin B, which could be good candidates for custom-made natural adjunct cultures suitable for particular traditional cheeses and intended to improve the safety of raw milk cheeses. Whether or not the particular bacteriocin genes are also expressed in situ in cheeses and thus play a certain protection role against the development of potentially pathogenic bacteria needs to be elucidated.

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2.2 *In-situ* INHIBITION OF *Staphylococcus aureus* BY LACTIC ACID BACTERIA CONSORTIA FROM TWO TRADITIONAL SLOVENIAN RAW MILK CHEESES

In-situ inhibicija *Staphylococcus aureus* s strani združb mlečnokislinskih bakterij tradicionalnih slovenskih sirov

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Mlečnokislinske bakterije, ki so naravno prisotne v tradicionalnih sirih in proizvajajo bakteriocine, predstavljajo neizčrpen vir mikrobov z zaščitnim potencialom. Učinkovitost nekaterih od teh bakteriocinov proti stafilokokom je že bila opisana. Prisotnost genov za bakteriocine s potencialno proti-stafilokokno aktivnostjo so že ugotovili v dveh tradicionalnih sirih iz surovega mleka, v siru Tolminc in Kraškem ovčjem siru. Iste gene za bakteriocine so zasledili tudi v kultivabilnih združbah mlečnokislinskih bakterij, izoliranih iz sirov na gojiščih Rogosa, M17 in CATC. Cilj raziskave je bil pojasniti, ali so mikrobne združbe, v katerih so bili ugotovljeni geni za bakteriocine, dejansko sposobne proti-stafilokokne aktivnosti v mleku in/ali siru. V ta namen smo izbrali različne mikrobne združbe glede na raznovrstnost odkritih genov za bakteriocine v njih in jih uporabili v preskusih inhibicije *Staphylococcus aures* v mleku in siru. V mleku, kjer smo sledili temperaturno-časovnemu režimu izdelave tradicionalnih sirov, so vse izbrane mikrobne združbe sirov učinkovito zavrle rast *Staphylococcus aureus* za približno 2 do 3 log ke. V siru je bila inhibicija stafilokokov manj izrazita, saj se je njihovo število zmanjšalo za približno 1,5 log ke. Izključili smo inhibicijo zaradi mlečne kisline, ali so za inhibicijo odgovorni bakteriocini, bo potrebno še dokazati.

Ključne besede: tradicionalni siri, varnost živil, združba MKB, proti-stafilokokna aktivnost

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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 Matijašić 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" (Trmčić 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 (Trmčić 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 (Trmčić 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 (Trmčić 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 (Trmčić 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						Nis	Lac481	AcidB	HelvJ	PlnA
	A	B	P	L50A	L50B	Cyt					
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 approximately10^2 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 10g of cheese or milk as described by Bogovič Matijašić 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-

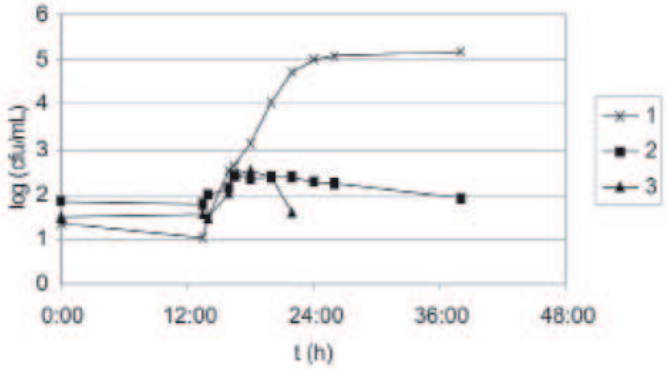

Figure 1. Growth of *Staphylococcus aureus* in milk with or without the presence of "Tolminc" (T2) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

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)

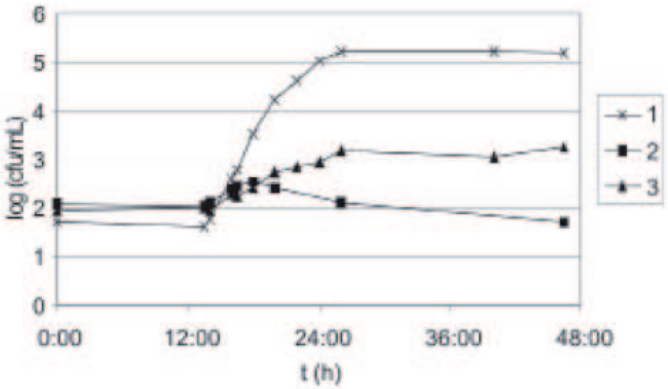

Figure 2. Growth of *Staphylococcus aureus* in milk with or without the presence of "Kraški ovčji sir" (K3) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

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

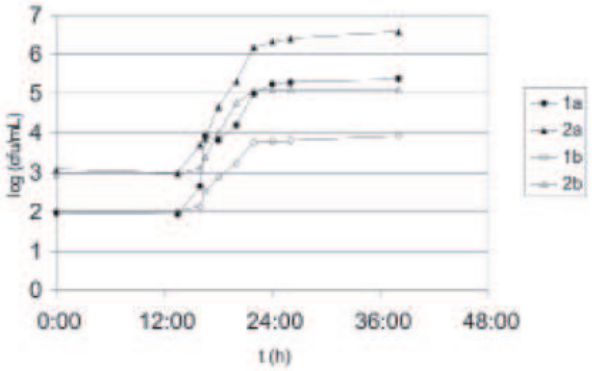

Figure 3. Growth of *Staphylococcus aureus* in milk in the presence of lactic acid: starting inoculation 10^2 cfu/mL non-adjusted pH level (1a), starting inoculation 10^3 cfu/mL non-adjusted pH level (2a), starting inoculation 10^2 cfu/mL pH level adjusted to 5.0 (1b), starting inoculation 10^3 cfu/mL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

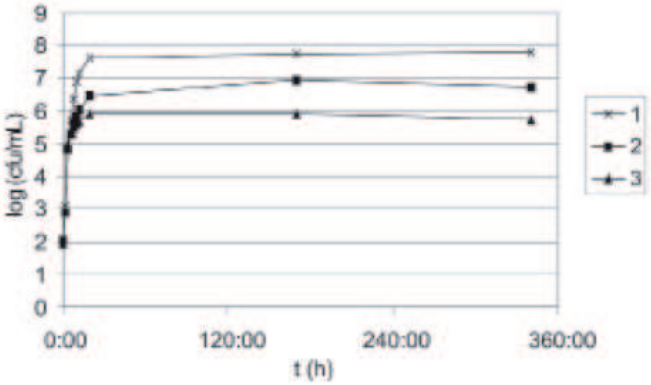

Figure 4. Growth of *Staphylococcus aureus* in cheese with or without the presence of "Tolminc" (T2) cheese microbial consortia: microbial consortia not present (1), lactobacilli consortia present (2), complete cheese microbial consortia present (3)

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 observation 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 mater 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 phenomena, 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

Acknowledgements

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2.3 COMPETITIVE ADVANTAGE OF BACTERIOCINOGENIC STRAINS WITHIN LACTIC ACID BACTERIA CONSORTIUM OF RAW MILK CHEESE

Kompetitivna prednost bakteriocinogenih sevov znotraj združbe mlečnokislinskih bakterij sira iz surovega mleka

Aljoša Trmčić, Tanja Obermajer, Andreja Čanžek Majhenič, Bojana Bogovič Matijašić in Irena Rogelj

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Prisotnost genskih determinant za različne bakteriocine je bila že raziskana v tradicionalnih slovenskih sirih iz surovega mleka Tolminc in Kraški ovčji sir. Ti geni so bili prisotni tudi v kultivabilni mikrobni populaciji. V tem delu smo skušali ugotoviti, ali se prisotnost genskih determinant za bakteriocine v mikrobni združbi odraža tudi v njeni protimikrobni aktivnosti. Poleg tega smo želeli ugotoviti, ali imajo sevi, ki nosijo genske determinante za bakteriocine, kakšno kompetitivno prednost pri rasti v mikrobni združbi. Mikrobno združbo iz sira Tolminc smo zaporedno precepljali v mleku in na koncu preverili njeno protimikrobno aktivnost in prisotnost genskih determinant za bakteriocine. S primerjavo rezultatov pred in po precepljanju smo ugotovili, da se večina sevov, ki nosijo genske determinante za bakteriocine, ni uspela ohraniti znotraj združbe. Sevi, ki so se uspeli ohraniti, so vsebovali genske determinante za enterocine P, L50B in citolizin. Protimikrobna aktivnost pred in po precepljanju se ni bistveno spremenila in je ne moremo pripisati kateremu od preiskovanih bakteriocinov.

Ključne besede: tradicionalni sir, bakteriocinski geni, bakteriocini, protimikrobna aktivnost, kompeticija

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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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2.4 CHARACTERIZATION OF BACTERIOCINOGENIC STRAINS OF LACTIC ACID BACTERIA FROM TRADITIONAL SLOVENIAN CHEESE 'TOLMINC'

Opis bakteriocinogenih sevov mlečnokislinskih bakterij iz tradicionalnega slovenskega sira Tolminc

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Namen te raziskave je bil raziskati, ali so v tradicionalnem slovenskem siru Tolminc prisotne mlečnokislinske bakterije, ki proizvajajo znane bakteriocine. Prisotnost genskih determinant za različne bakteriocine je bila v tej vrsti sira dokazana že v predhodnih raziskavah, kakor tudi prisotnost bakteriocinskih genov ter protimikrobna aktivnost kultivabilne mikrobne populacije. Ker ni bilo mogoče potrditi povezave med prisotnimi genskimi determinantami za bakteriocine in dejansko protimikrobno aktivnostjo mikrobne združbe, smo v tej raziskavi preverili še iste lastnosti na nivoju posameznih bakteriocinogenih sevov. Podobno kot v predhodnih rezultatih so enterokoki in njihovi bakteriocini prevladali v mikrobni združbi sira. Nobeden od izoliranih sevov ni zaviral rasti *Staphylococcus aureus*, medtem ko so posamezni sevi inhibirali ostale indikatorske seve. Večina osamljenih sevov je vsebovala genske determinante za citolizin. Na osnovi ugotavljanja genskih determinant za bakteriocine, protimikrobne aktivnosti, fenotipizacije s sistemom PhP (PhenePlate^{TM}) in PCR identifikacije smo opazili nekatere podobnosti med izolati rodu *Enterococcus*.

Ključne besede: tradicionalni sir, bakteriocinski geni, bakteriocini, enterocini, enterokoki, protimikrobna aktivnost

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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 ^{kp}	1 ^{kp}	0	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G0x30/3	1 ^{kp}	/	/	/	/	/	/	/
G0x30/4	2 ^{kp}	1 ^{kp}	0	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G0x42/1	1 ^{kp}	1 ^{kp}	0	0	1 ^{kp}	2 ^{kp}	1 ^{kp}	1 ^{kp}
G0x42/2	1 ^{kp}	0	0	0	1 ^{kp}	1 ^{kp}	0	1 ^{kp}
G0x42/9	1 ^{kp}	1+4 ^{kp*}	6 ^{k}	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G0xR/1	5 ^{kp}	0	0	0	4 ^{k**}	0	1 ^{kp}	4 ^{k**}
G0xR/2	5 ^{kp}	/	/	/	/	/	/	/
G10x30/4	2 ^{k}	1+6 ^{kp*}	7 ^{k}	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G10x42/1	1 ^{kp}	/	/	/	/	/	/	/
G10x42/2	2 ^{kp}	/	/	/	/	/	/	/
G10x42/3	1 ^{kp}	1+4 ^{kp*}	6 ^{k}	0	1 ^{kp}	5 ^{kp}	1 ^{kp}	1 ^{kp}
G10x42/3.1	6 ^{kp}	5 ^{kp}	4 ^{kp}	0	1 ^{kp}	3 ^{kp}	3 ^{kp}	0
G10x42/5	2 ^{kp}	/	/	/	/	/	/	/
G10x42/6	1 ^{k}	/	/	/	/	/	/	/
G10x42/8	2 ^{kp}	/	/	/	/	/	/	/
G10x42/9	2 ^{kp}	1+4 ^{kp*}	6 ^{k}	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G10x42/10	1 ^{kp}	/	/	/	/	/	/	/
G10x42L/1	/	2 ^{kp}	/	/	/	/	/	/
G10x42L/2	/	2 ^{kp}	/	/	/	/	/	/
G10x42L/3	/	2 ^{kp}	/	/	/	/	/	/
G10x42L/4	1 ^{kp}	1+4 ^{kp*}	6 ^{kp}	0	1 ^{kp}	1 ^{kp}	1 ^{kp}	1 ^{kp}
G10x42L/5	/	2 ^{kp}	/	/	/	/	/	/
Ec. faecium ATCC 19434	8 ^{kp}	6 ^{kp}	6 ^{kp}	0	6 ^{kp}	3 ^{kp}	3 ^{kp}	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

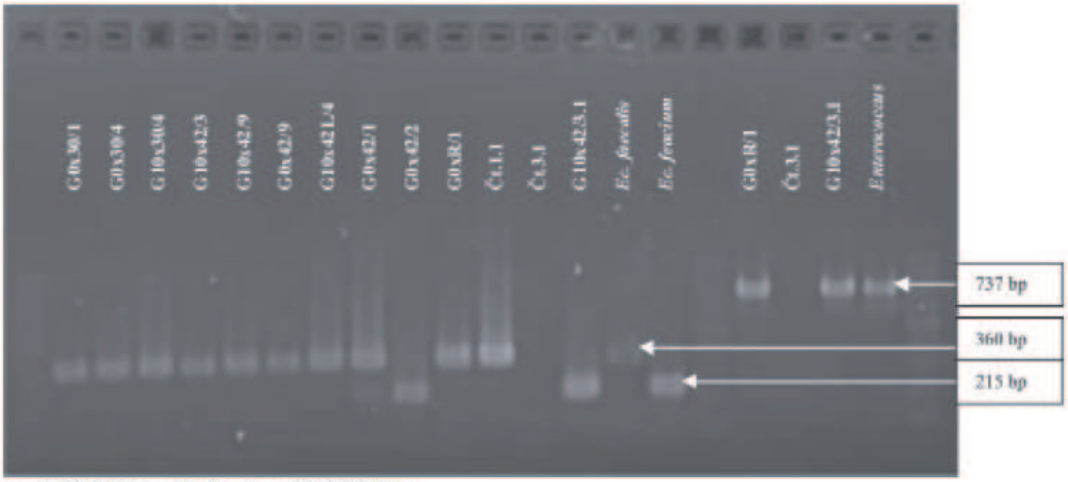
Isolate designation Oznaka izolata	Presence of gene determinant for bacteriocin Prisutnost genske determinante za bakteriocin									
	Enterocin						Nizin/Nisin	Lakticin 481 Lacticin 481	Acidocin B	Plantaricin A
	A	B	P	L50A	L50B	Cytolysin Citolizin				
G0x30/1	-	-	-	-	+	+	-	-	-	-
G0x30/4	-	-	-	-	-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

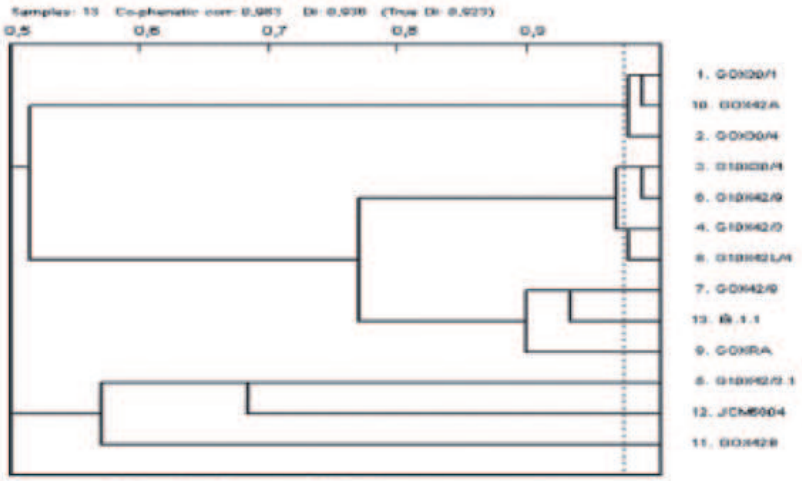


Figure 2. Dendrogram of PhP classification of enterococci
Slika 2. Dendrogram enterokoka svrstanih u PhP grupe

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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2.5 EXPRESSION OF NISIN GENES IN CHEESE – A QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION APPROACH

Izražanje genov nizina v siru – pristop s kvantitativno verižno reakcijo s polimerazo v realnem času

Aljoša Trmčić, Christophe Monnet, Irena Rogelj in Bojana Bogovič Matijašić

Journal of Dairy Science 94, 77-85 (2011b)

Vloga bakteriocinov v različnih okoljih še ni bila popolnoma pojasnjena, v veliki meri zaradi težav, ki so povezane z njihovim odkrivanjem. Nizin, protimikrobni peptid, ki ga proizvaja *Lactococcus lactis,* ima že dolgo zgodovino varne uporabe v živiliskih izdelkih in je bil preučen s številnih aspektov genetike, biosinteze, imunosti, regulacije in načina delovanja. Še vedno pa so nekateri mehanizmi, ki so povezani z dinamiko izražanja genov za nizin, nepojasnjeni. Zelo malo raziskav izražanja bakteriocinov je bilo narejenih v kompleksnih okoljih, kot je naprimer sir. Glavni cilj te študije je bil ugotoviti, ali se geni za nizin izražajo tudi v siru med zorenjem. V ta namen smo na podlagi testiranja rasti v mleku in bakteriocinske aktivnosti izbrali sev *L. lactis* M 78, izolat iz surovega mleka, ki producira nizin A. Izražanje vseh 11-ih genov, vključenih v biosintezo nizina, smo spremljali v siru podobnem mediju, s pomočjo reverzne transkripcije in PCR v realnem času. Skupno RNA smo osamili neposredno iz modelnega sira, po metodi brez predhodnega ločevanja bakterijskih celic. Sev M 78 je dobro rastel v eksperimentalnem siru ter izločal nizin, ki ga je bilo mogoče izmeriti že po štirih urah fermentacije. Prisotnost nizina kot aktivatorja je spremenila tako izražanje genov nizina kot končno akumulacijo aktivnega nizina. Na osnovi razlik v izražanju genov kot funkcije časa smo posamezne gene lahko razvrstili v štiri skupine: *nisA, nisFEG, nisRK in nisBTCIP*. Na osnovi rasti producenta nizina, aktivnosti nizina, funkcije genov nizina in njihove lokacije smo določili korelacije, ki pomagajo pri razlagi regulacije biosinteze in imunosti. Ta študija je prva, pri kateri so bili intenzivno kvantificirani transkripti genov za bakteriocin v siru podobnem mediju.

Ključne besede: nizin, izražanje genov, reverzna transkripcija in verižna reakcija v realnem času, sir

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Expression of nisin genes in cheese—A quantitative real-time polymerase chain reaction approach

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ABSTRACT

The role of bacteriocins in different environments has not been thoroughly explained, mainly because of the difficulties related to the detection of their production. Nisin, an antimicrobial peptide produced by *Lactococcus lactis* has a long history of safe use in food products and has been studied from many aspects of genetics, biosynthesis, immunity, regulation, and mode of action. Still, some aspects concerning the dynamics of nisin gene expression remain unknown, especially in complex media like cheese. The main objective of the present study was to quantify in a cheese-like medium the expression of nisin genes in *L. lactis* M78, a well-characterized nisin A producer isolated from raw milk. The expression of all 11 genes involved in nisin biosynthesis was evaluated during cheese production by real-time reverse transcription-PCR. Total RNA was extracted from cheeses using a direct extraction method without prior separation of microbial cells. The M78 strain grew well in experimental cheeses, producing detectable amounts of nisin after 4 h of fermentation. The presence of nisin as an activator modified both the expression of nisin genes and the accumulation of active nisin. Four groups could be distinguished based on gene expression as a function of time: *nisA*, *nisFEG*, *nisRK* and *nisBTCIP*. Based on nisin-producing strain growth, nisin activity, function of nisin genes, and their location, correlations were established that contribute to the explanation of regulation of nisin biosynthesis and immunity. This study is the first in which the evolution of bacteriocin gene transcripts has been quantified rigorously in a cheese-like medium.

Key words: nisin, gene expression, real-time reverse transcription-polymerase chain reaction, cheese

INTRODUCTION

Bacteriocin-producing lactic acid bacteria are gaining trust for use as starter cultures and as means for improving the safety of fermented foods. The role of bacteriocins in different environments and control of the mechanisms of bacteriocin production and activity, however, is still not thoroughly explained, mainly because of the difficulties related to their detection in complex environments. Nisin, a ribosomally synthesized lantibiotic naturally produced by several strains of *Lactococcus lactis*, is one of the best characterized lactic acid bacteria bacteriocins. The potential of nisin-producing starters to control undesirable microorganisms in cheese has been already demonstrated (Maisnier-Patin et al., 1992; Yezzi et al., 1993; Rodríguez et al., 1998; Bouksaim et al., 2000; Ayad, 2009). Several variants of nisin are known, with nisins A and Z being the most common, differing in one nucleotide and in one AA respectively. The nisin gene cluster is located on a large conjugative transposon and is composed of 11 genes, *nisABTCIPRKFEG* (Figure 1), involved in complex biosynthesis, regulation, and immunity. Among these genes, *nisA* encodes a nisin A precursor peptide of 57 AA residues (pre-nisin), which is a structural peptide (pro-nisin) attached to a leader peptide; *nisB* and *nisC* encode modification enzymes (dehydration and cyclization) involved in post-translational modification of pro-nisin; *nisT* encodes a transport protein involved in secretion of modified pre-nisin outside the cell (ABC transporter); *nisP* encodes a membrane-bound serine protease which cleaves pre-nisin, releasing the active pro-nisin; *nisI* encodes a lipoprotein involved in self-protection against nisin; *nisFEG* encode transport proteins also involved in self-protection (ABC transporter); and *nisR* and *nisK* encode a response regulator and a histidine kinase which form a two-component signal transduction system involved in regulation of nisin biosynthesis activated by nisin. Transcription of nisin genes is facilitated from 3 major promoter sites of which the promoter proceeding *nisR* is constitutive, whereas the *nisA* and *nisF* promoters are controlled by the 2-component regulatory system NisRK. An alter-

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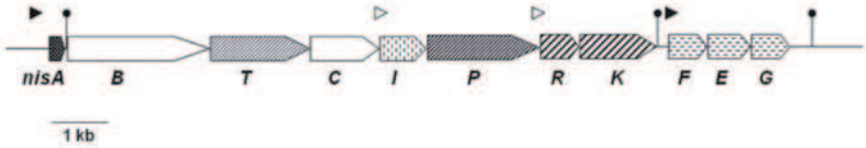

Figure 1. Organization of genes involved in nisin production, regulation, and immunity. Black and white triangles indicate nisin-inducible and constitutive promoters, respectively. Hairpins indicate putative transcription terminator loops. Genes are as follows: *nisA*, nisin structural gene; *nisB* and *nisC*, post-translational modification; *nisT*, translocation; *nisP*, activation; *nisR* and *nisK*, regulation; *nisI* and *nisFEG*, immunity.

native induction system exists for the *nisA* promoter that functions independently of NisRK. In fact, the galactose metabolite of the Leloir pathway can induce *nisA* transcription from the same start site. In addition to the *nisR* promoter, another constitutive promoter has been identified before *nisI*. The function of this promoter may be to establish a basal nisin immunity before the cell encounters nisin for the first time (Li and O'Sullivan, 2002, 2006). For a complete review of nisin biosynthesis, immunity, regulation, mode of action, and engineering, see Lubelski et al. (2008).

The structure of the nisin cluster where most of the genes are overlapping (GenBank accession numbers X68307, L16226, X76884, and U17255) indicates translational coupling (Kuipers et al., 1993). However, *nisA* is separated from the rest of the cluster by an inverted repeat which may act either as a transcription terminator or translation processing site (Engelke et al., 1992; Kuipers et al., 1995; Dodd et al., 1996). Two TCT-N_8-TCT repeats are located at the positions −39 to −26 and −107 to −94 upstream of the *nisA* transcription start site. A single TCT-N_8-TCT repeat is also located at the positions −39 to −26 upstream of the *nisF* transcription start site. Although the nisR can initiate transcription by binding to one of the TCT-N_8-TCT repeats, it needs 2 repeats for optimal binding, which is the reason for weaker expression of *nisFEG* than *nisA* (de Ruyter et al., 1996; Chandrapati and O'Sullivan, 1999, 2002; Penna et al., 2005). The linear response of the *nisA* and *nisFEG* promoters in response to nisin, together with the constitutive nature of the *nisR* promoter has been demonstrated (Kuipers et al., 1995; de Ruyter et al., 1996).

Kuipers et al. (1995) detected expression controlled by the *nisA* promoter to be 50 times higher for *nisA* than for the genes located after the inverted repeat that separates *nisA* from the other *nis* genes. On the other hand, Runar Ra and Saris (1995) indicated that all nisin genes are transcribed as 2 large polycistronic transcripts, *nisZBTCIPRK* and *nisFEG*, regulated by 2 nisin-dependent promoters (Ra et al., 1996). It is possible that polycistronic transcripts are cleaved during further processing into separate single-gene transcripts (Buchman et al., 1988; Steen et al., 1991; Engelke et al., 1992). Higher stability of *nisA/Z* transcripts can be another reason for more elevated concentrations of them (Runar Ra and Saris, 1995; Li and O'Sullivan, 2006). Using immunoblotting, Engelke et al. (1994) showed that the peptide encoded by *nisA* is present in the cell before the peptide encoded by *nisB*. According to the speculations of De Vuyst and Vandamme (1992), this may explain why nisin activity coincides more with expression of *nisB* than with expression of *nisA*.

The main objective of the present study was to quantify in a cheese-like medium the expression of bacteriocin genes, using a nisin producer as a model organism. We have chosen a nisin producer because nisin genes have been well characterized, and also because it has already been demonstrated that nisin can be produced in situ in cheese. However, a similar approach may also be used for other bacteriocins. In addition, despite several studies on nisin, the time-dependent expression of all 11 genes involved in nisin biosynthesis and immunity has not yet been reported. Expression of the nisin genes was compared with the growth of producer strain, acid production, and accumulation of active nisin in cheese.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The nisin-producing strain *L. lactis* M78, an isolate from raw milk (Samelis et al., 2009), was kindly provided by J. Samelis (National Agricultural Research Foundation, Dairy Research Institute, Ioannina, Greece). It was confirmed as a nisin A producer by sequence analysis of the entire nisin gene cluster (GenBank accession number HM219853). The nisin-susceptible strain *Lactobacillus sakei* ATCC 15521 was used as the indicator strain in biological assay for nisin quantification. *Lactoccocus lactis* S3 (UMR GMPA, INRA, Thiveval-Grignon, France), which does not produce nisin, was used as a negative control. *Lactococcus* and *Lactobacillus* strains were routinely grown under static conditions at 30°C in

M17 lactose broth (Terzaghi and Sandine, 1975) and in De Man, Rogosa, and Sharpe (MRS) broth (De Man et al., 1960), respectively.

Cheese Production

Experimental cheeses were made using ultrafiltered milk (5.5% total fat) produced in UMR1253 (STLO, INRA, Rennes, France), as described by Hannon et al. (2006) and stored at −20°C. Cheese cultures were prepared by inoculation of sterile (100°C/10 min) reconstituted skim milk (100 g/L; Difco Laboratories, Detroit, MI) with M78 strain and incubation at 30°C for 15 h. Ultrafiltered milk was inoculated at a concentration equivalent to 10^7 cfu/mL and divided into 2 parts. One part was supplemented with 1.2 IU/mL of nisin A (Sigma, St-Quentin-Fallavier, France). After the addition of 60 μL/L of chymosin (Maxiren 180, DSM Food Specialties, Delft, the Netherlands), each part was divided into 15 aliquots to make 15 small model cheeses of 70 g. Coagulation of ultrafiltered milk resulted in model cheeses being formed without release of the whey. Model cheeses were incubated at 30°C for 24h, which is near the optimal growth temperature of the nisin-producing strain M78. After 6 h of fermentation, the cheeses were transferred onto grids in sterile crystallizing basins and the incubation continued at 30°C.

Cheese Analysis

Cheeses with and without added nisin were sampled at 4, 6, 9, 12, and 24 h. At each sampling, 3 entire cheeses were taken as 3 separate parallels and each of them was analyzed. Besides RNA extraction, the bacterial counts and nisin production were determined, and pH was measured using a Blue line 27 surface electrode (Schott, VWR, Fontenay-sous-Bois, France).

Bacterial Growth. One gram of cheese was homogenized with 9 mL of 2% trisodium citrate dihydrate (pH 7.5) using a mechanical blender (Ultra-Turrax T25, Ika Labortechnik, Staufen, Germany). The resulting suspension was 10-fold serially diluted in tryptone-salt broth (9.5 g/L) and plated in triplicate on M17 agar plates. Colonies were enumerated after 48 h of incubation at 30°C.

Nisin Production. The procedure introduced for nisin quantification in cheese was based on previously published methods (Tramer and Fowler, 1964; Wolf and Gibbons, 1996; Pongtharangkul and Demirci, 2004). Cheese extracts were prepared by homogenizing 1 g of cheese in 1 mL of 0.02*M* HCl using a mechanical blender. The cheese homogenates were heated in a water bath at 90°C for 5 min. After cooling to room temperature, they were centrifuged (10 min at 5,000 × *g*) and the resulting supernatants were filter-sterilized (0.22 μm). Cheese extracts were obtained also from cheeses produced with strain S3, which does not produce nisin. For the standard curve, standard samples were prepared from cheese homogenates supplemented with a known amount of nisin. Agar plates for nisin activity assay were prepared from 2 layers of MRS agar; the base layer contained 1.5% of agar-agar, and the cover layer 0.75% of agar-agar. In addition, the cover layer was supplemented with 1% (wt/wt) of Tween 40. After sterilization (120°C/20 min) and cooling to 55°C, the base layer (20 mL) was poured into the plates (90 mm in diameter) and left to solidify and dry (12 h) at room temperature. The cover soft agar was incubated at 55°C (12 h) and before being poured (20 mL) over the base layer, it was inoculated with 2% of a fresh culture (18 h) of the indicator strain *L. sakei* ATCC 15521. After solidification of the soft agar at room temperature (2 h), 5-mm wells were punched into the cover layer using the back end of a Pasteur pipette. The wells were immediately filled with 70 μL of cheese extract and plates placed at 4°C for a 24-h pre-diffusion step. After pre-diffusion, the plates were incubated at 30°C for 24 h and the diameter of the inhibition halos measured using a caliper horizontally and vertically to the nearest 0.1 mm.

Expression of Nisin Genes. The priority of cheese sampling was to get cheese samples through the first step of extracting RNA. Extraction of RNA from cheeses was done as described previously (Monnet et al., 2008), except that the amount of cheese processed in each tube was increased to 500 mg, using 7-mL bead-beating tubes that contained 3.2 g of zirconium beads (diameter, 0.1 mm; Biospec Products, Bartlesville, OK). After addition of 5 mL of TRIzol reagent (Invitrogen, Cergy Pontoise, France), the tubes were vigorously shaken in a bead beater (Precellys24 Dual, Saint-Quentin-en-Yvelines, France) by using two 20-s mixing sequences at a speed of 6,500 rpm. The tubes were cooled on ice for 5 min after each mixing sequence. After the bead-beating procedure, the tubes were placed at −80°C until all of the samples for RNA extraction had been collected. To ensure a sufficient amount of extracted RNA, the content of 2 tubes was subsequently pooled and processed using 15-mL Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany), and RNA was purified using a single RNeasy spin column (Qiagen, Courtaboeuf, France). The RNA was eluted from spin columns with 30 μL of RNase-free water.

Purified RNA was quantified at 260 nm using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA integrity number

Table 1. Sequences and target genes of primers (F = forward, R = reverse) used in this study

Gene	Gene product	Primer sequence (5′→3′)	Reference
16S rRNA	16S ribosomal RNA	F: GCTCACCAAGGCGATGATACATA R: ACCAACGTTCTTCTCTACCAACA	Monnet et al., 2008
23S rRNA	23S ribosomal RNA	F: ACAGGATAGGTAGGAGCCATT R: CGATTATGCCAGCGGGTTAG	Monnet et al., 2008
nisA	Nisin structural gene	F: TCAGGTGCATCACCACGCATTA R: TTACGTGAATACTACAATGACAAGTTGCT	This study
nisB	Nisin biosynthesis protein	F: GGGAGAGTTGCCGATGTTGT R: TAAAGCCACTCGTTAAAGGGCAAT	This study
nisT	Nisin transport ATP-binding protein	F: CAACTTTCAGGAGGTCAGTGG R: AGCTACAGGATCGAGTGCAG	This study
nisC	Nisin biosynthesis protein	F: CAAATCGGAGAATCAGATGTCTCAAT R: GCTAAGATACAGCCCACTCCAG	This study
nisI	Nisin immunity protein	F: CTGAAGTACGTGTGTTTGATTCAGTTAG R: TCGCCATAATCCCATTCCGTC	This study
nisP	Nisin leader peptide-processing serine protease	F: TGCACCGAGTGTATTTGAGGATGT R: GCAGGAGCATAAATTGCATCCG	This study
nisR	Nisin biosynthesis regulatory protein	F: GAGAGGAACGCAATAAACATGCAG R: GTTTGACCATTGACACAGACTCG	This study
nisK	Nisin biosynthesis sensor	F: CGAACGTGATATGAAACACTACCC R: ACTAGGTACATCCGACTTGACATAA	This study
nisF	Nisin transport/immunity protein	F: TGGATCCTGATGGTATTGCGG R: AGCTACTTTACTTATTTCGTGCAACTG	This study
nisE	Nisin transport/immunity protein	F: TTGATTTGGCTAAGAGGTATACTAGCG R: ATAAAGGGTAAATTCCAAGAAGCTGC	This study
nisG	Nisin transport/immunity protein	F: GGCCAATCGTTTCCAAGAAATAAATGT R: CCCTACTGCCTGTGAAACTCC	This study

was determined with the 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) using RNA 6000 NANO chips according to the manufacturer's instructions. The concentration of extracted RNA was adjusted to 25 ng/μL, and DNase treatment was performed using the TURBO DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. Absence of DNA contamination in RNA samples was confirmed with non-reverse transcribed samples (controls). Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Cergy Pontoise, France) with random hexamer priming and RNaseH treatment. The reaction mixture contained 200 ng of DNase-treated RNA in the final volume of 20 μL. The reverse transcription procedure was performed according to the manufacturer's recommendations.

Oligonucleotide primers were designed using the LightCycler probe design software (v1.0; Roche Applied Science, Mannheim, Germany) and synthesized by Eurogentec (Seraing, Belgium). The thermocycling program consisted of initial denaturation at 95°C for 8 min, followed by 45 cycles of denaturation (95°C/10 s), annealing (60°C/7 s), and extension (72°C/6 s). Fluorescence acquisition (530 nm) was done at the end of each extension. After real-time PCR, a melting curve analysis was performed by measuring fluorescence during heating from 65 to 95°C at a transition rate of 0.1°C/s. Quantification cycle ($\mathbf{C_q}$) values were determined with LightCycler software (version 3.3), using the second derivative method. Standard curves were generated by plotting C_q values as a function of the log cDNA concentrations. From the slopes of the standard curves, the PCR efficiency (E) was calculated for each primer pair using the following formula: $E = 10^{-1/slope}$ (Pfaffl, 2004). The genes investigated in the present study and the corresponding primer pairs are presented in Table 1. Real-time PCR measurements of each sample were performed using 1/5 and 1/10 dilutions of the reverse-transcribed RNA. The resulting C_q values were used to calculate the expression of selected genes by relative quantification using a reference gene and a calibrator (Eq. 3.5; Pfaffl, 2004). We tested 16S rRNA and 23S rRNA respectively to be used as reference genes. The last sampling (24 h), corresponding to the cheese without added nisin, was chosen as the calibrator. Absence of inhibitors in both reverse transcription and real-time PCR was confirmed by comparison of standard curves obtained with serially diluted samples before and after reverse transcription.

Statistical Analyses

At each sampling time, 3 separate cheeses were sampled. For the determination of nisin activity, each cheese was used to produce one cheese extract and one end result. For the determination of nisin gene expression, each cheese was used to obtain one RNA extract and real-time PCR analyses were performed using 2

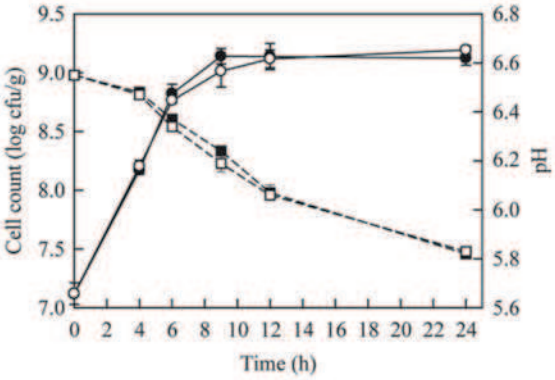

Figure 2. Dynamics of growth of *Lactococcus lactis* M78 and of pH in the model cheeses. Results are expressed as mean values of 3 repetitions. Closed and open symbols correspond to cheeses with and without addition of 1.2 IU/mg of nisin, respectively. Circles correspond to cell counts and squares to pH.

dilutions of the cDNA. Results from the 3 replicate cheeses were used to calculate mean values and standard deviations, which represented both the methodological and the experimental errors.

RESULTS

Bacterial Growth and Nisin Production in Milk and Cheese

Expression of nisin genes was measured during 24 h of manufacturing cheese with and without addition of nisin. Growth of *L. lactis* M78 was similar in cheeses with and without added nisin (Figure 2). Exponential phase was observed until 6 h of fermentation with growth rate constants 0.94 and 0.91/h, respectively for cheeses with and without added nisin. After about 12 h, *L. lactis* M78 reached the stationary phase in both types of experimental cheeses. Final cell count in cheeses with and without added nisin was 9.12 ± 0.06 and 9.19 ± 0.04 log cfu/g, respectively. The pH value at 24 h was 5.83 ± 0.1. Nisin production was very different between the 2 types of experimental cheeses (Figure 3). As expected, addition of a small amount of nisin (1.2 IU/g) increased production of nisin.

Extraction of RNA from Cheese and Real-Time Reverse Transcription-PCR Analysis

A prerequisite for successful expression quantification is the extraction of good quality RNA from cheese. The yield of RNA after extraction was 4.9 (±0.7) $\times 10^{-6}$ ng/cfu and 2.4 (±0.2) $\times 10^{-6}$ ng/cfu for samples obtained at 4 and 24 h, respectively. The RNA integrity number values of the RNA samples were always above 8.5, which indicates that RNA was not degraded and was, thus, appropriate for real-time reverse transcription-PCR analysis. The absence of contaminating DNA in RNA samples was confirmed by the absence of amplification in samples that were not subjected to reverse transcription. The absence of inhibitors of reverse transcription and of amplification reactions in the samples was confirmed by comparison of the standard curves obtained using serial dilutions of samples both before and after reverse transcription, which had identical slopes and intercepts. The efficiency of the real-time PCR amplifications was always at least 94% and not more than 100%. The C_q values of the non-template controls were always above 38 cycles, and more than 10 cycles above the C_q values of the samples with the lowest target concentration. No significant variation of 16S and 23S rRNA abundance in the 2 experimental cheeses (the amount of RNA that was reverse-transcribed was standardized) existed and the 16S rRNA gene was used as the reference gene in expression quantification. Although we did not calculate the detection limit we always worked within the range of standard curves developed for this study.

Expression of Nisin Genes During Cheese Manufacture

Relative expression ratios of *nis* genes in cheeses with and without added nisin are presented in Figure 4. From these data, 4 expression groups of nisin genes can

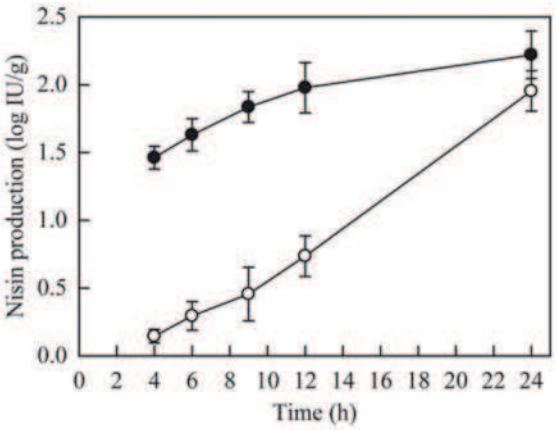

Figure 3. Nisin production by *Lactococcus lactis* M78 in the model cheeses. Results are expressed as mean values of 3 repetitions. Closed and open symbols correspond to cheeses with and without addition of 1.2 IU/mg of nisin, respectively.

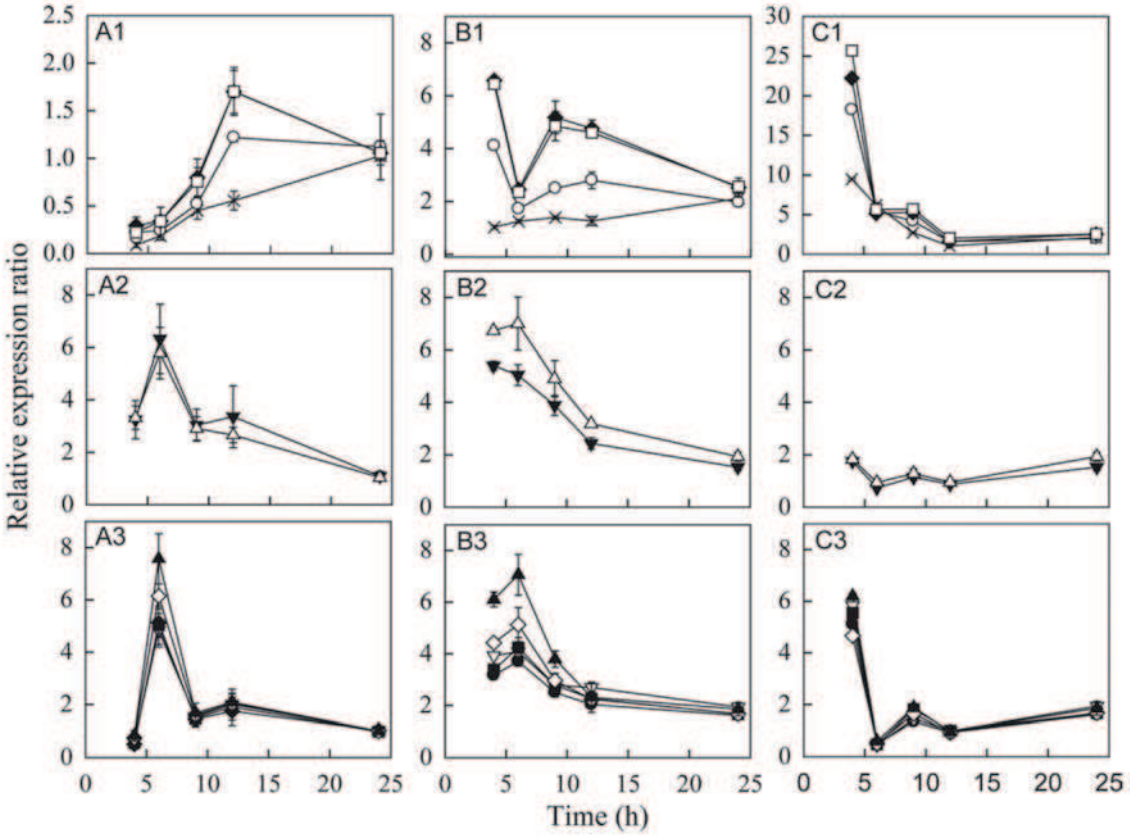

Figure 4. Evolution of the relative expression ratio of nisin genes in model cheeses without (A) or with (B) addition of 1.2 IU/g of nisin, and ratio between the 2 types of cheeses (C). Symbol designations are as follows: 1: *nisA* (×), *nisF* (○), *nisE* (♦), and *nisG* (□); 2: *nisR* (▼) and *nisK* (△); 3: *nisB* (●), *nisT* (▽), *nisC* (■), *nisI* (◊), and *nisP* (▲). Expressions are relative to the sample at 24 h without added nisin (calibrator), and error bars represent the standard deviations of 3 biological repetitions. When error bars are not visible, they fall within the symbol.

be distinguished: *nisA*, *nisFEG*, *nisRK*, and *nisBTCIP*. Expression of the *nisA* gene is higher in the presence of external nisin. The highest difference was at 4 h of fermentation, which corresponds to a 10-fold difference in expression. The ratio decreased gradually to a 2-fold difference at 24 h of fermentation (Figure 4, C1). The 3 immunity genes *nisFEG* showed a similar expression evolution as a function of time, whereas different profiles were observed between fermentations with or without added nisin. In cheeses without addition of nisin, an increase from 4 to 12 h and a decrease at 24 h was observed (Figure 4, A1). In the presence of nisin, expression determined at 4 h was higher by factors from 18 to 26 (Figure 4, C1). For each of the 2 types of fermentation, the genes *nisRK* showed a similar evolution of expression as a function of time (Figure 4, A2 and B2). A slight difference in *nisRK* expression was observed at 4 h, where it was approximately 2 times higher in cheeses with added nisin (Figure 4, C2). For the last group of nisin genes (*nisBTCIP*), expression reached its maximum at 6 h of fermentation (Figure 4, A3 and B3). At 4 h, expression in cheeses with added nisin was about 5 times higher than in cheeses without added nisin (Figure 4, C3).

DISCUSSION

The aim of the present study was to evaluate the expression of the genes involved in the production of nisin, a well-characterized bacteriocin, in cheese. We used a nisin-producing strain, *L. lactis* M78, which was isolated from raw milk. This strain was found to

demonstrate better growth and acidifying activity in milk and cheese than the other 2 nisin-producers tested (results not shown), which are generally regarded as weak acid producers and unable to coagulate milk (Froseth and McKay, 1991; De Vuyst and Vandamme, 1992; Takala and Saris, 2007). The fact that this strain is proteinase positive (A. Trmčić, unpublished data) and able to ferment lactose (Samelis el al., 2009) may explain its ability to grow well in milk and cheese. The growth was also expected to be influenced by nisin that was added in a commercial preparation form to one series of experimental cheeses. According to Rauch et al. (1994), nisin is supposed to prolong the lag phase of growth without affecting the growth rate or cell yield of nisin-producing strains. This phenomenon was not observed in our study because our test strain entered the exponential growth phase immediately after inoculation in the milk, with or without nisin supplementation.

The second and main effect expected from addition of nisin to the cheese-milk was stimulation of its own production by nisin producer present in cheese. Nisin is a known self-inducer and this characteristic was also confirmed in our study. Nisin activity at the first sampling (4 h) was about 10-times higher when nisin was present in cheese-milk at inoculation. The concentration of added nisin (1.2 IU/g, which is equivalent to 0.08 log IU/g) was below detection limit and was, thus, negligible in comparison to that of the nisin produced by strain M78, at least after 4 h of fermentation. We also observed a strong correlation between production of nisin and expression of its genes. In cheese with added nisin, an overexpression of the nisin genes *nisABTCIPFEG* was detected. This overexpression was the highest at 4 h of fermentation and from that point on, it varied considerably as a function of time. Four different groups were distinguished according to expression as a function of time: *nisA*, *nisBTCIP*, *nisRK*, and *nisFEG*.

Genes *nisFEG* are under the control of a nisin-dependent promoter and this is in agreement with our results. The presence of nisin in cheese stimulated the initial expression of *nisFEG* by 18 to 26 times, and expression was at least 2-times higher by the end of the experiment. Additionally, in cheese with no nisin added, an increase of *nisFEG* expression was observed from 4 to 12 h. This was probably due to bacterial production of nisin and its accumulation in cheese. The genes *nisFEG* encode for nisin immunity proteins; therefore, the likely explanation is that higher concentrations of nisin also demand a higher protection of the nisin-producing strain and, consequently, a higher production of immunity proteins.

The expression of the second nisin-dependent promoter, located upstream of *nisA*, was strongly induced by the presence of nisin in cheese. In contrast to the expression stimulation of *nisFEG*, where expression increased during the first 12 h of fermentation, *nisA* showed an increase in expression during the entire 24 h of fermentation. Although in both types of cheeses the increase of *nisA* expression was observed, in those without added nisin the increase was more pronounced (10-fold) compared with the 2-fold increase in cheeses with added nisin. Some other studies reported that expression of *nisA* and production of nisin were most extensive during the exponential growth phase and ceased completely in the stationary phase (Engelke et al., 1994; Takala and Saris, 2007). However, the results of this study showed different dynamics. Both *nisA* expression and nisin production by *L. lactis* M78 were still observed during the stationary phase and even increased in cheeses without added nisin. Expression of *nisA* and production of nisin at 4 h of fermentation were about 10-fold higher in cheeses with added nisin. A linear correlation between the expression of *nisA* and nisin activity in cheeses could be observed throughout the entire experiment. Linear correlation is presented by the equation $y = 0.735x$, with y representing the relative expression ratio and x representing nisin activity (log). Such a correlation was expected because the *nisA* gene encodes for the structural protein that is converted to active nisin. Kuipers et al. (1995) and de Ruyter et al. (1996) also observed a linear correlation between expression and activity of nisin in the study on *nisA* promoter activity in the presence of nisin.

All of the genes involved in the formation of active nisin, together with the immunity gene *nisI*, form a cluster of overlapping genes, which are located downstream of *nisA* (Kuipers et al., 1993; Li and O'Sullivan, 2006). The gene overlapping and, consequently, the transcription coupling, also were confirmed in this study by the equal expression dynamics of these genes. Additionally, the expression dynamics of *nisBTCIP* genes differed from the expression of *nisA*, which points out the presence of individual *nisA* transcripts besides large polycistronic transcripts, as already reported by Runar Ra and Saris (1995) and Ra et al. (1996). The transcription of *nisA* into 2 different types of transcripts may be due to the inverted repeat that is present between *nisA* and *nisB*, and may cause a limited read-through during transcription from the *nisA* promoter (Kuipers et al., 1995; de Ruyter et al., 1996). Steen et al. (1991) also mentioned the possibility of *nisA* being released from a larger polycistronic transcript after processing, where the inverted repeat may also have a role. Dependence of *nisBTCIP* genes on the induction by nisin was confirmed by the results of measured expression at 4 h of fermentation, when the levels of expression were 4- to 6-times higher in cheeses with added nisin,

compared with those without added nisin. Maximum expression was reached after 6 h of fermentation. The point of maximum expression does not seem to affect the correlation between *nisA* expression and accumulation of active nisin as would be expected (De Vuyst and Vandamme, 1992; Engelke et al., 1994). No obvious correlation between nisin production and expression of *nisB* was found, although the expression dynamics of *nisB* were completely comparable with those reported by Engelke et al. (1994). Although the experimental design did not allow us to make absolute comparisons among expressions of different nisin genes, we could estimate the absolute expression from the C_q values. The C_q values for the *nisA* gene were always the lowest of all examined nisin genes, which means that this transcript was the most abundant one. Several mechanisms may explain this observation. One reasonable conclusion would be that *nisA* transcripts are more stable than the other nisin gene transcripts (De Vuyst and Vandamme, 1992; Li and O'Sullivan, 2006). The promoter upstream of *nisA* can also be activated by galactose or lactose, which can account for higher expression of this gene in milk and cheese (Chandrapati and O'Sullivan, 1999; Penna et al., 2005; Li and O'Sullivan, 2006). Lower expression of *nisFEG* genes compared with *nisA* expression is consistent with the results of de Ruyter et al. (1996) and the fact that the promoter upstream of *nisF* contains only one of 2 binding sites present in the nisin-dependent promoter located upstream of *nisA* (Chandrapati and O'Sullivan, 2002). A second constitutive promoter located upstream of *nisI* has been reported, enabling nisin-producing strains to establish immunity before the presence of nisin in the environment (Li and O'Sullivan, 2002, 2006). Because our sampling started during the exponential growth phase, the nisin present at this time already acted as an inducer and might, thus, mask any transcription initiation from this constitutive promoter. In fact, no difference of expression dynamics could be observed between *nisIP* and *nisBTC*.

Genes *nisRK* encode for regulatory proteins that transfer the signal from extracellular nisin to the promoters upstream of *nisA* and *nisF*. These regulatory proteins genes have to be expressed constantly, to enable the nisin-producing strain to respond to the presence of nisin in the surrounding environment. Because of this, *nisRK* genes are under the control of a nisin-independent promoter. But this constitutive expression was only partly confirmed in the present study because the number of transcripts at the beginning and the end of the experiment was about 2-times higher in the cheeses with added nisin. In addition, a significant variation of *nisRK* transcript quantity as a function of time was found. If this is not a result of a change in RNA stability, it could raise some doubt about the exclusive constitutive expression of these 2 genes. The study of Ra et al. (1996) showed that these genes may be part of a large *nisZBTCIPRK* transcript. In that case, *nisRK* could be transcribed as 2 different transcripts, which might explain the observed variation of transcript quantity as a function of time. Furthermore, it was noticed that, except at 4 h, the transcription profile of *nisRK* was similar to that of *nisBTCIP*.

CONCLUSIONS

In the present study, we succeeded in following the expression of 11 nisin genes from *L. lactis* M78 in model cheeses by real-time reverse transcription-PCR. This study is the first one in which the evolution of transcripts has been quantified rigorously in a cheese-like medium. Beside the contribution to the explanation of regulation of nisin biosynthesis and immunity, this study presents an example of what could be done in the future to better understand the role of bacteriocins in different environments and the physiology of microorganisms growing in cheese.

ACKNOWLEDGMENTS

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3 DISCUSSION AND CONCLUSIONS (RAZPRAVA IN SKLEPI)

3.1 DISCUSSION

3.1.1 Comparison of different DNA extraction procedures

With advances in molecular biology and microbial ecology we have gained numerous new tools that are useful in the analysis of complex microbial populations. But still most of the studies describing the search for specific genetic determinants like bacteriocin gene determinants are mainly limited to the screening of individual isolates. Two exceptions are Moschetti et al. (2001) and Bogovič Matijašić et al. (2007) that reported successful detection of specific bacteriocin sequences in total bacterial DNA extracted directly from cheese. Both studies were limited to qualitative analysis of cheese microbiome (DNA), demonstrating the presence of bacteriocinogenic strains in cheese. The approach used in the present study is unique as we used a molecular method for the initial screening of cheese microbiome for bacteriocin determinants, followed by the search for interesting bacteriocinogenic strains by cultivation methods. This procedure is very specific, applicable only when searching for strains that produce already known bacteriocins. Its main advantage is better efficiency, since we can limit our search to the samples of cheese already proven to contain the gene determinants of bacteriocins which we are interested in.

Testing different procedures of total bacterial DNA extraction from cheese we found out that the choice of diluent for homogenization of cheese is very important ($p<0,005$) (Annex A). Using sodium citrate solution we were able to obtain the highest concentration of extracted DNA. Sodium citrate is one of the solutions recommended by International Dairy Federation for microbiological analysis of cheese (IDF standard 122B:1992). This is probably because of chelating properties of sodium citrate, which enables good disruption of cheese matrix and consequently effective release of the present microbiota into the solution. The more effective the release of microbiota into the solution is, the more this microbiota and total DNA from it is representative for the analysed cheese. We came to similar conclusions based on detected gene determinants in isolated microbial consortia. The highest number of gene determinants was detected in individual cheeses homogenised before DNA extraction in sodium citrate.

The method used for DNA extraction from cheese homogenate also had a minor influence (marginal statistical significance, $p\leq0,05$) on DNA extraction efficiency (Annex A). The extraction with the commercial Wizard® Genomic DNA Purification Kit (Promega) proved to be more successful than the extraction with phenol-chloroform-isoamyl alcohol (Cocolin et al., 2001). It would be expected that the likelihood of detection of certain gene

determinant depends mostly on bacterial DNA concentration. In our research this was confirmed only when sodium citrate was used for homogenization of cheese. Even though the extraction yield of DNA was usually better when commercial kit was used, compared to the phenol-chloroform-isoamyl alcoholprotocol, the latter proved to be better concerning the heterogeneity, i. e. the number of detected bacteriocin gene determinants, and stability of the extracted DNA. Amagliani et al. (2007) also reported lower success of amplification of specific sequences when DNA was extracted using commercially available extraction kits rather than conventional DNA extraction method with phenol-chloroform-isoamyl alcohol. Besides the two methods we used in this study, Mohar Lorbeg (2008a) tested six additional methods and came to the same conclusion. Namely, methods based on phenol-chloroform-isoamyl alcohol extraction resulted in more representative DNA, as evaluated with PCR using specific oligonucleotide primers for *Enterococcus* (*Ec*.) genus. Beside that Mohar Lorbeg (2008b) also evaluated, in her unpublished experiments, the extracted DNA with universal oligonucleotide primers for quantification of total DNA according to Barakat et al. (2000). When comparing the results from the two reactions it turned out that reaction for genera *Enterococcus* was in some cases negative even though the reaction with universal primers indicated a high concentration of extracted DNA. From these observations we can presume that the extraction of DNA from individual strains of the microbial population is much dependent on the method of extraction, since DNA from different strains may not be equally represented inside the total extracted DNA. Similar conclusions can be made also on the basis of our results, which showed that higher concentration of DNA did not necessarily coincided with the diversity of detected representatives. In general, the extraction of higher concentrations of DNA with better purity, which is with lower amount of potential PCR inhibitors, demands longer, multistep extraction procedures (Wilson, 1997).

3.1.2 The presence of bacteriocins' gene determinants in cheese and isolated microbial consortia

The strains of *Enterococcus* genera are very well represented in traditional Slovenian cheeses (Čanžek Majhenič et al., 2005; Katana, 2001), which is also reflected in the high detection frequency of enterocin gene determinants. From the bacteriocins of lactobacilli, gene determinants of plantaricin A were detected in all nine cheeses, indicating that *Lactobacillus* (*Lb*.) *plantarum* is also well represented in traditional Slovenian cheeses. This was already reported for Kraški ewe's cheese (Čanžek Majhenič et al., 2007).

Since production technology of Kraški and Tolminc cheese varieties involves cooking of the curd at 45°C and the use of thermophilic starter cultures is allowed, it would be reasonable to examine the presence of gene determinants for bacteriocins of *Streptococcus* (*Str*.) *thermophilus,* usually named thermophilins. Another group of species which is

among the most common ones in Slovenian and other traditional raw milk cheeses is *Lb. casei* group (Čanžek Majhenič et al., 2007; Pisano et al., 2007; Wouters et al., 2002). Bacteriocins described so far for this group are lactocin 705 (Cuozzo in sod., 2003), bacteriocin 217 (Lozo in sod., 2004), bacteriocin SJ (Lozo in sod., 2007), bacteriocin UB9 (Fira in sod., 2008), paracin 1.7 (Ge in sod., 2009) and bacteriocin HL32 (Pangsomboom in sod., 2009). We were unable to find oligonucleotide primers for these bacteriocins in the available literature. The one set of primers we designed for lactocin 705, on the basis of sequence available in GenBank were not very successful since the product turned out to be non-specific.

Most of the bacteriocin gene determinants were detected in both DNA from cheeses and DNA from microbial cheese consortia. From this observation we can presume that most of the gene determinants are present in a cultivable LAB population of cheeses. However, some gene determinants, for example the ones for nisin, were only detected in DNA extracted directly from cheeses (T2, T3, and T4). A reason for this can be that target DNA sequences were extracted from the non-cultivable bacteria. On the contrary, the observation of gene determinants for nisin in the DNA from microbial consortia only can be explained with low concentration of lactococci in cheese that carry nisin genes (Čanžek Majhenič et al., 2007; Gobbetti et al., 1999). Lactococci are mesophillic LAB that don't grow well at high temperatures of curd cooking applied in the hard cheese production (Beresford and Williams, 2004; O'Sullivan D, 2000). Similarly, we detected gene determinants for enterocin L50A in microbial consortia of four cheeses and helveticin J gene determinants in microbial consortia of three cheeses but not directly in cheeses. Here, the concentration of target DNA in the cheeses was probably also below detection limit while cultivation of microbiota on suitable solid growth media leaded to the enrichment of viable cell population containing the target DNA.

The diversity of detected gene determinants was generally higher in samples of Tolminc cheeses compared to the samples of Kraški ewe's cheese. The reasons for this may be found in the characteristics of milk used for cheese production (animal species, geographic region) as well as in the technological procedure. Results for cheeses K1, K2 and K3 indicate that technology of cheese production most probably involved thermal treatment of raw milk which basically inactivated most of the naturally present microbiota. The specification of the technological protocol for production of cheese Kraški ewe's cheese otherwise permits the thermal treatment of raw milk.

3.1.3 Antimicrobial activity of isolated microbial consortia

Even though the results showed lower diversity of bacteriocin gene determinants in Kraški ewe's cheese than in Tolminc cheese, there was no obvious difference in the antimicrobial activity of their microbial consortia (Annexes B and C). The antimicrobial activity was never detected with the supernatants, obtained after cultivation of consortia in liquid growth media (MRS, M17), or with the concentrated supenatants. Obviously the concentration of synthesized and excreted bacteriocins or their stability was poor. When testing microbial consortia with a spot test where the bacteria are applied directly on the surface of the agar, the bacteriocins accumulate locally around the spot and thus can reach much higher concentrations than in liquid media. Another well known fact is that bacteriocin activity can be lost during acquisition and storage due to their adsorbtion to the surface of producer bacteria or degradation by proteolytic enzymes. Moretro et al. (2000) also reported that bacteriocins were accumulated in the liquid media to lesser extent than in the solid media, and that they were under constant process of degradation. The results obtained from testing the cultivable microbial consortia showed that the number of detected bacteriocin gene determinants alone did not correlate with the actually observed antimicrobial activity of this consortia, or that the presence of a wide range of bacteriocin genes did not assure a wide range of inhibition. This finding was also tested in a separate experiment (Trmčić et al., 2010a) where we examined the *in situ* anti-staphylococcal activity in milk and cheese. We tested activity of microbial consortia from two cheeses, of which the first one (T2) had the highest number of detected bacteriocin gene determinants and the second one (K3) the lowest. Even though the microbial consortia from cheese T2 was more effective at inhibiting staphylococci, we could not connect this activity to the action of any bacteriocins. These results raised the question about how individual bacteriocinogenic strains within the complex microbial population produce bacteriocins and whether these strains are represented in a sufficient number within the population to be able to exert detectable bacteriocin activity. To get at least a partial answer to these questions we subsequently propagated mixed microbial consortium of cheese T2, composed of three consortia grown on three different media (Rogosa, M17 and CATC), in milk and followed the changes of profile of detected bacteriocin gene determinants and antimicrobial activity during this process (Trmčić et al., 2011a).

3.1.4 The effect of microbial consortium propagation on the presence of bacteriocin gene determinants and its antimicrobial activity

During the subsequent propagation of T2 consortium in milk, the number of detected bacteriocin gene determinants was reduced from initial eight to three which indicates that the strains containing most of these bacteriocins' genes determinants were unable to successfully multiply and persist within the microbial consortium. On the other hand,

strains containing gene determinants for enterocins P, L50B and cytolysin were able to persist during propagation and in some cases even proliferated. During cheese production, enterococci are usually present in high concentrations in the beginning phases while are outgrown by lactobacilli in the later phases. Evidently, by daily propagation of T2 consortium we stimulated the growth of enterococci within the population, which can be the reason for the absence of gene determinants for plantaricin A. The gene determinants for plantaricin A were, apart from the determinants for enterocin P and cytolysin, the most common in both cheese and corresponding microbial consortia (Trmčić et al., 2008). Besides the differences in detected bacteriocin gene determinants, the changes in antimicrobial activity were also observed during propagation, both reflecting the changes in microbiota composition. In contrast to the original microbial consortia, after propagation there was no strong inhibition of enterococci, the only antimicrobial activity preserved was against indicator strains *Listeria* (*L.*) *monocytogenes* (Annex E), *L. innocua* and *Lb. sakei.* The obtained inhibitory spectrum is comparable to the antimicrobial activity of strain *Ec. faecium* ATCC 19434 (Annex F), which produces enterocins A, B and P. Besides above mentioned strains this bacteriocinogenic strain also inhibited all three indicator strains from *Enterococcus* genus (*Ec. faecalis* ATCC 19433, *Ec. faecium* CCM 4647, *Ec. durans* CCM 5612). The antimicrobial activity obtained with *Ec. faecium* ATCC 19434 are in accordance with the results of Park et al. (2003) obtained with the same strain. Since this strain produces additional two bacteriocins whose gene determinants were not detected in propagated microbial consortium and since there were also other bacteriocins' genes determinants present in DNA of propagated microbial consortium, we decided to continue with the analysis of the antimicrobial activity and of the presence of bacteriocin gene determinants on the level of individual strains, representatives of the original and propagated microbial consortia of cheese.

3.1.5 The presence of bacteriocin gene determinants in individual isolates and their antimicrobial activity

We isolated individual colonies that exhibited inhibition of indicator strains *L. monocytogenes* and *Lb. sakei* on M17 (30°C, 42°C) as well as on modified MRS agar, The only two strains isolated from modified MRS agar, generally intended for lactobacilli, turned out to grow better on M17 plates and were finally identified as *Enterococcus* strain. There were a lot of reports on bad selectivity of LAB growth media (Menendez et al., 2001). We expected from the chosen growth media primarily to support growth of different groups which they were formulated for, in order to obtain as many microbiota representatives as possible. The selectivity was not so important in this context. The only growth medium used in this study that was found very selective was CATC for enterococci. During our work we established that *Ec. faecium* strains (G0x42/2 and

G10x42/3.1) grew very poorly or not at all on CATC media (Annex J). This is a problem already mentioned by Reuter (1992). Because of this, an unintentional selection of enterococci occurred on CATC growth media.

We found particular similarities among the strains when examining the antimicrobial activity against indicator strains *L. monocytogenes* and *Lb. sakei*. As the double inhibition halos of protein nature were detected with both individual strains and with propagated mesophilic consortium (Trmčić et al., 2010b, 2011a, Annexes E and H), we presumed that this microbial consortium was predominantly composed of only a few strains which we were able to isolate from the consortium. Among ten representative isolates, we identified by species specific PCR eight as *Ec. faecalis* and two as *Ec. faecium.* The same two groups were identified using typization with PhP system. Within *Ec. faecalis* group we identified three smaller groups of related strains, while the three *Ec. faecium* strains together with *Ec. faecium* ATCC 19434 did not show any close relation.

When looking for mutual linkage between antimicrobial activity and detected bacteriocin gene determinants we were unable to come to any solid conclusions. All ten analysed strains of enterococci carried gene determinants for cytolysin, which is fairly uncommon for enterococci coming from cheese (Mohar Lorbeg, 2008; Katana Burja, 2006; Čanžek Majhenič et al., 2005), and even more uncommon for *Ec. faecium* species. De Vuyst et al. (2003) for example, identified among 37 tested enterococci strains only one *Ec. faecium* strain with gene determinants for cytolysin. Even more interesting is the fact that we identified in three strains of *Ec. faecalis*, gene determinants for enterocin L50B and in one of them additional determinants for enterocin A, B, P, L50A, which are characteristic for *Ec. faecium* species. There is a possibility that some isolates were not pure. Namely, when isolating individual strains from complex environments we can sometimes come across strains that grow in symbiosis and are despite thorough purification process very hard to separate. In case of G0x42/1, the multiplex PCR analysis revealed a positive result for species *Ec. faecalis* and *Ec. faecium*, which confirmed our anticipation that this culture was actually a mix of two strains, representatives of two species.

Among indicator strains used for testing antimicrobial activity there was also *Ec. faecium* ATTC 19434. Our intention was to see whether observed antimicrobial activity can be attributed to any bacteriocin(s) other than enterocin A, B or P. It turned out that all of the isolated strains demonstrated equal (1mm) inhibitions of this indicator strain (Annex G). Similar inhibition, demonstrated by all isolates, was observed against indicator strain *Ec. durans* CCM 5612. This inhibition was attributed to cytolysin because this was the only one whose genes were found in all isolated strains. Since cytolysin has also a haemolytic property we decided to test all isolates for haemolytic activity on solid growth media. The

results of De Vuyst et al. (2003) indicated that most of the strains containing gene determinants for cytolysin were also capable of β haemolysis. None of tested strains exhibited α or β haemolysis. Based on this observation we assumed that the inhibition demonstrated by isolated strains was not directly connected to cytolysin, but could be a result of genotypic/phenotypic feature that appears in combination with cytolysin.

The only isolate that demonstrated somehow different antimicrobial characteristics was G0xR/1 strain. The activity against *Ec. durans* CCM 5612 (Annex I) as well as against *Ec. faecium* ATCC 19434 (Annex G) was identical (4 mm). The protein nature of inhibition of the two indicator strains could not be clearly confirmed, using two proteolytic enzymes (chymotrypsin and proteinase K). Despite this, the appearance of the inhibition (very clear halos with well defined edge), indicated that it was a result of bacteriocin-like activity. Since only the gene determinants for cytolysin were detected in G0xR/1 strain, it was not possible to conclude which bacteriocins were responsible for the observed antimicrobial activity. The strain G0xR/1 was identified as *Ec. faecalis* and for this species there were at least five other enterocins described whose gene determinants were not looked for in our study (Galvez in sod., 1998; Eguchi in sod., 2001; Franz in sod., 2002; Martín-Platero in sod., 2006).

3.1.6 The problem of isolating bacteriocinogenic strains from cheese

Of numerous bacteriocin gene determinants identified within microbial consortia of cheese T2, we found only a few in the individual strains isolated from these consortia. The main reason for such results is probably the procedure used to isolate these strains. As we isolated microbial consortia and single strains only from countable agar plates (plates with 30-300 colonies), we limited the screening to the population which is most abundant in cheese and overlooked the strains that were less represented. We selected only the strains that exhibited inhibitory action towards indicator strains which is a relatively easy way of searching for bacteriocinogenic strains. It is however well known that the presence of genetic determinants for individual protein does not also assure its synthesis, and also the synthesised proteins are not necessarily excreted and active. That is why expression of bacteriocin genes is of key importance in search and application of interesting strains. The results of the study also showed that the ability to produce bacteriocins by itself does not necessarily assure a competitive advantage to such a strain. It has to be taken into consideration that traditional type cheeses, produced from raw milk, have a very diverse microbiota in which single strains hardly prevail. Inside microbial population there is a certain balance between different bacteriocinogenic strains, where both, ability to produce bacteriocins and ability to be resistant to bacteriocins of others, play a role. The strains inside a population that produce different bacteriocins inhibit each other to a certain extent

since ability to produce one bacteriocin does not bring resistance to other bacteriocins. In general, microbial ecology does not necessarily attribute the same importance to the role of bacteriocins in strains competitiveness as it does to the role of other microbial interactions, like competition for nutrients between representatives with the same environmental demands and others (Beresford et al., 2001; Bersford and Williams, 2004). A reason for that is probably also because there is a lack of studies on actual role of bacteriocins *in vivo*, in microbial ecosystems.

3.1.7 The problem of connecting detected bacteriocin gene determinants with observed antimicrobial activity

In this research, we were only rarely able to make concrete connections between exerted antimicrobial activity and detection of bacteriocin gene determinants. We encountered this problem when testing consortia as well as individual strains. The discrepancy between the number of detected gene determinants and the actual antimicrobial activity determined with bioassay shows the complexity that connects these two features. It has to be taken into consideration that we narrowed our search to bacteriocins that were described so far and were, according to the data of other researchers, expected to be found in these consortia. Bacteriocins selected for the screening probably present only a small part of all bacteriocins that can be produced by the members of these consortia. As a consequence, bacteriocin activity observed in bioassay can be a result of expression of bacteriocin genes that were not included in PCR analysis. In addition, many of isolates posess gene determinants for more than one bacteriocin, which may interact among each other, on the expression or final activity level. We are also limited with the selection of sensitive indicator strains and of proteolytic enzymes used for the confirmation of protein nature of inhibition, as different bacteriocins are inactivated by different proteolytic enzymes.

Biological methods for determination of bacteriocin activity (bioassays) also have several limitations especialy for application in complex media like cheese. Among others, there is high limit of detection, uneven distribution of bacteriocins in cheese matrix, adsorption of bacteriocins onto proteins, degradation of bacteriocins by extracellular and intracellular bacterial peptidases, which are released from lysed cells. In addition, we cannot detect any activity of individual bacteriocins when more than one is present in the sample. On the other hand the molecular methods have proved to be useful, as already reported in other similar studies (Moschetti et al., 2001, Bogovič Matijašić et al., 2007). Specific and sensitive molecular methods could be upgraded to allow detection of gene determinants that are also expressed in cheese matrix.

3.1.8 The application of the molecular methods for detection of bacteriocins' genes expression in cheese

Methods based on reverse transcription (RT) and real-time polymerase chain reaction (real-time PCR) are successfully being used for determination of gene expression. RT real-time PCR is a method which through quantification of gene transcription to messenger ribonucleic acids (mRNA), allows not only qualitative determination of gene expression but also quantitative. Despite numerous benefits of RT real-time PCR, the studies are limited to examination of cultures in liquid media and there are less examples of its use in complex samples (Ramiah et al., 2007; Granger et al., 2008). In our study of production of bacteriocins *in situ* in cheese the method proved to be useful, presenting a more specific alternative to bioassay methods. The success of the method is very much dependant on the quantity and quality of extracted RNA. RNA extraction from complex media like cheese is difficult because RNA itself is very unstable and additionally there is also a high content of proteins, fats, RNA degrading enzymes and residues of DNA which affect the RNA isolation. Extraction of RNA from cheese demands procedures, adapted to this rich matrix. Until now five methods were proposed (Bonaïti et al., 2006; Ulve et al., 2008; Monnet et al., 2008; Makhzami et al., 2008; Duquenne et al., 2010), of which the method developed by Monnet et al. (2008) stands out the most, since it is the only direct method, which means it doesn't demand cell separation from the cheese matrix.

Transcription and degradation of transcripts is terminated in the first step of the RNA extraction. This is achieved by immediate homogenisation of cheese sample in Trizol® reagent, disrupting the cheese matrix and cells with zirconium beads. The result obtained by this method gives a more real picture of transcription at a given time, compared to other methods that involve separation of cells from the cheese matrix. We decided to test this method of RNA extraction and try to quantify the expression of bacteriocin genes in cheese (Trmčić et al. 2011b). For this purpose we used a sterile cheese model inoculated with monoculture of strain *Lactococcus* (*Lac.*) *lactis* M 78, which produces bacteriocin nisin A (Annexes K and L).

The method described by Monnet et al. (2008) proved to be effective and applicable but, as expected, the final amount of extracted RNA was lower compared to the methods that involve separation of cells from cheese matrix. To acquire sufficient amount of RNA, in some instances, we performed the extraction in several parallels (500 mg) and combined them in the final step (elution with water). For the same reason, we started with the sampling when cell concentration reached 10^8 colony forming units (CFU) per gram of cheese. The total quantity of extracted RNA was between 1088 and 4625 ng. This quantity of extracted RNA was sufficient since we standardized the reverse transcription reaction to

200 ng. The manufacturer of reverse transcription kit (Invitrogen, 18080-051) claims that the reaction can even be performed with 1 pg of total RNA. The main problem in RNA extraction is maintaining the RNA stability during the procedure. The measurements of concentration on NanoDrop ND-1000 and RIN numbers (RNA Integrity Number) on 2100 Bioanalyzer demonstrated that the total extracted RNA was intact and suitable for analysis with RT real-time PCR. We also excluded the presence of any DNA contaminations or inhibitors of RT real-time PCR reaction in the RNA extracts. Even though the quantity of extracted RNA was relatively low in comparison to the quantities that could be obtained by the methods that involve separation of cells from cheese matrix, the real value of this method is representativeness of the final result. Representativeness means that the results reflect the actual state and this is by all means the main factor in choosing an appropriate method of analysis. In this study we confirmed applicability of the method of RNA extraction developed by Monnet et al. (2008).

No matter which method of RNA extraction is used, there are two major challenges in using the RT real-time PCR method in real cheese samples. The first one is how to achieve an accurate and reliable normalization of RT real-time PCR measurements in cheese samples where several microbial species are present. In relative quantification the measured values are usually normalised to the total cellular RNA (Ulve et al., 2008; Monnet et al., 2008; Makhzami et al., 2008). Since this approach is less applicable for real complex samples, in our case cheese, one or more reference genes are used instead (Duquenne et al., 2010). In our work and work of Monnet et al. (2008), the amount of 16S and 23S ribosomal RNA proved to be a good representative of total amount of RNA from corresponding strain and therefore were suitable for use as normalisation references in the studies with pure cultures. The problem is that cheeses, especially the ones produced from raw milk, contain numerous, more or less related strains that share common genetic features. Even if we knew exactly the microbial composition of each cheese it would be very hard to design oligonucleotide primers specific enough for a given bacteriocinogenic strain. The validation of reference genes within the experimental conditions used is the most important step in this kind of analysis. Instead of using these internal references, there is also a possibility of using external references in a form of exogenously spiked known quantities of RNA (DeCoste et al., 2011). The problem with this kind of normalisation is similar as the one with total RNA and absolute quantification. The results using this kind of normalisation provide information about gene expression of the entire strain population. It is however impossible to gain information on a single cell level if we do not know the number of cells inside this population. As already mentioned, this presents a problem when analysing cheese samples with several related strains.

At the moment it seems that the method which we successfully applied in the expression quantification in a pure culture cheese model, could serve in real cheese samples at most as a qualitative rather than quantitative method. These kinds of studies have been already performed in cheese (Bonaiti et al., 2006) and in other complex environments like river sediments (Nogales et al., 2002). The procedure of qualitative analysis could be similar as the one described in the publication Trmčić et al. (2008), with the difference that presence of gene determinants would not be determined in metagenomic DNA but in the cDNA derived from metagenomic RNA. The results of this kind of analysis would include not only the information about the presence of certain gene determinants, but also about its active expression in cheese. With this a second challenge is presented in using this method for analyzing real cheese samples. The microbial population in real cheese samples is in late stationary or constant die-off phase and lysis. Most of the hard and semi-hard cheese varieties are ripened for two or more months and minimal ripening period of traditional Slovenian cheeses is usually 60 days. During our work we detected transcription of nisin genes during 24 hours only, while in some other studies the successful transcript quantification was still obtained after 3, 7 and 14 days of fermentation/ripening period (Duquenne et al., 2010; Makhzami et al., 2008; Monnet et al., 2008). Ulve et al. (2008) performed their analysis during six months of ripening period but the quantity and quality of extracted RNA was already low after six months. They also reported that the process of cell lysis had a great effect on extracted RNA and that this was the reason for unsuccessful extraction from three commercial French cheeses. On the other hand Duquenne et al. (2010) managed to extract larger quantities of RNA from three commercially available semi-hard cheeses, but even though they demonstrated a good quality of extracted RNA they didn't perform any analysis to confirm the presence of mRNA within total RNA. A sufficient amount of mRNA can probably be extracted only from actively growing cells. It is also known that the expression of bacteriocin genes is mostly present during the second half of the exponential growth phase and it stops soon after the start of the stationary growth phase. Our results showed also a somewhat different expression dynamics for some of the genes (Trmčić et al., 2011b).

The results of determination of nisin gene expression during 24 h fermentation of cheese model are described in the publication Trmčić et al. (2011b). The set cheese model proved to be very useful. We have chosen nisin as a test bacteriocin because it is the most studied bacteriocin of LAB, its *in situ* production in cheese has already been established, it is produced in high amounts and its activity can be measured *in situ* relatively easy, using classical bioassay. There are 11 genes involved in nisin production, immunity and regulation. Like many researchers before, we proved the expression of nisin genes *in situ* in cheese (Maisnier-Patin et al., 1992; Yezzi et al., 1993; Rodriguez et al., 1998; Bouksaim et al., 2000; Rodriguez et al., 2005; Ayada, 2009). In addition, we supported and compared

the results of bioassay method with the results of RT real-time PCR analysis. We found a strong correlation between the results of both methods, which proves the tight relation between transcription of nisin genes and its final activity which is a result of its biosynthesis and excretion from the cells. Based on the transcription of nisin genes as a function of time we were able to distinguish among four groups of genes: *nisA*, *nisBTCIP*, *nisFEG* and *nisRK*. By comparison of the expression dynamics of these four groups of genes, their function and location in the gene cluster, the growth dynamics and antimicrobial activity of producer strain, we were able to prove some already known facts, like the fact that nisin acts as a *quorum sensing* pheromone and that it stimulates its own production (van der Meer et al., 1993; Kuipers et al., 1995; Ra et al., 1996; Kleerebezem, 2004). We also showed some correlations in the expression of individual nisin genes that can help explain regulation of biosynthesis and immunity of bacteriocin nisin. This study is the first one presenting the expression of all 11 nisin genes, and one of the first ones that presents rigorous, time dependent quantification of transcripts in a cheese-like medium.

3.2. CONCLUSIONS

According to our set hypothesis we came to the following conclusions:

- Traditional Slovenian cheeses produced from raw milk contain in their rich microbiota also LAB strains able to produce different bacteriocins.

- Microbiota of traditional Slovenian cheeses produced from raw milk contains LAB strains which collectively or individually inhibit outgrowth of potentially pathogenic *Staphylococcus aureus* in milk and cheese. We were unable to correlate observed inhibitory action with bacteriocin activity.

- *In situ* in milk, only a few bacteriocin producing strains of LAB demonstrated a competitive advantage in the microbial consortia. This observation may be partly a consequence of test conditions which favoured growth of enterococci over other groups of LAB within the microbial population.

- An approach based on molecular methods can provide a faster detection of strains that produce specific bacteriocins. The cultivation methods are however still needed when the end goal of detection is isolation of individual strains or discovery of the strains that produce new bacteriocins.

- Using bioassay we were unable to detect bacteriocin activity *in situ* in traditional cheeses, but it was proved to be successful in the model cheese inoculated with nisin A producing strain of *Lactococcus lactis*.

- The molecular method RT real-time PCR, together with the appropriate method of RNA extraction from cheese, proved to be useful for quantitative determination of bacteriocin gene expression in cheese that contained a monoculture of bacteriocin producing strain.

3.3 RAZPRAVA

3.3.1 Primerjava različnih postopkov izolacije DNA

Z razvojem molekularne biologije in mikrobne ekologije smo dobili številna nova orodja, ki so vse bolj uporabna tudi za analizo kompleksnih mikrobnih združb. Kljub temu so bile vsaj do sedaj objavljene raziskave večinoma omejene na iskanje specifičnih genskih determinant, kot so geni za različne bakteriocine, le v posameznih izolatih. Moschetti in sod. (2001) in Bogovič Matijašić in sod. (2007) so eni redkih, ki so poročali o postopku izolacije skupne deoksiribonukleinska kislina (DNA) neposredno iz sira ter uspešnem iskanju specifičnih sekvenc za bakteriocine v njej. Avtorji so se omejili zgolj na kvalitativno analizo, torej na dokazovanje prisotnosti bakteriocinogenih sevov v siru. Do danes pa še ni bilo objavljenih raziskav, kjer bi za iskanje bakteriocinogenih sevov v kompleksnem okolju in mikrobnih združbah uporabili podoben pristop kot v tej raziskavi, to je molekularno metodo kot začetno presejalno metodo ter jo kombinirali z iskanjem zanimivih bakteriocinogenih izolatov z gojitvenimi tehnikami. Ta postopek je sicer zelo specifičen, tako da je uporaben le za iskanje sevov, ki proizvajajo posamezne že znane bakteriocine, omogoča pa bolj učinkovito iskanje zanimivih izolatov, saj se lahko omejimo na tiste vzorce sira ali katerega drugega medija, pri katerem so genske determinante za iskane bakteriocine zagotovo prisotne.

Pri preizkušanju različnih postopkov izolacije skupne DNA iz sira smo ugotovili, da je zelo pomembna ($p<0,005$) izbira prave raztopine za homogenizacijo vzorcev sira (Priloga A). Z uporabo raztopine natrijevega citrata smo uspeli pridobiti najvišje koncentracije skupne DNA. Natrijev citrat med drugim tudi International Dairy Federation priporoča za mikrobiološke analize sira (IDF standard 122B:1992). Razlog za to je najverjetneje v tem, da deluje kot kelatno sredstvo in ima zelo dobro sposobnost razbijanja sirnega matriksa ter posledično dobrega sproščanja prisotne mikrobne združbe v raztopino. Boljše kot je sproščanje mikrobnih celic v raztopino, bolj je ta mikrobna združba oziroma skupna DNA iz te združbe, reprezentativna za analizirani sir. Do podobnih zaključkov smo prišli tudi na podlagi rezultatov iskanja genskih determinant v osamljeni mikrobni združbi. Največje število prisotnih genskih determinant pri posameznih sirih smo ugotovili z uporabo natrijevega citrata.

Na učinkovitost izolacije DNA je v manjši meri vplival tudi sam postopek izolacije iz homogenata sira (mejna statistična značilnost, $p\leq0,05$) (Priloga A), pri čemer se je izolacija s komercialnim kompletom Wizard® Genomic DNA Purification Kit (Promega) izkazala za nekoliko bolj uspešno kot izolacija po postopku z fenol-kloroform-izoamilnim alkoholom (Cocolin in sod., 2001). Pričakovali bi, da je verjetnost za to, da v siru potrdimo

prisotnost posamezne genske determinante, odvisna predvsem od koncentracije skupne bakterijske DNA iz obravnavanega sira. V naši raziskavi smo ta pričakovanja potrdili le v primeru uporabe natrijevega citrata za homogenizacijo sira. Čeprav je bil izplen DNA pri izolaciji s komercialnim kompletom praviloma boljši kot pri izolaciji s fenol-kloroform-izoamilnim alkoholom pa se je slednja izkazala za boljšo kar zadeva raznolikosti ali stabilnosti DNA in kar se je pokazalo v številu odkritih bakteriocinskih genov. Tudi Amagliani in sod. (2007) so poročali o slabši uspešnosti pomnoževanja specifičnih sekvenc z uporabo komercialno dostopnih kompletov za izolacijo DNA v primerjavi s konvencionalno izolacijo DNA s fenolom in kloroformom. Mohar Lorbeg-ova (2008a) je poleg dveh metod, ki smo jih uporabili sami, preizkusila še šest drugačnih metod in prišla do podobnih zaključkov, da izolacija po postopku s fenol-kloroform-izoamilnim alkoholom daje najbolj primerno DNA, kar je ovrednotila z verižno reakcijo s polimerazo (PCR) in specifičnimi začetnimi oligonukleotidi za rod *Enterococcus* (*Ec.*). Poleg tega je Mohar Lorbeg (2008b) v neobjavljenih poskusih ovrednotila izolirano DNA še z univerzalnimi začetnimi oligonukleotidi za kvantifikacijo DNA (Barakat in sod., 2000). Pri primerjavi rezultatov obeh reakcij PCR se je izkazalo, da so reakcije za rod *Enterococcus* lahko negativne tudi, če reakcija z univerzalnimi začetniki kaže višje koncentracije izolirane DNA. Iz tega sledi, da je izolacija DNA iz posameznih sevov, ki sestavljajo mikrobno združbo, lahko različno učinkovita ob uporabi različnih postopkov izolacije, posledično pa je tudi DNA teh posameznih sevov različno zastopana znotraj skupne izolirane DNA. Do podobnih zaključkov smo prišli tudi v naši raziskavi, saj večja koncentracija DNA ni zagotavljala tudi zastopanosti DNA iz večjega števila predstavnikov mikrobiote. Na splošno so za izolacijo večjih količin DNA, ki je obenem tudi čistejša oziroma vsebuje manj potencialnih inhibitorjev reakcije PCR, potrebni daljši, večstopenjski postopki izolacije (Wilson,1997).

3.3.2 Prisotnost genskih determinant za bakteriocine v osamljeni mikrobni združbi in v siru

Vrste iz rodu *Enterococcus* so v slovenskih tradicionalnih sirih zelo dobro zastopani (Čanžek Majhenič in sod. 2005; Katana, 2001), kar se odraža tudi v pogostosti odkrivanja genskih determinant za enterocine. Med bakteriocini laktobacilov smo genske determinante za plantaricin A detektirali v vseh devetih sirih, kar prav tako kaže na dobro zastopanost bakterije *Lactobacillus* (*Lb*.) *plantarum* v slovenskih tradicionalnih sirih, o čemer so poročali že Čanžek Majhenič in sod. (2007).

Glede na to, da tehnologija izdelave obeh preiskovanih tradicionalnih sirov vključuje dogrevanje sirnega zrna do 45 °C in da za njihovo izdelavo dovoljujejo uporabo termofilne starterske kulture, bi bilo smiselno preveriti prisotnost genskih determinant za različne termofiline, ki jih lahko vsebujejo sevi *Streptococcus* (*Str*.) *thermophilus*. Prav tako bi bilo

smiselno preveriti prisotnost genskih determinant za bakteriocine, ki jih proizvajajo vrste iz skupine *Lb. casei* saj so tako v slovenskih (Čanžek Majhenič in sod., 2007) kot ostalih tradicionalnih sirih (Pisano in sod., 2007; Wouters in sod., 2002) najbolj zastopane vrste mlečnokislinskih bakterij. Bakteriocini, ki so bili do sedaj opisani za skupino *Lb. casei* so lactocin 705 (Cuozzo in sod., 2003), bakteriocin 217 (Lozo in sod., 2004), bakteriocin SJ (Lozo in sod., 2007), bakteriocin UB9 (Fira in sod., 2008), paracin 1.7 (Ge in sod., 2009), bakteriocin HL32 (Pangsomboom in sod., 2009). V dostopni literaturi nismo uspeli najti začetnih oligonukleotidov za omenjene bakteriocine. Edini par začetnih oligonukleotidov, ki smo ga sestavili za laktocin 705, na osnovi sekvence dostopne v GenBank, ni bil uporaben, saj se je pomnožek izkazali za nespecifičnega.

Večino genskih determinant za bakteriocine smo detektirali tako v skupni bakterijski DNA sira kot v DNA osamljene mikrobne združbe, iz česar lahko sklepamo, da je bil izvor teh genov v kultivabilni populaciji mlečnokislinskih bakterij (MKB) iz sira. Nekatere genske determinante, kot so na primer tiste za nizin, pa smo vendarle ugotovili samo v DNA, pridobljeni neposredno iz sira (T2, T3, T4). Razlog za to je lahko, da so tarčne sekvence DNA izvirale iz nekultivabilnih bakterij. Primer, ko smo gene za nizin ugotovili v DNA iz mikrobne združbe, ne pa v DNA neposredno iz sira, pa je mogoče pojasniti z nizko koncentracijo laktokokov z geni za nizin v siru (Čanžek Majhenič in sod., 2007; Gobbetti in sod. 1999). Laktokoki so namreč mezofilne MKB, ki pri višjih temperaturah, uporabljenih pri dogrevanju sirnega zrna, slabo rastejo (Beresford in Williams, 2004; O'Sullivan D, 2000). Podobno smo odkrili tudi determinante za enterocin L50A v mikrobnih združbah štirih sirov in za helveticin J v mikrobnih združbah treh sirov, ne pa v sirih samih. Tudi tu je bila verjetno koncentracija tarčne DNA iz sira pod mejo detekcije, medtem ko je kultivacija mikrobne populacije na ustreznem trdnem gojišču lahko DNA močno pomnožla.

Na splošno je bila raznolikost detektiranih genskih determinant za bakteriocine večja pri sirih Tolminc kot pri Kraških ovčjih sirih. Razlogi za to so lahko v osnovni surovini (živalska vrsta, geografsko poreklo), kakor tudi v tehnološkem postopku. Pri sirih K1, K2 in K3 kažejo rezultati na to, da je tehnologija izdelave teh sirov najverjetneje vključevala toplotno obdelavo mleka, ki je že v osnovi inaktivirala večji del naravno prisotne mikrobiote. Specifikacija za izdelavo Kraškega ovčjega sira toplotno obdelavo mleka sicer dovoljuje.

3.3.3 Protimikrobna aktivnost osamljene mikrobne združbe

Kljub temu, da smo v Kraških ovčjih sirih ugotovili manj genskih determinant za bakteriocine kot v tolminskih sirih, se med obema vrstama sira niso pokazale večje razlike

v dejanski protimikrobni aktivnosti mikrobnih združb sira, ki smo jo preverjali z biološkimi testi (Prilogi B in C). Z nobenim od supernatantov, pridobljenih po gojenju združb v ustreznih gojiščih (MRS in M17), kljub koncentriranju nismo uspeli potrditi inhibitornega delovanja, kar je verjetno posledica nizke koncentracije sintetiziranih bakteriocinov ali slabe stabilnosti. Pri testiranju združb z metodo lise na agraju, kjer bakterije nanesemo neposredno na agar, pride med inkubacijo do lokalnega kopičenje bakteriocina v neposredni bližini lise in je koncentracija verjetno znatno večja kot v supernatantih, ki jih testiramo na podoben način. Prav tako je znano, da aktivnost bakteriocinov lahko izgubimo med pripravo (bakteriocini naprimer lahko ostanejo vezani na površini bakterij, ki jih proizvajajo; lahko jih razgradijo proteolitični encimi) in hranjenjem supernatantov. O tem, da se v tekočem gojišču bakteriocini ne kopičijo v takšni meri kot v trdnem, in da so pod stalnim vplivom razgradnje, so poročali tudi Moretro in sod. (2000). Iz rezultatov, ki smo jih dobili z osamljenimi mikrobnimi združbami sirov, smo zaključili, da samo število genskih determinant za bakteriocine ne pove veliko o njihovi protimikrobni aktivnosti oziroma ne zagotavlja širokega spektra inhibicije. To predpostavko smo testirali tudi v dodatnem poskusu (Trmčić in sod., 2010a), kjer smo preverili anti-stafilokokno aktivnost *in situ* v mleku in siru. Preverili smo aktivnost dveh mikrobnih združb sirov, od katerih je bila prva (T2) takšna z največjim številom genskih determinant za bakteriocine, druga (K3) pa takšna z najmanjšim. Čeprav je mikrobna združba sira T2 bolj izrazito zavirala stafilokoke, te aktivnosti nismo mogli povezati z aktivnostjo bakteriocinov. Ti rezultati so izpostavili vprašanje, do kakšne mere posamezni bakteriocinogeni sevi znotraj kompleksne združbe izločajo bakteriocine in ali so zastopani znotraj te združbe v zadostnem številu, da pridejo proizvedeni bakteriocini do izraza. Z namenom dobiti vsaj delni odgovor na to vprašanje smo mešano mikrobno združbo sira T2 zaporedno precepljali v mleku in spremljali, kako se tekom postopka spreminja profil detektiranih genskih determinant za bakteriocine in protimikrobna aktivnost (Trmčić in sod., 2011a).

3.3.4 Vpliv zaporednega precepljanja osamljene mikrobne združbe na prisotnost genskih determinant za bakteriocine in njeno protimikrobno aktivnost

Tekom zaporednega precepljanja celotne mikrobne združbe sira T2 se je število detektiranih genskih determinant za bakteriocine zmanjšalo na tri od prvotnih osem, kar kaže na to, da se sevi, ki so vsebovali večino genskih determinant za bakteriocine, niso prav uspešno razmnoževali znotraj mikrobne združbe in se je njihov delež zmanjšal. Na drugi strani so se sevi, ki so vsebovali strukturne gene za enterocin P, L50B in citolizin, uspeli ohraniti med postopkom precepljanja in se je v nekaterih primerih njihov delež celo povečal. Za dinamiko rasti posameznih skupin mlečnokislinskih bakterij med izdelavo sira je značilno, da se v začetnih fazah poveča koncentracija enterokokov, ki jih med zorenjem

prerastejo laktobacili. Pri dnevnem precepljanju mikrobne združbe smo očitno spodbujali prav rast enterokokov na račun drugih predstavnikov združbe, kar je mogoča razlaga za to, da na koncu nismo uspeli detektirati genskih determinant za plantaricin A. Genske determinante za plantaricin A so bile poleg determinant za enterocin P in citolizin najbolj pogoste tako v osamljeni mikrobni združbi kot v siru (Trmčić in sod., 2008). Poleg razlik v zastopanosti genskih determinant so se spremembe sestave mikrobne združbe med precepljanjem pokazale tudi v protimikrobni aktivnosti. Za razliko od prvotne mikrobne združbe se po precepljanju ni pokazala močna inhibicija enterokokov, ohranila se je le protimikrobna aktivnost proti indikatorskim sevom vrst *Listeria* (*L.*) *monocytogenes* (Priloga E), *L. innocua* in *Lb. sakei.* Dobljeni rezultati so primerljivi s protimikrobno aktivnostjo seva *Ec. faecium* ATCC 19434 (Priloga F), ki proizvaja enterocine A, B in P. Ta bakteriocinogeni sev je poleg prej omenjenih treh sevov zaviral še vse tri indikatorske seve iz rodu *Enterococcus* (*Ec. faecalis* ATCC 19433, *Ec. faecium* CCM 4647, *Ec. durans* CCM 5612). Dobljeni rezultati protimikrobne aktivnosti seva *Ec. faecium* ATCC 19434 so skladni z rezultati, ki so jih z istim sevom dobili Park in sod. (2003). Glede na to, da omenjeni bakteriocinogeni sev proizvaja še dodatna dva bakteriocina, za katere nismo našli genskih determinant v DNA mikrobne združbe po zaporednem precepljanju, obenem pa je ta mikrobna združba vsebovala še druge genske determinante za bakteriocine, smo delo nadaljevali tako, da smo prisotnost genskih determinant za bakteriocine in protimikrobne aktivnosti preverili še na nivoju posameznih bakteriocinogenih sevov, ki jih je vsebovala mikrobna združba pred in po zaporednem precepljanju.

3.3.5 Prisotnost genskih determinant za bakteriocine pri posameznih osamljenih sevih in njihova protimikrobna aktivnost

Iz kolonij, ki so inhibirale indikatorska mikroorganizma *L. monocytogenes* in *Lb. sakei,* smo osamili posamezne seve. Seve smo osamili tako z gojišča M17 (30°C, 42°C) kot z modificiranega gojišča MRS. Za edina dva seva, ki smo ju osamili z modificiranega gojišča MRS, ki je sicer namenjeno laktobacilom, smo ugotovili, da rasteta bolje na gojišču M17 in da sta predstavnika rodu *Enterococcus*. O slabi selektivnosti gojišč za MKB je bilo že veliko objavljenega (Menendez in sod., 2001), tako da smo od le-teh pričakovali predvsem, da bodo omogočili rast tistih skupin, katerim so namenjeni, in tako pridobili čim več različnih predstavnikov mikrobiote, ne pa da bodo zelo selektivna za ostale. Edino od uporabljenih gojišč, ki se je izkazalo za zelo selektivno, je bilo gojišče CATC za enterokoke. Pri predstavnikih vrste *Ec. faecium* (G0x42/2 in G10x42/3.1) smo tekom dela ugotovili, da zelo slabo oziroma ne rastejo na gojišču CATC (Priloga J). Ta problem je omenjal že Reuter (1992). Na gojišču CATC je tako prihajalo tudi do neželjene selekcije predstavnikov enterokokov. Pričakovali smo tudi, da bo veliko izolatov bakteriocinogenih sevov med seboj sorodnih ali celo identičnih. Podobnosti smo našli pri pregledu

protimikrobne aktivnosti proti indikatorskima mikroorganizmoma *L. monocytogenes* in *Lb. sakei*. Dvojne inhibicijske cone proteinske narave smo zasledili tako pri posameznih osamljenih sevih kot pri mezofilni mikrobni združbi med zaporednim precepljanjem (Trmčić in sod., 2010b, 2011a, Prilogi E in H), iz česar lahko sklepamo, da v tej mikrobni združbi prevladuje le nekaj sevov. Od desetih reprezentativnih sevov, ki smo jih osamili, smo s pomočjo PCR in specifičnih začetnikov za rod in vrsto identificirali osem izolatov kot *Ec. faecalis* in dva kot *Ec. faecium*. Isti dve skupini smo dobili tudi pri tipizaciji s pomočjo sistema PhP. Znotraj skupine *Ec. faecalis* smo z istim sistemom ugotovili tri manjše skupine, ki vsebujejo potencialno sorodne seve, medtem ko trije sevi iz skupine *Ec. faecium*, med katerimi je tudi *Ec. faecium* ATCC 19434, niso pokazali bližnje sorodnosti.

Pri iskanju povezav med protimikrobno aktivnostjo in prisotnimi geni za posamezne bakteriocine ni bilo mogoče priti do trdnih zaključkov. Vseh deset analiziranih sevov enterokokov je vsebovalo genske determinante za citolizin, kar je precej neznačilno za enterokoke iz sirov (Mohar Lorbeg, 2008; Katana Burja, 2006; Čanžek Majhenič in sod., 2005), še manj pa je značilno za vrsto *Ec. faecium*. De Vuyst in sod. (2003) so naprimer med 37 preiskovanimi sevi enterokokov našli en sam sev *Ec. faecium* z genom za citolizin. Še bolj zanimivo je, da smo pri treh sevih *Ec. faecalis* ugotovili prisotnost genov za enterocin L50B, pri enem sevu pa tudi enterocine A, B, P in L50A, ki so značilni za vrsto *Ec. faecium*. Obstaja verjetnost, da nekateri sevi niso bili čisti. Namreč, pri izolaciji posameznih sevov iz kompleksnih okolij včasih naletimo na takšne, ki rastejo v simbiozi in jih je kljub temeljitemu prečiščevanju zelo težko razdvojiti. Tako je analiza z multiplo PCR v primeru izolata G0x42/1 pokazala pozitivno reakcijo za vrsti *Ec. faecalis* in *Ec. faecium,* kar nakazuje na to, da je bila kultura dejansko mešanica dveh sevov, predstavnikov dveh vrst.

Med indikatorskimi sevi, ki smo jih uporabili za preverjanje protimikrobne aktivnosti posameznih izolatov, je bil tudi *Ec. faecium* ATTC 19434. Naš namen je bil, da preverimo, ali lahko morda opaženo protimikrobno aktivnost pripišemo kateremu od bakteriocinov, različnih od enterocinov A, B ali P. Izkazalo se je, da smo pri vseh izolatih lahko zasledili podobno inhibicijo (1 mm) tega indikatorskega seva (Priloga G). Podobno inhibicijo, ki so jo kazali vsi izolati, smo zasledili tudi proti indikatorskem sevu *Ec. durans* CCM 5612. Inhibicije smo pripisali citolizinu, saj je to edini od preiskovanih bakteriocinov, čigar genske determinante smo našli pri vseh izolatih. Ker ima citolizin tudi hemolitične lastnosti, smo se odločili, da pri vseh izolatih preverimo tudi produkcijo citolizina s pomočjo hemolitičnega testa na trdnem gojišču. Rezultati De Vuysta in sod. (2003) kažejo na to, da je večina sevov, ki nosijo genske determinante za citolizin, sposobnih tudi β hemolize. Pri nobenem od naših testiranih sevov nismo potrdili sposobnosti α ali β hemolize. Iz tega lahko sklepamo, da inhibicija, ki so jo izkazali izolati enterokokov,

najverjetneje ni neposredno povezana s citolizinom, je pa lahko rezultat genotipske/fenotipske lastnosti, ki se pojavlja v kombinaciji s citolizinom.

Edini izolat, ki je pokazal nekoliko drugačne protimikrobne lastnosti, je bil sev G0xR/1. Tako proti *Ec. durans* CCM 5612 (Priloga I) kot proti *Ec. faecium* ATCC 19434 (Priloga G) je bila aktivnost identična (4 mm). Proteinske narave inhibicije nobenega od omenjenih indikatorskih sevov nismo uspeli jasno potrditi z uporabo proteolitičnih encimov (kimotripsin in proteinaza K). Kljub temu je izgled same inhibicijske cone (izjemno čista z ostro definiranim robom) kazal na to, da je bila rezultat delovanja nekega bakteriocina. Ker smo pri sevu G0xR/1 zasledili le genske determinante za citolizin, ni mogoče sklepati, kateri bakteriocini bi lahko bili odgovorni za opaženo protimikrobno aktivnost. Sev G0xR/1 smo identificirali kot predstavnik vrste *Ec. faecalis*. Pri tej vrsti so opisali še najmanj pet enterocinov, ki jih v naši raziskavi nismo iskali (Galvez in sod., 1998; Eguchi in sod., 2001; Franz in sod., 2002; Martín-Platero in sod., 2006).

3.3.6 Problem osamitve bakteriocinogenih sevov iz sira

Od številnih genskih determinant, ki smo jih zasledili v mikrobni združbi sira T2, smo jih zelo malo našli tudi v posameznih sevih, osamljenih iz te združbe. Glavni razlog za takšne rezultate je najverjetneje postopek osamitve takšnih sevov. Ker smo namreč mikrobne združbe ali posamezne seve pridobivali samo s štev nih plošč (plošče s 30-300 kolonijami), smo s tem omejili iskanje na populacijo, ki je v siru najbolj številna, manj zastopane seve pa izgubili. Izbirali smo samo seve, ki so v biološkem testu pokazali zaviralni učinek proti indikatorskim sevom, kar je relativno enostaven način iskanja bakteriocinogenih sevov. Znano je namreč, da sama prisotnost genskih determinant za posamezni protein ne zagotavlja tudi njegove biosinteze, in da tudi sintetizirani proteini niso nujno tudi aktivni. Zato je izražanje bakteriocinskih genov ključnega pomena, tako pri iskanju zanimivih sevov kakor tudi za samo aplikacijo. Rezultati raziskave so tudi pokazali, da sposobnost izločanja bakteriocina takemu sevu sama po sebi še ne zagotavlja tudi kompetitivne prednosti. Potrebno je upoštevati, da imajo tradicionalni siri, ki so izdelani iz surovega mleka, izredno pestro mikrobioto, znotraj katere posamezni sevi težje prevladajo. Znotraj mikrobne združbe vlada ravnotežje med različnimi bakteriocinogenimi sevi, pri čemer igrata vlogo tako sposobnost proizvodnje bakteriocinov kakor tudi sposobnost rezistence proti drugim bakteriocinom. Sevi iste združbe, ki producirajo različne bakteriocine, se medsebojno tudi do neke mere inhibirajo, saj sposobnost proizvodnje določenega bakteriocina sevu ne prinese odpornosti proti drugim bakteriocinom. Na splošno v mikrobni ekologiji pomenu bakteriocinov za kompetitivnost sevov ne pripisujejo take vloge kot drugim mikrobnim interakcijam, kot so tekmovanje za hranila med predstavniki, ki imajo podobne okoljske zahteve in drugo (Beresford in sod., 2001; Bersford in

Williams, 2004). Razlog za to je gotovo tudi v pomanjkanju raziskav o dejanski vlogi bakteriocinov *in vivo*, v mikrobnih združbah.

3.3.7 Problem povezave med prisotnostjo genskih determinant za bakteriocine in dejansko protimikrobno aktivnostjo

V raziskavi smo le v posameznih primerih uspeli pokazati neposredno povezavo med protimikrobno aktivnostjo in prisotnimi geni za bakteriocine. S tem problemom smo se srečali tako pri testiranju združb kakor tudi posameznih sevov. Neskladnost med številom detektiranih genskih determinant in z biološkimi testi dejansko ugotovljeno protimikrobno aktivnostjo kaže na kompleksnost, ki obe lastnosti povezuje. Potrebno je upoštevati, da smo se pri iskanju omejili le na tiste bakteriocine, ki so bili do sedaj opisani in za katere je bilo sodeč po podatkih drugih raziskovalcev najbolj verjetno, da jih v taki združbi najdemo. Ti verjetno predstavljajo le manjši del vseh bakteriocinov, ki jih predstavniki take združbe lahko producirajo. Tako je bakteriocinska aktivnost, opažena v biološkem testu, lahko rezultat bakteriocinov, katerih genskih determinant nismo vključili v analizo s PCR. Poleg tega vsebuje veliko sevov genske determinante za več bakteriocinov, za katere ne vemo, kakšno je njihovo izražanje, oziroma kakšne so medsebojne interakcije pri njihovem izražanju, biosintezi ter izločanju. Tudi izbira indikatorskih sevov predstavlja problem, saj je le poznavanje njihove občutljivosti za iskane, znane in neznane bakteriocine zelo omejeno. Podobno velja tudi za proteolitične encime, ki se uporabljajo za potrditev proteinske narave inhibicije, saj različne bakteriocine inaktivirajo različni proteolitični encimi.

Biološki testi pri dokazovanju aktivnosti bakteriocinov imajo tudi vrsto drugih omejitev, ki pridejo do izraza v kompleksnih medijih, kot je sir. Med njimi so visok prag detekcije, neenakomerna razporeditev bakteriocinov v siru, adsorbcija bakteriocinov na beljakovine sira in s tem manjša razpoložljivost za indikatorski sev ali njihova razgradnja z ekstracelularnimi ali intracelularnimi bakterijskimi peptidazami, ki se sprostijo iz poškodovanih celic. Poleg tega ne moremo zaznati aktivnosti posameznih bakteriocinov, kadar jih je več vrst prisotnih v vzorcu, pač pa le celokupno aktivnost. Na drugi strani so se molekularne metode pri našem delu izkazale za uporabne, podobno kot so ugotavljali že v podobnih raziskavah (Moschetti in sod. 2001, Bogovič Matijašić in sod. 2007). Specifične in občutljive molekularne metode bi bilo smiselno nadgraditi tako, da bi omogočale odkrivanje tistih genskih determinant, ki se v siru tudi izražajo, ter se s tem vsaj delno izogniti uporabi manj specifičnih bioloških testov.

3.3.8 Uporaba molekularne metode za ugotavljanje izražanja genov za bakteriocine v siru

Za merjenje izražanja genov se zelo uspešno uporabljajo metode, ki temeljijo na obratni transkripciji (RT) in verižni reakciji s polimerazo v realnem času (real-time PCR). RT real-time PCR je metoda, ki preko kvantifikacije prepisov genov v informacijsko ribonukleinsko kislino (mRNA) omogoča ne samo kvalitativno temveč tudi kvantitativno ugotavljanje izražanja posameznih genov. Kljub številnim prednostim metode RT real-time PCR, so raziskave omejene predvsem na kulture v tekočih gojiščih, manj pa je primerov uporabe na kompleksnejših vzorcih (Ramiah in sod., 2007; Granger in sod., 2008). Pri našem eksperimentalenem delu ugotavljanja izražanja bakteriocinov *in situ* v siru se je metoda izkazala za uporabno, predvsem pa za bolj specifično alternativo biološkim metodam. Uspešnost metode je zelo odvisna od količine in kakovosti izolirane RNA, katere izolacija je precej zahtevna in je zaradi tega ta pristop manj razširjen. Osamitev RNA iz kompleksnega medija kot je sir je težavna ker je sama RNA zelo nestabilna, poleg tega pa so prisotne tudi velike količine beljakovin, maščob, encimov, ki razgrajujejo RNA, ter ostankov DNA. Osamitev RNA iz sira tako zahteva postopke ekstrakcije, ki so prilagojeni temu bogatemu matriksu. Do sedaj je bilo predlaganih pet metod (Bonaïti in sod., 2006; Ulve in sod., 2008; Monnet in sod., 2008; Makhzami in sod., 2008; Duquenne in sod., 2010) od katerih najbolj izstopa metoda, ki so jo razvili Monnet in sod. (2008), saj je edina neposredna metoda, kar pomeni, da ne zahteva predhodnega ločevanja celic iz sirnega matriksa.

Transkripcija in razgradnja transkriptov s strani prisotnih nukleaz se zaustavi v prvem koraku izolacije RNA. Slednje dosežemo s takojšnjo homogenizacijo sira v reagentu Trizol® ter razbijanjem sirnega matriksa in celic s pomočjo cirkonijevih kroglic. Rezultati, dobljeni s to metodo, dajo bolj realno sliko transkripcije v danem času kot postopki, ki vključujejo predhodno ločevanje celic od sirnega matriksa. Pri našem delu smo se odločili preveriti omenjeno metodo izolacije RNA in poskusili kvantificirati izražanje genskih determinant za bakteriocin v siru (Trmčić in sod., 2011b). V ta namen smo uporabili sterilni model sira inokuliran z monokulturo seva *Lactococcus* (*Lac.*) *lactis* M 78, ki proizvaja bakteriocin nizin A (Prilogi K in L).

Metoda, ki so jo opisali Monnet in sod. (2008), se je izkazala za učinkovito in uporabno, vendar pa je bil, kakor smo tudi pričakovali, končni izplen izolirane RNA nižji v primerjavi s tistim, ki bi ga dobili z metodami, ki vključujejo postopek ločevanja celic iz sirnega matriksa. Da smo pridobili dovolj RNA, smo v nekaterih primerih morali postopek izolacije izvajati v več paralelkah (po 500 mg sira), ki smo jih v končnem koraku (elucije z vodo) združili. Zaradi istega razloga smo tekom eksperimenta pričeli z vzorčenjem, ko je

koncentracija celic dosegla 10^8 kolonijskih enot na gram sira. Končni izplen RNA je bil med 1088 in 4625 ng. Takšna količina izolirane RNA je zadostovala, saj smo reakcijo obratne transkripcije standardizirali na količino 200 ng. Po zagotovilih proizvajalca kita za obratno transkripcijo (Invitrogen, 18080-051) je mogoče reakcijo izvesti že z 1 pg celokupne RNA. Ključni problem pri izolaciji iz sira je ohraniti stabilnost RNA med postopkom. Meritve koncentracije na aparaturi NanoDrop ND-1000 in vrednosti RIN (angl. RNA Integrity Number) s pomočjo aparata 2100 Bioanalyzer so pokazale, da je bila izolirana celokupna RNA nerazgrajena in primerna za analizo z RT real-time PCR. Prav tako smo izključili verjetnost prisotnosti DNA ali inhibitorjev reakcije RT real-time PCR. Kljub temu, da je količina izolirane RNA nekoliko nižja, v primerjavi s količino, ki bi jo lahko dobili s pomočjo metod, ki vključujejo ločevanje celic iz sirnega matriksa, je sama reprezentativnost končnih rezultatov tisto, kar daje tej metodi njeno pravo vrednost. Reprezentativnost končnih rezultatov pomeni, da ti rezultati predstavljajo dejansko stanje in to je vsekakor na prvem mestu pri izbiri primerne metode. V raziskavi smo potrdili uporabnost metode izolacije RNA, ki so jo opisali Monnet in sod. (2008).

Ne glede na uporabljeno metodo izolacije RNA, se pri uporabi metode RT real-time PCR v realnih vzorcih sira srečamo z dvema večjima izzivoma. Prvi je, kako doseči točno in zanesljivo normalizacijo RT real-time PCR meritev v vzorcih sira, kjer je prisotnih več mikrobnih vrst. Pri relativni kvantifikaciji izmerjene vrednosti običajno normalizirajo na celokupno RNA celic (Ulve in sod., 2008; Monnet in sod., 2008; Makhzami in sod., 2008). Ker je ta način manj uporaben za realne kompleksne vzorce, v našem primeru za sir, se namesto tega uporablja en ali več referenčnih genov (Duquenne in sod., 2010). Pri našem delu smo, podobno kot Monnet in sod. (2008) ugotovili, da so geni za 16S in 23S ribosomsko RNA konstitutivno izraženi in so zato uporabni kot zgoraj omenjene normalizacijske reference. Problem je v tem, da imajo siri, še posebej tisti narejeni iz surovega mleka, številne seve, ki so si med seboj bolj ali manj sorodni in imajo podobne genetske lastnosti. Tudi če bi natančno poznali sestavo mikrobne združbe sira, bi težko sestavili takšne začetne oligonukleotide, ki bi bili dovolj specifični za posamezen bakteriocinogeni sev. Validacija referenčnih genov znotraj vsakega testnega pogoja je vsekakor najpomembnejši korak pri analizah takšne vrste. Namesto uporabe teh internih referenc obstaja možnost uporabe eksternih referenc v obliki dodane, znane količine zunanje RNA (DeCoste in sod., 2011). Problem s takim načinom normalizacije je podoben kot s celokupno RNA in absolutno kvantifikacijo. Rezultati pri uporabi takšne normalizacije dajejo informacijo o izražanju genov na nivoju celotne populacije seva. Nemogoče pa je dobiti podatek na nivoju posamezne celice, če ne ugotovimo števila celic znotraj te populacije. Slednje je lahko težavno, če analiziramo vzorce sira, ki vsebujejo več sorodnih sevov.

Trenutno se zdi metoda bolj uporabna za kvalitativne kot kvantitativne namene, ko analiziramo realne vzorce sira. Takšne študije so že bile opravljene v siru (Bonaiti in sod., 2006) in ostalih kompleksnih okoljih kot so rečni sedimenti (Nogales in sod., 2002). Postopek kvalitativne analize bi lahko bil podoben postopku opisanemu v članku Trmčić in sod. (2008), s to razliko, da prisotnost genskih determinant ne bi ugotavljali v metagenomski DNA, temveč v komplementarni DNA (cDNA), pridobljeni iz metagenomske RNA. Rezultat takšne analize bi vseboval ne samo podatek o prisotnosti določenih genskih determinant, temveč tudi podatek o njihovem aktivnem izražanju v siru. Pri tem se pokaže drugi izziv pri uporabi metode za analizo realnih vzorcev sira. Mikrobna populacija v pravih vzorcih sira se nahaja v pozni stacionarni fazi rasti oziroma v konstantni fazi odmiranja in lize. Večina trdih in pol-trdih vrst sirov mora zoreti dva ali več mesecev in minimalen čas zorenja za večino tradicionalnih slovenskih sirov je 60 dni. Pri našem delu smo zadnje vzorčenje in izolacijo RNA opravili po 24 h fermentacije, drugi pa poročajo o uspešni kvantifikaciji transkriptov tudi po 3, 7 in 14 dneh fermentacije/zorenja (Duqunne in sod., 2010; Makhzami in sod., 2008; Monnet in sod., 2008). Ulve in sod. (2008) so opravili analize tekom šestih mesecev zorenja sira, vendar je bila količina in stabilnost izolirane RNA po šestih mesecih že slabša. Isti so poročali tudi o močnem negativnem vplivu lize celic na izolacijo RNA iz treh komercialnih francoskih sirov. Na drugi strani so Duqunne in sod. (2010) uspeli izolirati velike količine RNA iz treh komercialno dostopnih poltrdih sirov, vendar pa kljub temu, da so prikazali dobro stabilnost izolirane RNA, niso opravili nikakršne analize, s katero bi potrdili prisotnost mRNA znotraj celokupne RNA. Zadostne količine mRNA verjetno lahko pridobimo samo iz aktivno rastočih celic. Poleg tega je tudi znano, da je izražanje genov za bakteriocine največje v drugi polovici eksponentne faze rasti, kmalu po nastopu stacionarne faze rasti pa se ustavi. Naši rezultati so za nekatere gene pokazali tudi nekoliko drugačno dinamiko (Trmčić in sod., 2011b).

V članku Trmčić in sod. (2011b) so opisani rezultati merjenja izražanja genov za nizin med 24 h fermentacijo modelnega sira. Zastavljeni model se je izkazal za izjemno dobrega. Nizin smo se odločili uporabiti kot testni bakteriocin, ker je najbolj preučeni bakteriocin MKB, ker je potrjena njegova produkcija *in situ* v siru, ker se producira v dovolj velikih količinah in ker je mogoče njegovo aktivnost dovolj enostavno zasledovati *in situ* s pomočjo klasičnih bioloških metod. V proizvodnjo, imunost in regulacijo produkcije nizina je vključenih 11 genov. Tako kot že številni pred nami, smo potrdili izražanje nizina *in situ* v siru (Maisnier-Patin in sod., 1992; Yezzi in sod., 1993; Rodriguez in sod., 1998; Bouksaim in sod., 2000; Rodriguez in sod., 2005; Ayada, 2009), s to razliko, da smo uporabo bioloških testov podprli in primerjali tudi z rezultati RT real-time PCR analize. Med rezultati obeh metod smo potrdili močno korelacijo, kar priča o tesni povezanosti transkripcije genskih determinant nizina in njegovo končno aktivnostjo. Na osnovi

dinamike transkripcije genov nizina kot funkcije časa smo uspeli razvrstiti gene v štiri skupine: *nisA*, *nisBTCIP*, *nisFEG* in *nisRK*. S primerjavo dinamike izražanja omenjenih skupin genov, njihove funkcije in lokacije v genski gruči ter rastne dinamike in protimikrobne aktivnosti testnega seva, smo uspeli potrditi nekatera že znana dejstva, kot je naprimer to, da nizin deluje kot quorum sensing feromon in spodbuja svojo lastno proizvodnjo (van der Meer in sod., 1993; Kuipers in sod., 1995; Ra in sod., 1996; Kleerebezem, 2004). Prav tako pa smo pokazali nekatere korelacije, ki lahko pomagajo pri razlagi regulacije biosinteze in imunosti bakteriocina nizina. Ta študija je prva, ki je vključevala analizo izražanja vseh 11 genov nizina, obenem pa je ena prvih, ki predstavlja intenzivno, časovno odvisno kvantifikacijo transkriptov v siru podobnem mediju.

3.4. SKLEPI

Na osnovi postavljenih hipotez smo prišli do naslednjih zaključkov:

- Tradicionalni slovenski siri, izdelani iz surovega mleka, vsebujejo v svoji pestri mikrobni združbi tudi seve MKB, ki proizvajajo različne bakteriocine.

- Mikrobna združba tradicionalnih slovenskih sirov, izdelanih iz surovega mleka, vsebuje seve MKB, ki skupno ali posamično zavirajo razrast potencialno patogene *Staphylococcus aureus* v mleku in siru. Opažene zaviralne aktivnosti nismo uspeli povezati z bakteriocinsko aktivnostjo.

- Le redki sevi MKB, ki proizvajajo bakteriocine, so *in situ* v mleku pokazali kompetitivno prednost znotraj mikrobne združbe. To opažanje je delno lahko posledica testnih pogojev, ki so favorizirali rast enterokokov v škodo drugih skupin MKB.

- Uporaba pristopa, ki temelji na molekularnih metodah lahko omogoči hitrejše iskanje sevov, ki producirajo točno določene bakteriocine. Gojitvene metode so še vedno potreben, ko je končni cilj iskanja osamitv posameznih sevov ali odkrivanje sevov, ki proizvajajo nove bakteriocine.

- Z uporabo bioloških testov nismo uspeli zasledili bakteriocinske aktivnosti *in situ* v tradicionalnih sirih, izkazali pa so se za uspešne v modelnem siru, inokuliranem s sevom *Lactococcus lactis*, ki proizvaja nizin A.

- Molekularna metoda RT real-time PCR v povezavi s primerno metodo za izolacijo RNA iz sira, se je izkazala za uporabno pri kvantitativnem ugotavljanju izražanja genov za bakteriocin v siru, ki vsebuje monokulturo bakteriocinogenega seva.

4 SUMMARY (POVZETEK)

4.1 SUMMARY

Most of traditional cheeses and dairy products in general are made of raw milk, whose chemical and microbial composition are, besides the technological procedure, the main parameters determining the specific characteristics of the final product. In the raw milk and in the final products made from it, the presence of undesired or even pathogenic strains can present a certain risk for the consumers. To address this problem, the use of commercial starter and protective cultures became very popular. In the production of traditional cheeses however the addition of the commercial cultures may negatively influence the characteristic sensory properties. The basic idea of this study was therefore to examine the possibility of application of the representatives of the natural microbiota of particular traditional cheeses instead of commercial LAB cultures, in order to increase the safety of such products. For this purpose, we intended to analyse the natural microbiota of individual type of traditional cheese, focusing on potential bacteriocin producing LAB, and to select the candidates with wide spectrum bacteriocin activity which could be used to produce starter cultures that are ‘tailor made’ for individual traditional cheese. The application of such starter cultures would relieve most of the problems arising from traditional technology (raw milk) and still preserve specific properties of traditional cheese including sensory ones. The term 'bacteriocins' represents a large and diverse group of ribosomaly synthesized antimicrobial proteins and peptides which can be found in Gram positive and in Gram negative bacteria. It is believed that their major role is to prevent other bacteria from growing in the environment. Bacteriocins can differ in structure, molecular mass, biochemical properties, spectrum of activity, mode of action and location of genes. Bacteriocins of LAB are classified into three main groups, modified bacteriocins or lantibiotics, non-modified (thermo-stabile) bacteriocins and large (thermo-labile) bacteriocins. Each of these groups is further divided into several types or sub-groups. Bacteriocin genes are organised in conserved regions which are located either in plasmids or in bacterial chromosome, as a mobile or non-mobile genetic element. Most of them are organised in operons that contain several genetic determinants, like structural pre-peptide gene, immunity protein gene and other genes, which are involved in transport and processing of bacteriocin. The main target of bacteriocins is usually the cell membrane, but there are also reports of other target structures like cell wall and cell proteins. Bacteria that produce bacteriocins have developed system of defence against action of its own bacteriocin, referred to as immunity. When bacteria are resistant against the action of bacteriocins produced by other bacterial strains, it is referred to as resistance. In raw milk and cheese several bacteriocins can be found since the microbiota is very diverse. Traditional Slovenian hard and semi-hard cheeses are mostly inhabited by *Lactobacillus*

(*Lb*.) and *Enterococcus* (*Ec*.) genus, and to lesser extent by *Lactococcus* (*Lac*.) and *Streptococcus* (*Str*.). In this work we studied traditional Slovenian cheese Tolminc (cows' milk, five samples) and Kraški ovčji sir (ewes' milk, four samples). The idea was to extract total deoxyribonucleic acid (DNA) from individual cheeses and to search by PCR for gene determinants of selected LAB bacteriocins typically produced by the species commonly found in raw milk cheeses. On the basis of testing of several different procedures for total DNA extraction from cheese we concluded that the most important factor ($p<0,005$) is the solution used for homogenisation of cheese. The most effective was found sodium citrate solution. The protocol for extracting DNA from cheese homogenate had smaller effect on the quantity of extracted DNA ($p\leq0,05$). Extraction with commercial Wizard® Genomic DNA Purification Kit (Promega) was more effective in terms of DNA quantity, while the procedure with phenol-chloroform-isoamyl alcohol yielded DNA that performed better in PCR reactions and in many cases demonstrated a higher diversity of detected bacteriocin gene determinants. The gene determinants were not only determined in total DNA extracted from cheese, but also in microbial consortia of cultivable strains isolated on partially selective growth media CATC, Rogosa and M17 which support growth of enterococci, lactobacilli and lactococci with other cocci form LAB group, respectively. In total DNA extracted from cheese as well as in DNA from cheese consortia of cultivable bacteria we searched for specific sequences of 19 different LAB bacteriocins using PCR. The observation that most of the bacteriocin gene determinants were detected either in total DNA and DNA from consortia, or in none of them, leaded us to conclusion that the source of the target gene determinants was mostly the cultivable bacterial population of cheese. In addition to detection of gene determinants for individual bacteriocins we also examined the actual antimicrobial activity of microbial consortia. Using the agar diffusion method we tested pure microbial consortia as well as supernatants, obtained after their cultivation in corresponding liquid media (MRS, M17). Despite the fact that Kraški ewe's cheese contained less bacteriocin gene determinants than Tolminc cheese, there were no significant differences between them in antimicrobial activity. It seems that the number of bacteriocins' gene determinants alone does not tell much about actual antimicrobial activity of bacteria that posses these determinants. We tested this conclusion in an additional experiment *in situ* in milk and in cheese, in which anti-staphylococcal activity of two microbial consortia was examined. Two consortia were selected on the basis of the number of detected bacteriocins' gene determinants, where the first one (T2) contained the highest, and the second one K3 the lowest number. Although anti-staphylococcal activity of microbial consortium T2 was more pronounced, we were unable to link this inhibition directly with bacteriocin activity. According to our hypothesis we continued the study of eventual competitive advantage of bacteriocinogenic strains within microbial population. For this purpose, the entire microbial consortia of cheese T2, consisting of individual consortia from three different growth media (CATC, Rogosa, M17), was consecutively

propagated (1% inoculum) for 10 days in sterile milk. The incubation with parallel samples was carried out at 30°C and at 42°C. After five and ten passages we determined the microbial composition and the presence of bacteriocins' gene determinants directly in fermented milk and in the cultivable microbial consortia. During propagation the number of bacteriocin gene determinants was reduced from initial eight to three, which indicates that share of strains that contained bacteriocin gene determinants was reduced within the microbial population. On the other hand, the strains possessing bacteriocin gene determinants for enterocin P, L50B and cytolysin were able to persist during propagation process. It seems that with daily propagation we stimulated the part of microbial population represented by enterococci. Besides changes in the number of detected bacteriocin gene determinants we observed also changes in antimicrobial activity of the microbial consortia. Unlike initial microbial consortia, the consortia obtained after propagation did not exhibit any more strong antimicrobial activity against enterococci. Antimicrobial activity was only preserved against indicator strains *Listeria* (*L.*) *monocytogenes*, *L. innocua* and *Lb. sakei. L. monocytogenes* and *Lb. sakei* were also used as indicator strains in the antimicrobial activity testing of individual isolates, from initial consortia and from consortia obtained after ten days of propagation. Isolates from both consortia were found very similar, which indicated that individual strains were predominant to some extent, probably also due to certain competitive advantage. Two isolates produced bacteriocin(s) different from those selected for the screening. Only a few of several bacteriocin gene determinants found in cheese consortia, were detected also in individual isolates. The main reason for this is probably in the procedure of selection of these strains. Namely, since the strains were isolated from the countable plates (30-300 colonies), we obviously favoured the strains which were predominant. In most cases we were unable to link the antimicrobial activity with the presence of bacteriocin gene determinants. We came to similar conclusion for both, isolated microbial consortia and single isolated strains. This discrepancy between detected bacteriocin gene determinants and observed antimicrobial activity (bioassays) shows the complexity that links these two properties together. Due to numerous limitations of bioassays for detection of bacteriocin activity, of which low specificity is the major problem, we decided to introduce an alternative method for determination of expression of bacteriocins in cheese, based on reverse transcription real-time PCR. We tested the method using a sterile cheese model inoculated with a monoculture of *Lac. lactis* M 78, which produces nisin A. The method of direct ribonucleic acid (RNA) extraction from cheese proved to be successful. Even though the quantity of extracted RNA was relatively low in comparison to the quantities that could be obtained by the methods that involve separation of cells from cheese matrix, the real value of this method is representativeness of the final result. Representativeness means that the results reflect the actual state and this is by all means the main factor in choosing an appropriate method of analysis. In this study we confirmed applicability of the method of

RNA extraction developed by Monnet et al. (2008). We successfully used RNA extraction procedure together with the reverse transcription real-time PCR to follow expression of bacteriocin gene determinants in cheese model, nevertheless there are two major challenges presented when using reverse transcription real-time PCR method in real cheese samples. The first one is how to achieve an accurate and reliable normalization of reverse transcription real-time PCR measurements in cheese samples where several microbial species are present. Each of the three methods usually applied for normalisation of data has certain limitations when using in real cheese samples. Using total cellular RNA or exogenously spiked RNA for normalisation in relative quantification presents a similar problem. Results obtained by this kind of normalisation provide information about gene expression of the entire strain population. To gain information on a single cell level, the number of cells of the strain tested inside the population has to be determined. This is however not easy in complex samples (cheese) since they contain several related strains that can't be distinguished by cultivation methods. The third and probably the best method of normalisation is to find a suitable constitutively expressed reference gene. This is also a demanding task since cheeses, especially those produced from raw milk contain numerous strains that share common genetic features. Even if we knew exactly the microbial composition of each cheese it would be very hard to design oligonucleotide primers specific for a given bacteriocinogenic strain. The second challenge in using reverse transcription real-time PCR in real cheese samples is the fact that microbial population in cheese is in its late stationary or death phase of growth where transcription of genes, quantity and quality of extracted RNA is scarce. Namely, expression of bacteriocin genes usually stops at the beginning of stationary growth phase. It is however worth to mention that in our study the results showed a somewhat different expression for some of the nisin genes. The study of expression of 11 nisin genes in cheese has confirmed in general some already known facts and also established some correlations that can contribute to the explanation of regulation of nisin biosynthesis and immunity. The conclusions of our entire work are the following: (a) the traditional Slovenian cheeses do contain strains of LAB which produce different bacteriocins, but only a few of these strains display a competitive advantage over the rest of microbial population; (b) these cheeses also contain strains of LAB which collectively or individually inhibit outgrowth of potentially pathogenic bacterium *Staphylococcus aureus* in milk and cheese, but we were unable to link observed inhibitory action with bacteriocin activity; (c) molecular approach can provide a faster detection of strains that produce specific bacteriocins, but classical bioassay approach is still needed when the end goal of detection is isolation of individual strains or discovery of the strains that produce new bacteriocins; (d) reverse transcription real-time PCR proved to be useful for quantitative determination of bacteriocin gene expression in cheese that contained a monoculture of bacteriocin producing strain.

4.2. POVZETEK

Večina tradicionalnih sirov in drugih tradicionalnih mlečnih izdelkov je izdelanih iz surovega mleka, ki je s svojo kemijsko in mikrobiološko sestavo poleg tehnološkega postopka izdelave glavni parameter, ki določa karakteristične lastnosti izdelka. Poleg zaželjene mikrobne združbe se v surovem mleku zelo pogosto pojavljajo tudi sevi, ki lahko v končnem izdelku predstavljajo potencialno nevarnost za potrošnika. V proizvodnji sira se je zato razširila uporaba komercialnih zaščitnih starterskih kultur, ki pa so pri izdelavi tradicionalnih izdelkov nezaželjene, ker lahko negativno vplivajo na značilne senzorične lastnosti. Osnovna ideja raziskave je, da bi bilo ta problem mogoče reševati tako, da bi posameznim tradicionalnim sirom namesto komercialnih kultur dodajali izbrane predstavnike njihove, naravno prisotne mikrobne združbe. V ta namen bi bilo potrebno analizirati naravno mikrobioto posameznih vrst sirov ter poiskati naprimer seve, ki lahko producirajo bakteriocine s širokim spektrom delovnja, ter iz njih sestaviti starterske kulture, ki so 'po meri' posamezne vrste sira. Uporaba takšne straterske kulture bi lahko omilila večino težav, ki spremljajo tradicionalno tehnologijo (surovo mleko) in obenem omogočila ohranitev specifičnih lastnosti tradicionalnega sira, vključno s senzoričnimi. Pod pojmom 'bakteriocini' se skriva velika in raznolika skupina protimikrobnih proteinov oziroma peptidov, ki jih najdemo tako pri po Gramu pozitivnih kot pri po Gramu negativnih bakterijah. Opisujejo jih kot ribosomsko sintetizirane proteine oziroma peptide, ki jih bakterija proizvaja z namenom, da prepreči rast drugih bakterij v okolici. Bakteriocini se med seboj razlikujejo v strukturi, molekulski masi, biokemijskih lastnostih, spektru aktivnosti, mehanizmu delovanja, pa tudi lokaciji genov. Bakteriocine mlečnokislinskih bakterij (MKB) razvrščajo v tri glavne skupine, modificirani bakteriocini oziroma lantibiotiki, ne-modificirani (toplotno stabilni) bakteriocini in veliki (toplotno občutljivi) bakteriocini. Vsaka od teh skupin se deli še v tipe ali podskupine. Geni za posamezne bakteriocine so organizirani v zelo dobro ohranjenih regijah, ki se lahko nahajajo na plazmidih ali na bakterijskem kromosomu, kot mobilni ali nemobilni genski elementi. Večinoma so ti elementi organizirani v obliki operonov, ki vsebujejo več genskih determinant, kot so strukturni gen prepeptida, gen proteina imunosti in ostali geni, ki so vključeni v procesiranje in transport bakteriocinov. Za bakteriocine je običajna tarča celična membrana, poročali pa so tudi o drugih možnih tarčnih strukturah. Bakterije, ki sintetizirajo bakteriocine, so razvile sistem zaščite pred delovanjem lastnega bakteriocina, ki ga opisujejo kot imunost. Kadar so bakterije odporne proti baktericinom drugih bakterijskih sevov, pa govorimo o rezistenci. Bakteriocini, ki jih lahko najdemo v surovem mleku in sirih, so zelo raznovrstni, saj je tudi mikrobiota raznovrstna. Slovenske tradicionalne trde in poltrde sire naseljujejo v največjem številu vrste rodova *Lactobacillus* (*Lb*.) in *Enterococcus* (*Ec*.), med običajnimi predstavniki pa najdemo tudi bakterije rodov *Lactococcus* (*Lac*.) in *Streptococcus* (*Str*.), čeprav v manjših koncentracijah. V tem delu smo raziskali tradicionalna slovenska sira Tolminc (iz kravjega mleka, 5 vzorcev) in

Kraški ovčji sir (4 vzorci). Ideja je bila, da iz posameznih tradicionalnih sirov najprej izoliramo celokupno bakterijsko deoksiribonukleinsko kislino (DNA), ter s pomočjo verižne reakcije s polimerazo (PCR) in specifičnih začetnih oligonukleotidov poiščemo prisotnost genskih determinant za nekatere najbolj razširjene bakteriocine MKB. Ko smo preiskušali različne postopke izolacije skupne DNA iz sira, smo ugotovili, da je najbolj pomembna ($p<0,005$) izbira prave raztopine za homogenizacijo vzorcev sira. Za najprimernejšo se je pokazala raztopina natrijevega citrata. Sam postopek izolacije DNA iz homogenata sira je imel manjši vpliv na količino izolirane DNA ($p\leq0,05$). Izolacija s komercialnim kompletom Wizard® Genomic DNA Purification Kit (Promega) se je izkazala za nekoliko učinkovitejšo, kar zadeva količino izolirane DNA, medtem ko smo z izolacijo po postopku s fenol-kloroform-izoamilnim alkoholom pridobili DNA, s katero je bolje potekala PCR in je bila pogosto večja tudi raznolikost genskih determinant za bakteriocine. Prisotnosti genskih determinant nismo ugotavljali samo v skupni bakterijski DNA, pridobljeni neposredno iz sira, ampak tudi v mikrobnih združbah, osamljenih na delno selektivnih gojiščih CATC, Rogosa in M17, ki podpirajo rast enterokokov, laktobacilov oziroma laktokokov in drugih kokov iz skupine MKB . Tako v DNA izolirani neposredno iz sirov kot v DNA izolirani iz osamljenih mikrobnih združb smo s pomočjo PCR iskali genske determinante za 19 bakteriocinov MKB. Večino genskih determinant za bakteriocine smo zasledili tako v skupni DNA sira kot v DNA osamljene mikrobne združbe, ali pa v nobeni od njih, zato smo sklepali, da je njihov vir predvsem kultivabilna bakterijska populacija sira oziroma da je med kultivabilnimi bakterijami iz sira veliko takih, ki vsebujejo genske determinante za preiskovane bakteriocine. Odkritje genskih determinant za posamezne bakteriocine pa še ne zagotavlja, da se taki geni tudi dejansko izražajo in da so bakteriocini tudi aktivni, zato smo preverili tudi bakteriocinsko aktivnost mikrobnih združb. Uporabili smo metodo neposredne inhibicije na trdnem gojišču, pri čemer smo nanašali tako čiste mikrobne združbe kot supernatante, dobljene po njihovi kultivaciji v ustreznem tekočem gojišču (MRS, M17). Kljub temu, da so vsebovali vzorci Kraškega ovčjega sira manj in vzorci Tolminskega sira več genskih determinant za bakteriocine, se pri preverjanju protimikrobne aktivnosti osamljenih mikrobnih združb niso pokazale večje razlike med njimi. Kaže, da samo število genskih determinant za bakteriocine ne pove veliko o dejanski protimikrobni aktivnosti bakterij, ki takšne determinante vsebujejo. To ugotovitev smo preverjali tudi v dodatnem poskusu, kjer smo *in situ* v mleku in siru testirali proti-stafilokokno aktivnost mikrobnih združb sirov, v katerih smo ugotovili najvišje (T2) in najnižje (K3) število genskih determinant za bakteriocine. Čeprav je bila proti-stafilokokna aktivnost mikrobne združbe sira T2 bolj izrazita, te aktivnosti nismo mogli neposredno povezati z aktivnostjo bakteriocinov. Raziskavo smo, skladno s postavljenimi hipotezami, nadaljevali tako, da smo celotno mikrobno združbo iz sira T2, sestavljeno iz posameznih združb z različnih gojišč, dnevno precepljali (1% inokulum) 10 dni zapored v sterilnem mleku. Z zaporednim precepljanjem v mleku, pri čemer smo eno paralelko inkubirali pri 30°C, drugo pa pri 42°C, smo želeli

preveriti morebitno kompetitivno prednost bakteriocinogenih sevov znotraj mikrobne združbe. Po petih in desetih precepljanjih smo preverili sestavo mikrobne združbe ter prisotnost genskih determinant v fermentiranem mleku in v osamljeni mikrobni združbi. Med precepljanjem se je število ugotovljenih genskih determinant za bakteriocine zmanjšalo na tri od prvotnih osem, kar kaže na to, da se je delež sevov z geni za bakteriocine znotraj mikrobne združbe zmanjšal. Na drugi strani so se sevi, ki vsebujejo gneske determinante za enterocin P, L50B in citolizin, uspeli ohraniti med postopkom zaporednega precepljanja. Razlog za takšne rezultate je najverjetneje v tem, da smo z enodnevnim precepljanjem spodbujali del mikrobne združbe, ki ga predstavljajo enterokoki. Poleg razlik v številu genskih determinant so se pokazale tudi spremembe v protimikrobni aktivnosti mikrobne združbe. Za razliko od prvotne mikrobne združbe se po precepljanju ni več pokazala močna inhibicija enterokokov, ohranila se je le protimikrobna aktivnost proti indikatorskim sevom vrst *Listeria* (*L.*) *monocytogenes*, *L. innocua* in *Lb. sakei.* Indikatorska seva *L. monocytogenes* in *Lb. sakei* smo uporabili tudi pri iskanju posameznih izolatov s protimikrobno aktivnostjo. Posamezne seve smo izolirali tako iz prvotne mikrobne združbe, kot iz tiste po 10-ih dneh precepljanja. Izolati iz obeh združb so si bili zelo podobni, kar kaže na to, da so posamezni sevi do neke mere prevladovali, verjetno tudi zaradi določene kompetitivne prednosti. Med izolati sta bila dva takšna, ki najverjetneje tvorita bakteriocine, za katere genskih determinant nismo našli oziroma še niso poznani. Od številnih genskih determinant, ki smo jih ugotovili v mikrobnih združbah sira, smo jih samo nekaj uspeli potrditi tudi pri posameznih izolatih. Glavni razlog za takšne rezultate je najverjetneje postopek iskanja takšnih sevov, ki daje prednost bolj številčnim sevom, ki na številnih ploščah (30-300 kolonij) prevladujejo. Protimikrobne aktivnosti v večini primerov ni bilo mogoče neposredno povezati s prisotnostjo genov za bakteriocine, kar velja tako za osamljene mikrobne združbe kot za posamezne izolate. Neskladnost med številom ugotovljenih genskih determinant in protimikrobno aktivnostjo kaže na kompleksnost, ki obe lastnosti povezuje. Zaradi številnih omejitev bioloških testov za ugotavljanje bakteriocinske aktivnost, med katerimi velja omeniti na prvem mestu nespecifičnost, smo se odločili uvesti alternativno molekularno metodo obratne transkripcije s PCR v realnem času (RT real-time PCR) za dokazovanje izražanja bakteriocinov v siru. Metodo smo preverili na sterilnem modelu sira, inokuliranega z monokulturo seva *Lac. lactis* M 78, ki proizvaja bakteriocin nizin A. Metoda neposredne izolacije ribonukleinske kisline (RNA) iz sira, ki smo jo uporabili, se je izkazala za uspešno. Čeprav ima to pomanjkljivost, da je končna količina izolirane RNA nizka, je reprezentativnost končnih rezultatov tisto, kar daje metodi pravo uporabnost. Reprezentativnost končnih rezultatov pomeni, da ti rezultati predstavljajo dejansko stanje in to je vsekakor na prvem mestu pri izbiri primerne metode. Postopek izolacije RNA skupaj z metodo RT real-time PCR smo uspešno uporabili za merjenje izražanja genskih determinant v modelnem siru, a kljub temu se srečamo z dvema večjima izzivoma, ko gre za uporabo metode v realnih vzorcih sira. Prvi je, kako doseči točno in zanesljivo

normalizacijo RT real-time PCR meritev v sirih, kjer je prisotnih več mikrobnih vrst. Vsaka od treh metod, ki so na voljo, ima svoje omejitve za uporabo v realnih vzorcih sira. Uporaba celokupne celične RNA ali dodajanje zunanje RNA za normalizacijo pri relativni kvantifikaciji predstavljata podoben problem. Rezultati, ki jih dobimo s takšno normalizacijo, dajejo informacijo o izražanju genov celotne populacije seva. Da bi dobili informacijo na nivoju posamezne bakterijske celice bakteriocinogenega seva, bi bilo potrebno vedeti število celic tega seva znotraj populacije, kar pa v kompleksnih vzorcih (sir) ni enostavno, saj vsebujejo vrsto sorodnih sevov, ki jih ni mogoče razlikovati z gojitvenimi metodami. Tretji in verjetno najboljši način normalizacije je, da najdemo primeren konstitutivno izražen referenčni gen. Tudi to je zelo zahtevno, saj imajo siri, še posebej tisti narejeni iz surovega mleka, številne seve, ki si delijo podobne genetske lastnosti. Tudi če bi točno poznali mikrobno sestavo sira, bi težko sestavili takšne začetne oligonukleotide za PCR, ki bi bili dovolj specifični za posamezni bakteriocinogeni sev. Drugi izziv pri uporabi teh metod merjenja izražanja bakteriocinskih genov v realnih vzorcih sira predstavlja dejstvo, da se mikrobna populacija v sirih nahaja v pozni stacionarni fazi rasti oziroma v fazi odmiranja, kjer sta transkripcija genov, stabilnost in količina izolirane RNA slabi. Izražanje genov za bakteriocine se po začetku stacionarne faze rasti običajno ustavi, čeprav so naši rezultati za nekatere gene nizina pokazali tudi nekoliko drugačno sliko. Študija ekspresije 11 genov za nizin v siru je na splošno potrdila nekatera že znana dejstva, prav tako pa je pokazala nekatere korelacije, ki lahko pomagajo pri razlagi regulacije biosinteze in imunosti tega bakteriocina. Skupni zaključki naše celotne raziskave so: (a) tradicionalni slovenski siri vsebujejo seve MKB, ki proizvajajo različne bakteriocine, vendar so le redki takšni, ki imajo kompetitivno prednost pred ostalo mikrobno populacijo; (b) ti siri prav tako vsebujejo MKB, ki skupno ali posamično zavirajo razrast potencialno patogene bakterije *Staphylococcus aureus* v mleku in siru, vendar opažene zaviralne aktivnosti nismo uspeli povezati z bakteriocinsko aktivnostjo; (c) molekularni pristop lahko omogoči hitrejše iskanje bakteriocinogenih sevov vendar pa je klasični pristop z biološkimi testi še vedno potreben, ko je končni cilj iskanja osamitev posameznih sevov ali odkrivanje sevov, ki proizvajajo nove bakteriocine; (d) RT real-time PCR se je izkazala za uporabno pri kvantitativnem ugotavljanju izražanja genov za bakteriocin v siru, ki vsebuje monokulturo bakteriocinogenega seva.

4.3 RESUMÉ

La plupart des fromages et produits laitiers traditionnels sont réalisés à partir de lait cru, qui est, de par sa composition chimique et microbiologique, le principal paramètre, avec les procédures technologiques, déterminant les caractéristiques du produit final. Parfois, des souches microbiennes ne faisant pas partie de la flore technologique peuvent poser un risque sanitaire pour les consommateurs. L'utilisation de ferments commerciaux peut limiter ce problème, mais ils ont généralement un effet défavorable sur les propriétés organoleptiques des fromages traditionnels. Dans cette étude, notre objectif était de déterminer l'intérêt d'utiliser, à la place de ferments commerciaux, des souches provenant de la flore microbienne des fromages Slovènes traditionnels. Pour cela, il est nécessaire d'analyser les populations naturelles des fromages et de sélectionner des souches productrices de bactériocines et pouvant servir à produire des ferments adaptés au type de fromage considéré. L'utilisation de ce type de ferment permettrait de résoudre la plupart des problèmes liés à la technologie traditionnelle (lait cru) tout en préservant les caractéristiques organoleptiques spécifiques de ces fromages.

Le terme "bactériocine" représente un groupe large et hétérogène de protéines antimicrobiennes et de peptides produits par des bactéries Gram-positives et Gram-négatives. Il s'agit de protéines et peptides à synthèse non ribosomique qui sont produits par les bactéries afin d'empêcher la croissance d'autres bactéries dans leur environnement. Les bactériocines peuvent différer en fonction de leur structure, masse molaire, propriétés biochimiques, spectre d'activité, mode d'action et localisation des gènes. Les bactériocines des bactéries lactiques sont classées selon trois groupes principaux, les bactériocines modifiées ou lantibiotiques, les bactériocines non-modifiés (thermostables) et les grandes bactériocines (thermolabiles). Chacun de ces groupes est subdivisé en sous-groupes. Les gènes permettant la synthèse des bactériocines sont regroupés dans des régions conservées sur des plasmides ou sur le chromosome, en tant qu'éléments génétiques mobiles on non mobiles. La plupart d'entre eux sont organisés sous forme d'opérons contenant plusieurs déterminants génétiques, tels que des gènes pré-peptide de structure, des gènes de protéine d'immunité, et d'autres gènes, impliqués dans le transport et la maturation de la bactériocine. Généralement, la principale cible des bactériocines est la membrane cellulaire, mais d'autres cibles ont aussi été reportées. Les bactéries productrices de bacteriocines ont développé des systèmes de résistance à l'action de leur propres bactériocines, nommés systèmes d'immunité. Lorsque les bactéries sont immunes à l'action de bactériocines produites par d'autres souches bactériennes, on parle de système de résistance. Les bactériocines présentes dans le lait cru et les fromages sont diverses, en raison de la grande variété au sein de la flore microbienne. Les fromages slovènes traditionnels à pâte dure ou semi-dure comportent en majorité des souches appartenant aux

espèces *Lactobacillus* (*Lb*.) et *Enterococcus* (*Ec*.), et dans une moindre mesure *Lactococcus* (*Lac*.) et *Streptococcus* (*Str*.).

Dans ce travail, nous avons étudié les fromages slovènes traditionnels nommés Tolminc (lait de vache, cinq échantillons) et Kraški ovčji sir (lait de brebis, quatre échantillons). L'idée de base était d'extraire l'ADN à partir de ces fromages et, par amplification par PCR avec des amorces spécifiques, de rechercher la présence de gènes de bactériocines caractéristiques des bactéries lactiques. Différentes procédures d'homogénéisation initiale du fromage et d'extraction d'ADN à partir des homogénéisats ont été testées. Trois types de tampon d'homogénéisation ont été utilisés : du phosphate dipotassique à 2% (poids/vol), du citrate trisodique dihydraté à 2% (poids/vol) et de la solution de Ringer diluée à ¼. Deux méthodes d'extraction d'ADN ont ensuite été employées. La première était basée sur l'utilisation de billes de zirconium/silice et de phénol-chloroforme-alcool isoamylique comme décrit par Cocolin et al. (2001) et la seconde utilisait un kit commercial de purification génomique (Wizard® Genomic DNA Purification kit, Promega). Nous avons observé que le facteur le plus important ($p<0,005$) était la solution d'homogénéisation, la solution de citrate trisodique étant la plus efficace. La méthode d'extraction d'ADN à partir des fromages homogénéisés avait un effet moindre sur la quantité d'ADN obtenue ($p<0,005$). L'extraction avec le kit Promega était plus efficace en terme de quantité d'ADN obtenue, tandis que la méthode au phénol-chloroforme-alcool isoamylique donnait des extraits qui permettaient de meilleures amplifications par PCR et qui, dans de nombreux cas, donnaient une diversité plus importante de détection de gènes de bactériocines.

La présence de déterminants génétiques n'a pas seulement été déterminée dans l'ADN issu des fromages, mais également dans les consortia microbiens isolés sur des milieux de culture partiellement sélectifs. Les fromages homogénéisés avec de la solution de Ringer à ¼ étaient dilués et ensemencés sur des géloses CATC (Citrate Azid Tween Carbonate), Rogosa et M17, utilisées respectivement pour les entérocoques, lactobacilles et bactéries lactiques mésophiles. Environ 300 colonies ont été récupérées à partir des géloses en utilisant 2 mL de solution de Ringer à ¼. L'ADN a été extrait à partir de chaque suspension cellulaire en utilisant le kit de purification Maxwell 16 Cell DNA (Promega). Dans l'ADN total extrait des fromages ainsi que dans l'ADN des consortia microbiens, nous avons recherché par PCR la présence de gènes spécifiques de 19 bactériocines de bactéries lactiques en utilisant des amorces adaptées. L'analyse des extraits d'ADN des 9 fromages pour la présence de gènes codant pour des bactériocines de bactéries lactiques a montré la présence, dans chaque fromage, d'entre un et neuf des gènes recherchés. Les gènes des entericines, à l'exception de ceux de l'entéricine 31 et de l'entéricine AS-48 (qui n'ont été détectés dans aucun échantillon) étaient fréquemment présents. Parmi les trois bactériocines typiquement produites par *Lactococcus lactis*, les déterminants de la nisine ont été détectés dans six fromages et ceux de la lacticine 481 dans deux fromages. Seuls

deux gènes de bactériocines de lactobacilles ont été détectés dans les fromages: les déterminants de plantaricine A, qui étaient présents dans tous les échantillons de fromage et ceux de l'acidocine B, présents dans seulement deux fromages. La plupart des résultats obtenus avec l'ADN extrait des fromages étaient identiques à ceux de l'ADN des consortia, ce qui indique que les souches productrices de bactériocines font partie de la population bactérienne viable. La détection de gènes de bactériocines ne signifie pas que ces gènes sont exprimés ou que les bactériocines sont actives, c'est la raison pour laquelle nous avons étudié l'activité antimicrobienne des consortia microbiens. Nous avons utilisé une méthode basée sur l'inhibition directe sur milieu gélosé (25 souches indicatrices), où nous avons déposé des surnageants obtenus après culture dans un milieu liquide (MRS, M17). Bien que les fromages de type Kraški ovčji sir contenaient moins de gènes de bactériocines que les fromages de type Tolminc, il n'y avait pas de différences significatives entre les deux du point de vue de leur activité antimicrobienne. Il semble que le nombre de gènes de bactériocines ne soit pas ou peu indicatif de l'activité antimicrobienne des bactéries comportant ces gènes.

Nous avons aussi testé cette conclusion dans une expérience où nous avons déterminé *in situ* l'acticité anti-staphylococcique de consortia microbiens. Les consortia microbiens issus des fromages dans lesquels le nombre de gènes de bactériocines détectés était le plus élevé (T2) et le plus bas (K3) ont été étudiés. Trois types d'expérience de compétition ont été menés. Dans le premier, nous avons déterminé l'effet de la présence des consortia des deux fromages sur la croissance de *Staphylococcus* (*S.*) *aureus* dans le lait. Dans le second, nous avons déterminé si la production d'acide lactique avait un effet sur la croissance de *S. aureus* dans le lait, et dans le dernier nous avons cherché à déterminer si la présence de consortia microbiens issus du fromage T2 avait un effet sur la croissance de *S. aureus* dans un fromage modèle. Les essais de compétition dans du lait ont été réalisés en appliquant les profils de temps/température de la fabrication des fromages de type Tolminc à l'exclusion de la période d'affinage. Les compétitions dans des fromages ont été réalisés de la même manière, mais en incluant une étape d'affinage de 15 jours. Pendant ces essais, l'activité de production de bactériocines était déterminée, soit directement sur les échantillons de fromages, soit dans différents extraits de fromage ou de lait. Cette étude a montré que tous les consortia microbiens étudiés avaient un effet marqué sur la croissance de *S. aureus* dans le lait et le fromage. Bien que les consortia du fromage Tolminc (T2) et Kraški ovčji sir (K3) inhibaient la croissance de *S. aureus* dans le lait et le fromage, il était impossible d'attribuer cette inhibition aux bactériocines pour lesquelles des gènes ont été détectés dans l'ADN extrait à partir des consortia. Les extraits obtenus à partir d'échantillons de lait et de fromages ne montraient pas de présence de composés inhibiteurs. La production de peroxyde d'hydrogène ou d'autres métabolites antimicrobiens n'a pas été évaluée, mais un effet lié à la production d'acides organiques et la baisse du pH a pu être exclu. Parmi les

mécanismes d'inhibition les plus probables, il pourrait y avoir des phénomènes liés à la nutrition, tel qu'une compétition ou la limitation en substrats.

Compte tenu de nos hypothèses, nous avons poursuivi cette étude en récupérant le consortium microbien du fromage T2, sous forme de consortia issus des différents milieux de culture, et en les propageant quotidiennement (inoculum de 1%) pendant 10 jours dans du lait stérile. En réalisant des propagations dans du lait, qui ont été effectuées à 30°C dans un essai et à 42°C dans un autre, nous souhaitions tester l'avantage compétitif des souches productrices de bactériocines au sein de la population microbienne. Après 5 et 10 propagations dans le lait, les consortia microbiens ont été isolés et des extractions d'ADN ont été réalisées. L'isolement de quatre consortia microbiens différents a été réalisé sur des géloses Rogosa (37°C), M17 (30 et 42°C) et CATC (37°C). L'activité antimicrobienne a été déterminée séparément pour chaque consortium, tandis que l'ADN total a été extrait à partir des quatre consortia puis mélangé. L'ADN a aussi été extrait à partir du consortium microbien original du fromage utilisé pour débuter les repiquages. L'analyse de l'activité antimicrobienne du consortium microbien initial, isolé à partir du fromage T2, envers 36 souches indicatrices n'a pas mis en évidence d'inhibition causée par des bactériocines. L'activité antimicrobienne la plus forte a été détectée avec un consortium microbien représenté par des lactobacilles, mais il était probablement dû à la production d'acides organiques. Nous avons observé une inhibition des souches indicatrices *Ec. durans* CCM 5612 et *Ec. faecium* CCM 4647 par le consortium de coques mésophiles (à partir de gélose M17 à 30°C). Il y avait aussi une légère réduction de ces inhibitions lorsqu'un traitement à la protéinase K a été appliqué, ce qui indique une possible implication d'un composé antimicrobien de nature protéique. La concentration de composé antimicrobien dans le surnageant n'était cependant pas suffisante pour être observée par les essais antimicrobiens. Le principal objectif de ce travail était de déterminer si les souches comportant les gènes de bactériocines ont un avantage compétitif dans des populations mixtes dans des conditions données et quelles sont les bactériocines potentiellement impliquées. Des repiquages successifs dans du lait à 30°C résultaient en une réduction du nombre de gènes détectés. Ainsi, après cinq repiquages, seulement deux bactériocines (enterocine P et cytolysine) parmi les cinq testées ont été détectées, et après 10 repiquages aucun d'eux. Les gènes pour ces deux bactériocines, l'entérocine P et la cytolysine, étaient cependant toujours détectés après 10 repiquages successifs à 42°C. Lorsque d'ADN était isolé à partir de consortia microbiens obtenus à partir de trois milieux gélosés différents (Rogosa, M17 et CATC), les déterminants génétiques pour l'entérocine L50B étaient aussi détectés, en plus des déterminants pour l'entérocine P et la cytolysine. Lors de la propagation, le nombre de gènes de bactériocines détectés diminuait de huit à trois, ce qui indique que la proportion de souches comportant les gènes de bactériocines diminuait au sein de la population, mais les souches qui comportaient les gènes pour l'entérocine P, L50B et la cytolysine étaient capables de persister lors de la propagation. L'explication de ce résultat

pourrait être qu'avec une propagation journalière nous avons stimulé la proportion de la population microbienne représentée par les entérocoques. En plus des changements dans les nombres de gènes de bactériocines détectés, il y avait aussi des changements dans l'activité antimicrobienne du consortium microbien. Contrairement au consortium initial, les consortia après propagation n'avaient pas de forte activité antagoniste envers les entérocoques. L'activité antagoniste n'était préservée qu'envers les souches indicatrices *L. monocytogenes* N°10 S 4ab, *Lb. sakei* ATCC 15521 et *L. innocua* ATCC 3090.

Les souches indicatrices *L. monocytogenes* et *Lb. sakei* ont également été utilisées pour isoler des souches individuelles présentant une activité antagoniste. Les souches ayant une activité antagoniste ont été isolées à la fois du consortium initial du fromage T2 et du consortium obtenu après repiquage dans du lait. Des dilutions des consortia microbiens ont été ensemencées sur de la gélose MRS modifiée (10% de sucres, 37°C) et M17 (30°C et 42°C). Après incubation, les colonies ont été recouvertes avec une surcouche de milieu gélosé inoculé avec des souches indicatrices. Les colonies entourées d'un halo d'inhibition clair ont été isolées et purifiées. A partir de l'origine (consortia microbiens avant et après propagation), de la croissance sur milieu gélosé (MRS, M17 et CATC) et des profils d'inhibition, nous avons sélectionné onze isolats pour des analyses complémentaires. Chacune des souches sélectionnées a été testée pour son activité antagoniste envers sept souches indicatrices (*L. monocytogenes* N°10 S 4ab, *S. aureus* ISS 464, *Lb. sakei* ATCC 15521, *L. innocua* ATCC 3090, *Ec. durans* CCM 5612, *Ec. faecalis* ATCC 19433, *Ec. faecalis* CCM 4647 et *Ec. faecium* ATCC 19434). L'ADN génomique de ces isolats a été extrait et utilisé pour la détermination, par PCR, des genres, espèces, et la présence des gènes correspondants aux différentes bactériocines. Nous avons également réalisé un phénotypage des isolats par PhP (PhenePlate^{TM}), qui est basé sur la mesure des cinétiques de réaction biochimique lors de la croissance dans des plaques de microtitration. Neuf des onze souches analysées par des PCR spécifiques d'espèces ont été identifiées comme *Ec. faecalis* et deux comme *Ec. faecium*. Ces deux mêmes groupes ont été identifiés avec le système PhP. Au sein du groupe *Ec. faecalis*, 3 sous-groupes ont été identifiés, alors que les deux souches d'*Ec. faecium* étaient très proches. Toutes les souches sélectionnées, à l'exception d'une (G0xR/1), avaient une activité antagoniste de nature protéique puisque la présence de protéases (chymotrypsine et protéinase K) supprimait l'activité antagoniste. Toutes les souches sauf une (G10x42/3.1) avaient les gènes de cytolysine et avaient la même efficacité envers les espèces du genre *Enterococcus*. Nous supposons que cette bactériocine est responsable de l'activité inhibitrice observée. Etant donné que la cytolisyne a également des propriétés hémolytiques, nous avons testé les souches pour cette propriété dans une gélose au sang et observé qu'aucune d'entre elles n'avait une activité hémolytique. A partir de ces résultats, nous pouvons conclure que la cytolysine n'est pas responsable de l'activité inhibitrice observée. Etant donné que les isolats de consortia microbiens avant et après propagation étaient très similaires, nous pouvons spéculer que des souches

individuelles étaient prédominantes, probablement en raison d'un certain avantage compétitif. Dans cette étude du microbiote du fromage T2 nous avons réalisé l'analyse complète de son potentiel antagoniste, en partant du fromage dans son ensemble jusqu'au niveau des souches, et du niveau génotypique au niveau phénotypique de l'activité antagoniste.

Parmi les nombreux gènes de bactériocines identifiés dans les consortia microbiens du fromage T2, nous n'en avons trouvé qu'une minorité dans les souches isolées à partir de ces consortia. La principale cause de ce résultat est probablement liée à la procédure d'isolement de ces souches. Etant donné que nous n'avons isolé des consortia microbiens et des souches individuelles uniquement à partir des boîtes de Pétri comptables (entre 30 et 300 colonies), nous avons limité notre recherche à la population la plus abondante dans le fromage, et perdu les souches moins représentées. Nous avons sélectionné uniquement les souches présentant une activité antagoniste envers les souches indicatrices, ce qui est un moyen relativement facile de rechercher des souches productrices de bactériocines. Il est connu que la présence de déterminants génétiques de certaines protéines n'assure pas nécessairement leur synthèse, et aussi que les protéines synthétisées ne sont pas nécessairement actives. C'est la raison pour laquelle l'expression de gènes de bactériocines a une importance clé dans la recherche et l'application de souches intéressantes. Les résultats de notre étude ont également montré que la capacité des souches à produire des bactériocines n'est pas forcément suffisante pour leur assurer un avantage compétitif. Il faut aussi prendre en considération le fait que les fromages traditionnels produits à partir de lait cru, ont un microbiote très diversifié dans lequel des souches individuelles prédominent rarement. Au sein de la population microbienne, il y a un certain équilibre entre les différentes souches productrices de bactériocines, qui dépend à la fois de la capacité à produire des bactériocines et de la capacité a résister aux bactériocines produites par les autres souches. Au sein d'une population, les souches produisant différentes bactériocines s'inhibent mutuellement jusqu'à un certain point, puisque la capacité à produire une bactériocine n'apporte pas de résistance aux autres bactériocines. En général, l'écologie microbienne n'attribue pas nécessairement la même importance au rôle des bactériocines pour la compétitivité des souches, qu'au rôle d'autres interactions telles que par exemple la compétition pour des nutriments. Ceci est probablement dû au manque d'études sur le rôle des bactériocines *in vivo*, dans les populations microbiennes.

Dans cette étude, nous n'avons que rarement été en mesure de faire des corrélations concrètes entre l'activité antagoniste et la présence de gènes de bactériocines. Nous avons eu ce problème à la fois pour des consortia et pour des souches individuelles. Ce manque de concordance entre le nombre de gènes détectés et l'activité antagoniste exercée réellement dans les tests d'inhibition montre que ces deux propriétés sont reliées de manière complexe. Il faut prendre en considération le fait que nous avons limité notre

recherche aux bactériocines décrites jusqu'ici et susceptibles d'être présentes dans ces consortia microbiens. Elles ne représentent probablement qu'une faible partie de toutes les bactériocines pouvant être produites par les membres de ces consortia. L'activité antagoniste observée dans nos tests peut aussi être le résultat de l'expression de bactériocines non étudiés dans les analyses par PCR. De plus, de nombreux isolats comportent des gènes correspondants à plusieurs bactériocines et nous ne savons pas comment ces gènes sont exprimés et quelles sont les interactions dans l'expression de tous ces gènes. Le choix des souches indicatrices peut aussi poser problème, car il n'y a qu'une connaissance limitée de leur sensibilité aux bactériocines. Un problème similaire concerne les protéases utilisées pour confirmer la nature protéique de l'inhibition; différentes bactériocines sont inactivées par différentes protéases. Les tests d'inhibition ont aussi d'autres limites pour détecter la production de bactériocines, notamment pour des milieux complexes tel que le fromage. Entre autre, il y a la limite de détection, qui est plutôt élevée, la répartition non homogène des bactériocines dans la matrice fromagère, l'adsorption des bactériocines sur les protéines, et leur dégradation par des peptidases bactériennes intra- et extra-cellulaires libérées à partir des cellules lysées. De plus, nous ne pouvons pas mesurer l'activité individuelle des bactériocines, mais uniquement l'activité globale. D'un autre coté, les méthodes moléculaires se sont avérées utiles, comme déjà mentionné dans d'autres études. Les méthodes moléculaires devraient certainement être améliorées afin de permettre la détection de gènes qui sont exprimés dans des matrices fromagères et ainsi d'éviter, au moins partiellement, l'utilisation de tests d'inhibition moins spécifiques.

En raison de nombreux inconvénients des tests d'inhibition, nous avons décidé de mettre en place une méthode de détermination de l'expression de gènes de bactériocines dans le fromage par Reverse Transcription et PCR en temps réel (RT-PCR en temps réel). La méthode a été testée en utilisant un fromage modèle stérile ensemencé par une culture pure de *Lac. lactis* M78, une souche productrice de nisine A. L'ARN total a été extrait en utilisant une méthode d'extraction directe sans séparation préalable des cellules de la matrice fromagère (Monnet et al., 2008). En plus de l'extraction d'ARN, les concentrations cellulaires, le pH et la production de nisine ont été mesurés. La souche *Lac. lactis* M78 se développait bien dans le fromage modèle, et produisait des niveaux détectables de nisine après 4 h de culture. La méthode d'extraction directe d'ARN s'est avérée efficace. Elle présente l'inconvénient de ne produire que de faibles quantités d'ARN, mais elle permet d'obtenir un extrait dont la composition est plus représentative de l'ARN des microorganismes présents dans le fromage. Même si nous avons utilisé avec succès la procédure d'extraction d'ARN en combinaison avec la RT-PCR en temps réel pour suivre l'expression de gènes de bactériocines dans un modèle fromager, il y a deux principaux défis pour appliquer cette méthode à des vrais échantillons de fromages. Le premier concerne la manière de normaliser les résultats des mesures de RT-PCR en temps réel dans

des échantillons où plusieurs espèces microbiennes sont présentes. Chacune des trois méthodes de normalisation disponibles présente des problèmes spécifiques lorsqu'elles sont appliquées à l'analyse de fromages. La normalisation par rapport à l'ARN total ou l'ajout d'un ARN exogène présentent un problème similaire. Les résultats ainsi obtenus fournissent une information sur l'expression du gène par rapport à l'ensemble de la population microbienne. Pour obtenir une information au niveau de la souche productrice de bactériocine considérée, il faudrait connaître le nombre exact de cellules de cette souche au sein de la population, ce qui est difficilement réalisable dans un milieu complexe tel que le fromage en raison de la présence d'autres souches ne pouvant être distinguées facilement sur des milieux gélosés. La troisième, et probablement la meilleure méthode de normalisation, serait d'utiliser un gène de référence exprimé de façon constitutive. Cette approche est cependant très délicate car les fromages, en particulier ceux produits à partir de lait cru, contiennent de nombreuses souches partageant des caractères génétiques communs. Même si nous connaissions exactement la composition microbienne de chaque fromage, il serait très difficile de trouver des amorces nucléotidiques suffisamment spécifiques pour une souche productrice d'une bactériocine donnée. Le second défi pour pouvoir appliquer la RT-PCR en temps réel pour suivre l'expression de gènes dans des vrais fromages est lié au fait que les populations microbiennes des fromages sont en fin de croissance, ou en phase de mortalité, où la transcription de gènes est probablement faible. Il en va de même de la quantité et la qualité de l'ARN extrait. L'expression de gènes de bactériocines s'arrête généralement au début de la phase stationnaire de croissance, mais nous n'avons pas observé cela pour certains des gènes correspondants à la nisine. L'étude de l'expression des 11 gènes de la nisine A dans du fromage a, dans l'ensemble, confirmé certains phénomènes déjà connus et également permis d'établir des corrélations contribuant à expliquer la régulation de la biosynthèse et l'immunité de la nisine. Comme d'autres équipes, nous avons mis en évidence une production de nisine dans le fromage, mais en plus, nous avons été en mesure de comparer les tests d'inhibition avec les mesures par RT-PCR en temps réel. Nous avons confirmé une forte corrélation entre les résultats des deux méthodes, ce qui montre la relation étroite entre la transcription des gènes de la nisine et l'activité finale. A partir de la transcription des gènes nisine en fonction du temps, nous avons été en mesure de distinguer quatre groupes de gènes : *nisA*, *nisBTCIP*, *nisFEG* et *nisRK*. En comparant les dynamiques d'expression de ces quatre groupes de gènes, leur localisation et leur fonction dans le groupe de gènes *nis*, leur dynamique de croissance et l'activité antimicrobienne de la souche productrice, nous avons souligné certains éléments déjà connus, comme le fait que la nisine agit comme une phéromone de *quorum sensing* qui stimule sa propre production. Cette étude est la première dans laquelle l'expression des 11 gènes de la nisine a été analysée, et également l'une des premières qui présente une quantification rigoureuse, en fonction du temps, de transcrits dans un milieu fromager.

Les principales conclusions de ce travail sont les suivantes : (a) les fromages Slovènes traditionnels contiennent des bactéries lactiques produisant différentes bactériocines, mais seulement une partie d'entre elles ont un avantage compétitif par rapport au reste de la population microbienne ; (b) ces fromages contiennent aussi des bactéries lactiques qui, individuellement ou collectivement, limitent la croissance de souches potentiellement pathogènes de *Staphylococcus aureus* dans le lait et le fromage, mais nous n'avons pas été en mesure de lier l'action antagoniste avec la production de bactériocines ; et (d) la RT-PCR en temps réel nous a permis de quantifier de l'expression de gènes de synthèse d'une bactériocine dans un fromage obtenu avec une culture pure d'une souche productrice de bactériocine.

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Beside Uroš Kren, Rok Erker is the oldest (vine) friend I have and he is only half of himself. The other half of him is his girlfriend Katja Povalej, she too is not a stand alone

type, Rok is there to make her whole (I'm sure they will make it official some day). Rok and Katja are the two persons who besides my mother (of course) can test my nerves the most. I guess the closer the person is the more it can get to you and these two are really, really close. They are my number one fans! I hope it is not too cocky for me to say that, but we are each other fans and we cheer for one another, I think we are way too young to have been born such a long time ago. I thank them for being next to me because as much as they know how to bring on the stress (unintentionally) they can take it away ten times faster (unintentionally). Thank you again. Since the apple does not fall far from the tree I have to mention Rok's family, family Erker/Pečenko, especially Na-Taša who has followed my career's ups and downs from the beginning. They have taken me in as a family member long time ago and I think the bond is growing stronger with every passing day. I thank them from the bottom of my hart for ALL the support and words of encouragement during these long years of my study/work.

One thing is to make visits as a family and a completely different thing is to actually live with me, for this you have to be a very special person. I think we both sure seem special under pressure. Nevertheless I want to thank my mother for all of her support during my education and life in general. She always encouraged me and my sister to have a good education and she always provided everything to have it. This was never easy for her being a single parent and I'm truly grateful that she went the extra mile for it at the beginning when it was the hardest. She too invested a lot of herself into this. When talking about investments I cannot go on without thanking my uncle Boban and aunt Darja for making my first year of this higher education tuition free, but most of all I want to thank them for all the moral support, the name 'Inđinjer' will forever ring in my ears (but this is something that will have to change some time soon).

I would also like to thank my father who went his separate way a long time ago and since then I really, really don't know much about his life in all the hidden corners of the world. The thing is he never truly left us he was always in touch, supporting us somewhere from the backstage. I thank him for this small part he had in my life, I'm sure he wanted to make this part bigger but it would just complicate things for us and I thank him very much for that, for not complicating things too much.

Nothing is as complicating as it seems when you have a sister, especially if you have an older sister to protect you and guide you. Well, she never had to do much she just stood there as a good role model. I thank my sister for that, for being a good role model and believing in me. My sister is not just about believing, she is also about doing. Of all the things she did for me the most pretentious one would have to be playing a role of my pocket lector. Without her I don't write, I don't read or speak English, I barely manage Slovene language without her. I thank her for that and also for leaving some space for excessively poetic, rule bending, half incoherent side of me.

Today, I am the man I want to be (not really, but I can walk on my own towards anything I want) and also my sister has her own children to play with and take care of. The minute I finished writing this dissertation she gave birth to a beautiful baby boy named Erik. So, I think it was written in the stars that this dissertation would be dedicated to him, his sister

Lana and all the generations to come. I hope that they will all write their own doctoral dissertation acknowledgements some day ..... not really, I just want us all to be happy.

In conclusion of my long acknowledgements I would just like to say

FOR ME A LOT OF TIMES BELIEVING IN MYSELF IS JUST A PROJECTION OF SOMEONE ELSE BELIEVING IN ME, AND I THANK EACH AND EVERY ONE OF YOU RESPONSIBLE FOR MAKING THIS PROJECTION POSSIBLE AND STRONG AS IT IS.

ANNEXES (PRILOGE)

Annex A (Priloga A)

Statistical analysis of data (Trmčić et al., 2008)

The concentration results of extracted DNA, obtained by different procedures of direct extraction from cheese (Trmčić et al., 2008), were statistically analysed. Taking in to account that cheeses are inhabited by a similar concentration of LAB (approximately 10^9 cfu/g) we made an assumption that there is no difference in total amount of DNA in each cheese that cam be extracted. In accordance with this assumption each cheese sample was regarded as a repetition and we performed statistical analysis of complete two-factor experiment with two fixed factors being evaluated. The first factor were different buffers being used for homogenisation of cheeses and the second factor being evaluated were different final procedures of DNA extraction from cheese homogenates. Computer program used for statistical analysis of data was SAS/STAT (SAS Institute inc.), more specifically the general linear modelling procedure (GLM). With analysis of variance during GML procedure we were looking for statistically significant differences between different treatments, statistically significant effect of one or the other factor and statistically significant interactions between the factors. All claims are made based on 95% confidence interval.

Statistična obdelava podatkov (Trmčić in sod., 2008)

Rezultate koncentracij izolirane DNA, ki smo jih dobili pri različnih postopkih neposredne izolacije iz sira, smo statistično obdelali. Glede na to, da sire naseljuje podobna koncentracija MKB (okoli 10^9 ke/g) smo naredili predpostavko, da med devetimi vzorci sira ni razlik v količini skupne DNA, ki je prisotna v siru in se jo lahko iz njega izolira. Ob takšni predpostavki, da so vzorci sirov ponovitve, smo naredili statistično analizo popolnega dvofaktorskega poskusa z dvema fiksnima preučevanima dejavnikoma. Prvi preučevani dejavnik je bila uporaba različnih raztopin za homogenizacijo sira in drugi preučevani dejavnik postopek končne izolacije DNA iz homogenata. Računalniški program, ki smo ga uporabili za statistično analizo podatkov je SAS/STAT (SAS Institute inc.) in sicer GLM postopek programa (angl. general linear modeling procedure). Z analizo variance znotraj GLM postopka smo iskali prisotnost statistično značilnih razlik med posameznimi obravnavanji, prisotnost statistično značilnega vpliva enega in drugega dejavnika ter prisotnost statistično značilne interakcije med dejavnikoma. Vse trditve, ki izhajajo iz statistične analize podatkov, so postavljene na osnovi 95% intervala zaupanja.

Copy of variance analysis from SAS/STAT program (SAS Institute Inc.)

Izpis analize variance programa SAS/STAT (SAS Institute inc.)

Legend (Legenda):

puf … Buffer for homogenisation of cheese (Raztopine za homogenizacijo sira)

1 … 2% Dipotassium hydrogen phosphate (2% Di-kalijev hidrogen fosfat)

2 … 2% Trisodium citrate dihydrate (2% Natrijev citrat dihidrat)

3 … Quarter-strength Ringer's solution (Fiziološka raztopina)

iz … Final procedure of DNA extraction (Postopek končne izolacije DNA)

1 … Commercial genomic DNA extraction kit- Wizard® Genomic DNA Purification Kit, Promega (Komercialni kompleta za izolacijo genomske DNA, Promega)

2 … Phenol-chloroform-isoamyl alcohol procedure – Cococlin et al., 2001 (Postopek s fenol-kloroform-izoamilnim alkoholom - Cococlin in sod., 2001)

Report copy of analysis where we took in to account the interaction between the two factors - puf*iz (Izpis analize pri katerem smo upoštevali interakcije med obema dejavnikoma – puf*iz)

The SAS System 11:10 Thursday, June 5, 2008 5

The GLM Procedure

Class Level Information

Class	Levels	Values
puf	3	1 2 3
iz	2	1 2

Number of observations 54

The SAS System 11:10 Thursday, June 5, 2008 6

The GLM Procedure

Dependent Variable: konc konc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	6451.93858	1290.38772	4.00	0.0041
Error	48	15468.11349	322.25236		
Corrected Total	53	21920.05207			

R-Square	Coeff Var	Root MSE	konc Mean
0.294340	112.8053	17.95139	15.91361

Source	DF	Type I SS	Mean Square	F Value	Pr > F
puf	2	4935.334519	2467.667260	7.66	0.0013
iz	1	1352.150856	1352.150856	4.20	0.0460
puf*iz	2	164.453206	82.226603	0.26	0.7758

Source	DF	Type III SS	Mean Square	F Value	Pr > F
puf	2	4935.334519	2467.667260	7.66	0.0013
iz	1	1352.150856	1352.150856	4.20	0.0460
puf*iz	2	164.453206	82.226603	0.26	0.7758

Report copy of analysis where we neglected the interaction between the two factors
(Izpis analize pri katerem smo zanemarili vpliv interakcij med dejavnikoma)

The SAS System 11:10 Thursday, June 5, 2008 7

The GLM Procedure

Class Level Information

Class	Levels	Values
puf	3	1 2 3
iz	2	1 2

Number of observations 54

The SAS System 11:10 Thursday, June 5, 2008 8

The GLM Procedure

Dependent Variable: konc konc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	6287.48538	2095.82846	6.70	0.0007
Error	50	15632.56670	312.65133		
Corrected Total	53	21920.05207			

R-Square	Coeff Var	Root MSE	konc Mean
0.286837	111.1121	17.68195	15.91361

Source	DF	Type I SS	Mean Square	F Value	Pr > F
puf	2	4935.334519	2467.667260	7.89	0.0010
iz	1	1352.150856	1352.150856	4.32	0.0427

Source	DF	Type III SS	Mean Square	F Value	Pr > F
puf	2	4935.334519	2467.667260	7.89	0.0010
iz	1	1352.150856	1352.150856	4.32	0.0427

Report copy of comparison of each factor

(Izpis primerjav enega in drugega dejavnika)

The SAS System 11:10 Thursday, June 5, 2008 9

The GLM Procedure
Least Squares Means

puf	konc LSMEAN	Standard Error	Pr > |t|
1	5.3544444	4.1676754	0.2048
2	28.5055556	4.1676754	<.0001
3	13.8808333	4.1676754	0.0016

iz	konc LSMEAN	Standard Error	Pr > |t|
1	20.9175926	3.4028927	<.0001
2	10.9096296	3.4028927	0.0023

Annex B (Priloga B)

Antimicrobial activity of isolated microbial consortia of cheese T2 and its concentrated supernatants, obtained after cultivation in liquide growth media (MRS, M17).

Protimikrobna aktivnost osamljenih mikrobnih združb sira T2 in njihovih koncentriranih supernatantov, dobljenih po njihovi kultivaciji v tekočih gojiščih (MRS, M17)

Indicator strains/ Indikatorski sevi	Isolated microbial consortia/ Osamljena mikrobna združba sira												
	CATC			Rogosa				M17 30°C			M17 42°C		
	M	S	NS	M	m	S	NS	M	S	NS	M	S	NS
C. perfringens 1B 1106	0	-	-	2	0	-	-	0	-	-	0	-	-
C. tyrobutyricum DSM 2637	0	-	-	8	1	-	-	0	-	-	0	-	-
Lb. sakei ATCC 15521	0	-	-	4	2	-	-	0	-	-	0	-	-
Lb. delbrueckii ssp. delbruecki ATCC 9649	0	-	-	4	8	-	-	0	-	-	0	-	-
Lb. johnsonii NCC 533	0	-	-	7	0	-	-	0	-	-	0	-	-
Lb. delbruecki ssp. bulgaricus ATCC 11842^T	0	-	-	1	0	-	-	0	-	-	0	-	-
Lb. paracasei ssp. paracasei DSM 5622	0	-	-	5	1	-	-	0	-	-	0	-	-
Lb. plantarum NCDO 1193	0	-	-	6	0	-	-	0	-	-	0	-	-
Lb. fermentum ATCC 14931	0	-	-	5	0	-	-	4	-	-	4	-	-
E. faecalis ATCC 19433	0	-	-	4	0	-	-	0	-	-	0	-	-
E. faecium CCM 4647	1	-	-	9	2	-	-	0	-	-	0	-	-
E. durans CCM 5612	2	-	-	6	0	-	-	0	-	-	0	-	-
L. monocytogenes N°10 S 4ab	0	-	-	13	4	-	-	0	-	-	0	-	-
L. innocua ATCC 33090	0	-	-	10	5	-	-	0	-	-	0	-	-
B. cereus CCM 2010	0	-	-	14	0	+	-	0	-	-	0	-	-
E. coli ATCC 11229	0	-	-	13	1	+	-	0	-	-	0	-	-
S. aureus ATCC 25923	0	/	/	6	3	-	-	0	/	/	0	/	/
S. aureus IM 131	0	/	/	11	3	-	-	0	/	/	0	/	/
S. aureus ATCC 19095	0	/	/	5	0	/	/	0	/	/	0	/	/
S. aureus ATCC 29213	0	/	/	17	4	-	-	0	/	/	0	/	/

» continues «
» se nadaljuje «

» continuation «
» nadaljevanje «

Indicator strains/ Indikatorski sevi	Isolated microbial consortia/ Osamljena mikrobna združba sira												
	CATC			Rogosa				M17 30°C			M17 42°C		
	M	S	NS	M	m	S	NS	M	S	NS	M	S	NS
S. aureus RN 4220	0	/	/	11	2	/	/	0	/	/	0	/	/
S. aureus WS 2605	0	/	/	4	1	/	/	0	/	/	0	/	/
S. aureus WS 2288	0	/	/	5	0	/	/	0	/	/	0	/	/
S. aureus WS 2438	0	/	/	6	0	/	/	0	/	/	0	/	/
S. aureus WS 2608	0	/	/	8	3	-	-	0	/	/	0	/	/
S. aureus WS 2286	0	/	/	9	0	/	/	0	/	/	0	/	/
S. aureus WS 2610	0	/	/	6	2	-	-	0	/	/	0	/	/
S. aureus WS 2787	0	/	/	7	5	-	-	0	/	/	0	/	/
S. aureus WS 2613	0	/	/	16	3	-	-	0	/	/	0	/	/
S. aureus WS 2898	0	/	/	4	0	/	/	0	/	/	0	/	/
S. aureus WS 2980	0	/	/	13	5	-	-	0	/	/	0	/	/
S. aureus WS 3543	0	/	/	5	0	/	/	0	/	/	0	/	/
S. aureus WS 2611	0	/	/	7	0	/	/	0	/	/	0	/	/
S. aureus Č.Sa.3.3	0	/	/	9	0	-	-	0	/	/	0	/	/
S. aureus Č.Sa.4.2	0	-	-	7	0	+	-	0	-	-	0	-	-
S. aureus Č.Sa.5.1	0	/	/	7	2	-	-	0	/	/	0	/	/

Legend:
M … Inhibition halo size (mm) when using isolated microbial consortium in spot-test/ Velikost inhibicijske cone (mm) pri difuzijskem-testu z osamljeno mikrobno združbo
m … Inhibition halo size (mm) when using isolated microbial consortia in spot-test with modified MRS medium/ Velikost inhibicijske cone (mm) pri izvedbi difuzijskega-testa na modificiranem MRS gojišču
S … Presence (+) or absence (-) of inhibition halo when using concentrated supernatant of isolated microbial consortium in spot-test/ Prisotnost (+) ali odsotnost (-) inhibicijske cone pri izvedbi difuzijskega-testa s koncentriranim supernatantom osamljene mikrobne združbe
NS … Presence (+) or absence (-) of inhibition halo when using neutralised and concentrated supernatant of isolated microbial consortium in spot-test/ Prisotnost (+) ali odsotnost (-) inhibicijske cone pri izvedbi difuzijskega-testa z nevtraliziranim in koncentriranim supernatantom osamljene mikrobne združbe
… Inhibition halo was clear with well defined edge/ Inhibicijska cona je čista z ostrim in dobro definiranim robom
/ … Spot-test was not carried out/ Difuzijski-test ni bil izveden

Annex C (Priloga C)

Antimicrobial activity of isolated microbial consortia of cheese K3 and its concentrated supernatants, obtained after cultivation in liquide growth media (MRS, M17).

Protimikrobna aktivnost osamljenih mikrobnih združb sira K3 in njihovih koncentriranih supernatantov, dobljenih po njihovi kultivaciji v tekočih gojiščih (MRS, M17)

Indicator strains/ Indikatorski sevi	Isolated microbial consortia/ Osamljena mikrobna združba sira												
	CATC			Rogosa				M17 30°C			M17 42°C		
	M	S	NS	M	m	S	NS	M	S	NS	M	S	NS
C. perfringens 1B 1106	0	+	-	1	0	-	-	2	-	-	1	-	-
C. tyrobutyricum DSM 2637	1	-	-	4	0	-	-	2	-	-	1	-	-
Lb. sakei ATCC 15521	0	-	-	3	0	-	-	0	-	-	0	-	-
Lb. delbrueckii ssp. delbruecki ATCC 9649	0	-	-	2	0	-	-	0	-	-	0	-	-
Lb. johnsonii NCC 533	1	-	-	1	0	-	-	0	-	-	1	-	-
Lb. delbruecki ssp. bulgaricus ATCC 11842^T	0	-	-	0	0	-	-	0	-	-	0	-	-
Lb. paracasei ssp. paracasei DSM 5622	0	-	-	3	0	-	-	2	-	-	0	-	-
Lb. plantarum NCDO 1193	0	-	-	3	0	-	-	0	-	-	0	-	-
Lb. fermentum ATCC 14931	0	-	-	6	0	-	-	2	-	-	0	-	-
E. faecalis ATCC 19433	0	-	-	4	0	-	-	2	+	-	2	+	-
E. faecium CCM 4647	2	-	-	6	0	-	-	3	+	-	2	-	-
E. durans CCM 5612	1	-	-	5	0	-	-	2	-	-	1	+	-
L. monocytogenes N°10 S 4ab	1	-	-	7	0	-	-	1	-	-	0	-	-
L. innocua ATCC 33090	1	-	-	7	0	-	-	1	-	-	0	-	-
B. cereus CCM 2010	0	-	-	8	0	-	-	2	-	-	0	-	-
E. coli ATCC 11229	1	+	-	5	0	-	-	2	+	-	1	-	-
S. aureus ATCC 25923	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus IM 131	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus ATCC 19095	0	-	-	/	0	+	-	5	-	-	0	-	-
S. aureus ATCC 29213	0	-	-	/	0	+	-	0	+	-	0	-	-

» continues «
» se nadaljuje «

» continuation «
» nadaljevanje «

Indicator strains/ Indikatorski sevi	Isolated microbial consortia/ Osamljena mikrobna združba sira												
	CATC			Rogosa				M17 30°C			M17 42°C		
	M	S	NS	M	m	S	NS	M	S	NS	M	S	NS
S. aureus RN 4220	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2605	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2288	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2438	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2608	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2286	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2610	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2787	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2613	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2898	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2980	1	-	-	/	0	+	-	0	-	-	1	-	-
S. aureus WS 3543	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus WS 2611	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus Č.Sa.3.3	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus Č.Sa.4.2	0	-	-	/	0	+	-	0	-	-	0	-	-
S. aureus Č.Sa.5.1	0	-	-	/	0	+	-	0	-	-	0	-	-

Legend:
M … Inhibition halo size (mm) when using isolated microbial consortium in spot-test/ Velikost inhibicijske cone (mm) pri difuzijskem-testu z osamljeno mikrobno združbo
m … Inhibition halo size (mm) when using isolated microbial consortia in spot-test with modified MRS medium/ Velikost inhibicijske cone (mm) pri izvedbi difuzijskega-testa na modificiranem MRS gojišču
S … Presence (+) or absence (-) of inhibition halo when using concentrated supernatant of isolated microbial consortium in spot-test/ Prisotnost (+) ali odsotnost (-) inhibicijske cone pri difuzijskem-testu s koncentriranim supernatantom osamljene mikrobne združbe
NS … Presence (+) or absence (-) of inhibition halo when using neutralised and concentrated supernatant of isolated microbial consortium in spot-test/ Prisotnost (+) ali odsotnost (-) inhibicijske cone pri izvedbi difuzijskega-testa z nevtraliziranim in koncentriranim supernatantom osamljene mikrobne združbe
… Inhibition halo was clear with well defined edge/ Inhibicijska cona je čista z ostrim in dobro definiranim robom
/ … Spot-test was not carried out/ Difuzijski-test ni bil izveden

Annex D (Priloga D)

Average counts of microorganisms in milk during propagation of T2 cheese consortia.

Povprečno število mikroorganizmov v mleku tekom zaporednega precepljanja mikrobne združbe sira T2.

Temperature of propagation/ Temperatura propagacije	Number of transfers/ Število precepljanj	Mesophilic LAB (cfu/mL)/ Mezofilni MKB (ke/mL)	Thermophilic LAB (cfu/mL)/ Termofilne MKB (ke/mL)	Enterococci (cfu/mL)/ Enterokoki (ke/mL)	Lactobacilli (cfu/mL)/ Laktobacili (ke/mL)	pH
At milk inoculation/ Ob inokulaciji mleka	0x	$8,2x10^4$	$1,1x10^5$	$9,8x10^3$	$3,7x10^5$	6,54
30°C	5x	$6,6x10^8$	$1,1x10^7$	$8,5x10^5$	$2,9x10^8$	4,25
	10x	$1,9x10^8$	$1,6x10^7$	$1,1x10^5$	$2,7x10^7$	4,12
42°C	5x	$2,7x10^7$	$3,2x10^7$	$3,0x10^4$	$2,6x10^7$	4,31
	10x	0 *	$3,6x10^7$	0 *	$1,7x10^7$	4,03

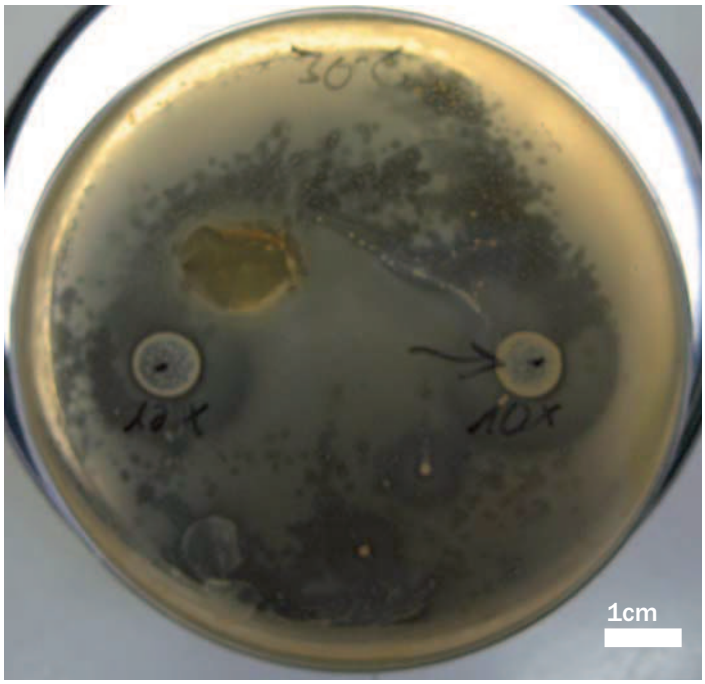
Legend: * ... No growth at dilution 10^{-1} and three repetitions

Legenda: * … Brez rasti pri razredčitvi 10^{-1} in treh ponovitvah

Annex E (Priloga E)

Antimicrobial activity of propagated mesophilic microbial consortia (ten transfers at 30°C) against *L. monocytogenes* N°10 S 4ab.

Protimikrobna aktivnost zaporedno precepljane mezofilne mikrobne združbe(deset precepljanj pri 30°C) proti *L. monocytogenes* N°10 S 4ab.



→ point of proteinase K application (mesto, kjer se je aplicirala proteinaza K)

Annex F (Priloga F)

Antimicrobial activity of bacteriocinogenic strains
Lac. lactis ssp. *lactis* M 78 and *E. faecium* ATCC 19434.

Protimikrobna aktivnost bakteriocinogenih sevov
Lac. lactis ssp. *lactis* M 78 in *E. faecium* ATCC 19434.

Indicator strains/ Indikatorski mikroorganizmi	Test strains/ Testni sevi	
	Lac. lactis ssp. *lactis* M 78	*E. faecium* ATCC 19434
S. aureus Č.Sa.4.2	2 [k]	0
S. aureus ISS 465	3 [k]	0
S. aureus ISS 464	5 [k]	0
S. aureus ISS 23	3 [k]	0
L. monocytogenes N°10 S 4ab	/	6 [kp]
L. innocua ATCC 33090	/	6 [kp]
E. faecalis ATCC 19433	/	3 [kp]
E. faecium CCM 4647	/	3 [kp]
E. durans CCM 5612	/	6 [kp]
Lb. sakei ATCC 15521	9 [k]	8 [kp]

Legend/Legenda:

2-8 … Inhibition halo size – mm (velikost inhibicijske cone - mm)

… Clear inhibition halo with sharp and well defined edge (Inhibicijska cona je čista z ostrim in dobro definiranim robom

k … Protein nature of inhibition was confirmed with chymotrypsin (Proteinska narava inhibicije je bila potrjena z uporabo kimotripsina)

p … Protein nature of inhibition was confirmed with proteinase K (Poteinska narava inhibicije je bila potrjena z uporabo proteinaze K)

/ … The spot-test was not performed (Difuzijski-test ni bil izveden)

Annex G (Priloga G)

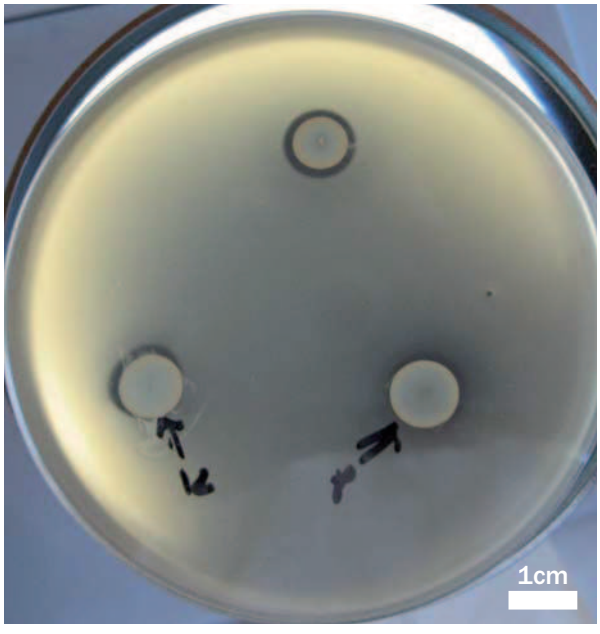
Antimicrobial activity of isolated strains against *Ec. faecium* ATCC 19434.

Protimikrobna aktivnost osamljenih sevov proti *Ec. faecium* ATCC 19434.

Isolated strains/ Osamljeni sevi	Indicator strain/ Indikatorski sev
	Ec. faecium ATCC 19434
G0x30/1	1 k p
G0x30/4	1 k p
G0x42/1	1 k p
G0x42/2	1 k p
G0x42/9	1 k p
G0xR/1	4 k **
G10x30/4	1 k p
G10x42/3	1 k p
G10x42/3.1	0
G10x42/9	1 k p
G1042L/4	1 k p
Čt.1.1	0
Čt.3.1	2 k p

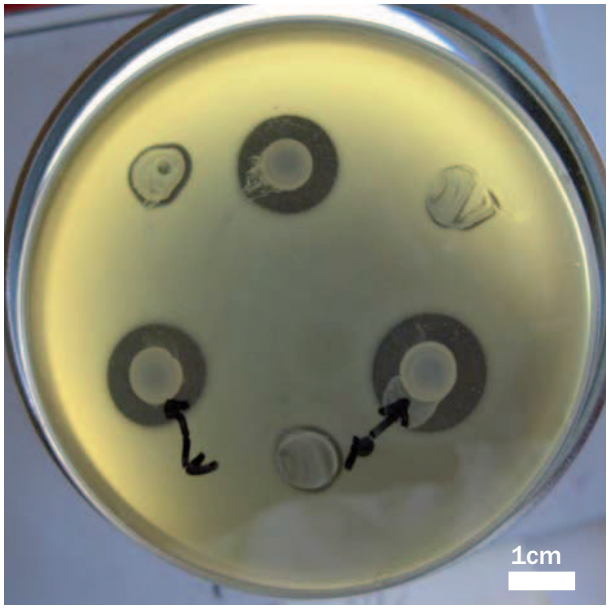
Legend/ Legenda:

0-4	…	Inhibition halo size - mm (velikost inhibicijske cone - mm)
	…	Clear inhibition halo with sharp nad well defined edge (Inhibicijska cona je čista z ostrim in dobro definiranim robom)
k	…	Protein nature of inhibition was confirmed with chymotrypsin (Proteinska narava inhibicije je bila potrjena z uporabo kimotripsina)
p	…	Protein nature of inhibition was confirmed with proteinase K (Proteinska narava inhibicije je bila potrjena z uporabo proteinaze K)
**	…	Very weak effect of chymotrypsin on antimicrobial activity of the strain (Zelo šibek vpliv kimotripsina na protimikrobno aktivnost izolata)



Antimicrobial activity of isolate G0x42/9 against *Ec. faecium* ATCC 19434.
K-presence of chymotrypsin, PK-presence of proteinase K.

Protimikrobna aktivnost izolata G0x30/1 proti *Ec. faecium* ATCC 19434.
K-prisotnost kimotripsina, PK-prisotnost proteinaze K.



Antimicrobial activity of isolate G0xR/1 against *Ec. faecium* ATCC 19434.
K-presence of chymotrypsin, PK-presence of proteinase K.

Protimikrobna aktivnost izolata G0xR/1 proti *Ec. faecium* ATCC 19434.
K-prisotnost kimotripsina, PK-prisotnost proteinaze K.

Annex H (Priloga H)

Antimicrobial activity of isolate G0x42/9 against *L. monocytogenes* N°10 S 4ab.

Protimikrobna aktivnost izolata G0x42/9 proti *L. monocytogenes* N°10 S 4ab.

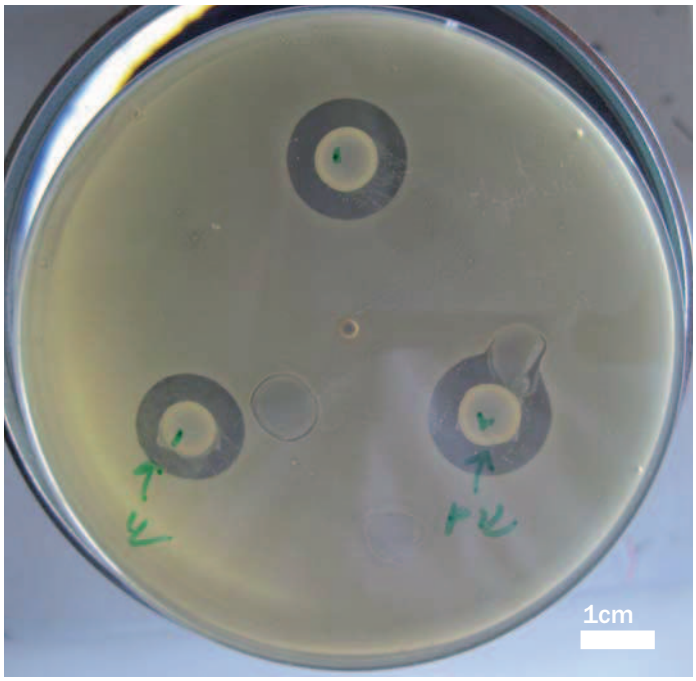


K ... presence of chymotrypsin (prisotnost kimotripsina)
PK ... presence of proteinase K (prisotnost proteinaze K)

Annex I (Priloga I)

Antimicrobial activity of isolate G0xR/1 against *E. durans* CCM 5612.

Protimikrobna aktivnost izolata G0xR/1 proti *E. durans* CCM 5612.

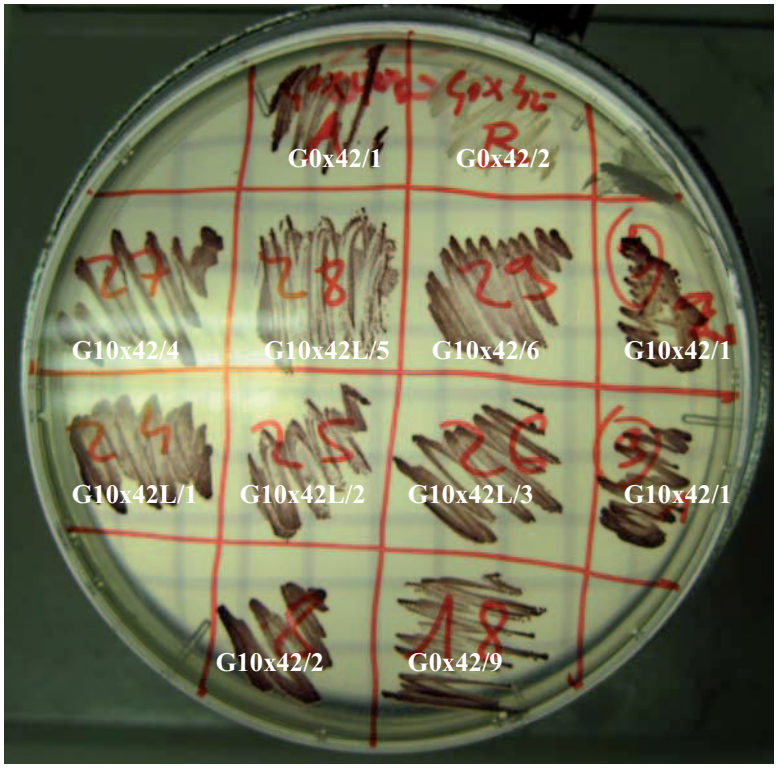


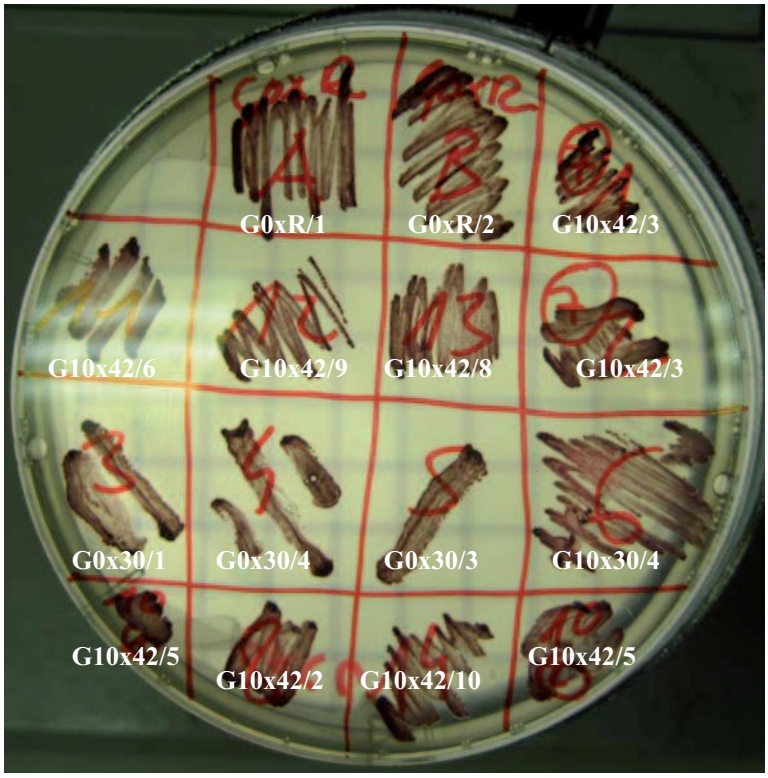
K ... presence of chymotrypsin (prisotnost kimotripsina)
PK ... presence of proteinase K (prisotnost proteinaze K)

Annex J (Priloga J)

Growth of isolated strains on solid CATC media.

Rast osamljenih sevov na trdnem gojišču CATC.





Annex K (Priloga K)

Lactococcus lactis subsp. *lactis* M 78 nisin biosynthetic gene cluster, complete sequence

Skupek genov za biosintezo nizina pri sevu *Lactococcus lactis* subsp. *lactis* M 78, celotna sekvenca

Display Settings: GenBank

Lactococcus lactis subsp. lactis nisin biosynthetic gene cluster, complete sequence

GenBank: HM219853.1

FASTA Graphics

Go to:

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  TITLE     Expression of nisin genes in cheese-A quantitative real-time
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  JOURNAL   J. Dairy Sci. 94 (1), 77-85 (2011)
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  TITLE     Direct Submission
  JOURNAL   Submitted (12-MAY-2010) Department of Animal Science - Chair of
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Annex L (Priloga L)

Complete nisin A gene cluster from *Lactococcus lactis* M 78 (HM219853)–obtaining the nucleic acid sequence and comparing it to other published nisin sequences (Trmčić et al., 2011c)

Celoten skupek genov nizina A pri sevu *Lactococcus lactis* M 78 (HM219853)-pridobitev nukleotidnega zaporedja in primerjava z ostalimi objavljenimi sekvencami nizina (Trmčić in sod., 2011c)

Complete nisin A gene cluster from *Lactococcus lactis* M78 (HM219853) – obtaining the nucleic acid sequence and comparing it to other published nisin sequences

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Running title: Obtaining and comparing nucleic acid sequence of nisin A

Number of pages: 13

Number of figures: 1

Number of tables: 1

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Abstract

Nisin is an antimicrobial peptide produced by *Lactococcus lactis*. It has a long history of safe use, mainly in food production. This bacteriocin has been studied from many aspects of genetics, biosynthesis, immunity, regulation and mode of action. The strain *Lactococcus lactis* M78 has already been described in previous studies as a good nisin A producer with equally good potential to be used in food production. The main objective of the present study was to determine the complete nucleic acid sequence of the nisin A gene cluster from this strain. This is the first time that all 11 genes that form the nisin A gene cluster were sequenced. The obtained sequence (GenBank: HM219853) was compared to other known nucleic acid sequences of bacteriocin nisin. The results of the comparison showed certain differences in sequences that might influence the structure and function of proteins involved in nisin biosynthesis, immunity and regulation.

Keywords: nisin, nucleic acid sequence, *Lactococcus lactis*

Introduction

Lactic acid bacteria that produce different bacteriocins are gaining trust for use as starter or protective cultures in the production of fermented foods. Nisin, a ribosomally synthesized lantibiotic is one of the most characterized LAB bacteriocins. There are several known variants of nisin naturally produced by several strains of *Lactococcus lactis* and *Streptococcus uberis*. The most common variants are nisins A and Z, which differ in one nucleotide and consequently in one AA. The nisin gene cluster is located on a large conjugative transposon, composed of eleven genes, *nisABTCIPRKFEG*, involved in complex biosynthesis, regulation and immunity (Li and O'Sullivan, 2002, 2006). Although nisin has been studied from all aspects of production, structure and function, a gap still exists in the published nucleic acid sequences. All the sequences of nisins A and Z that can presently be found in accessible databases feature clusters of at maximum five genes. Besides featuring less than half of the entire nisin A/Z cluster, the majority of these sequences is more than 15 years old and derived from several different strains. Basically, from all the published nisin A sequences we can assemble the complete cluster sequence only for *Lac. lactis* 6F3 (GenBank: X68307, X76884, U17225). Nowadays large quantities of complete microbial genomes are being deposited in public databases and still there is not one genome available that would feature an entire nisin A/Z cluster. The closest thing is the genome of *Lac. lactis subsp. lactis* KF147 (GenBank: NC_013656) which is deficient in the *nisT* gene and does not produce active nisin (Siezen et al., 2008). The sequence discussed in this paper presents complete information on nisin A genes in the same way as the gene sequences that are available for all the other newly discovered nisin variants, like nisin U and Q (Wirawan et al., 2006; Yoneyama et al., 2008). The strain to be used for sequencing was selected on the basis of its origin; it was isolated from raw milk used in the production of traditional Greek hard cooked cheese. It is a proteinase- and lactase-positive strain suitable for use in dairy production

(Giannou et al., 2008; Samelis et al., 2009). Strain *Lac. lactis subsp. lactis* M78 was confirmed as an active nisin A producer, active against several strains of *Listeria monocytogenes* and *Staphylococcus aureus*. This strain was used in a study on the expression of bacteriocin genes in cheese (Trmčić et al., 2011). Complete knowledge of the entire nisin sequence of this strain was prerequisite for conducting such a study. Since this strain has a unique capability of growing well in milk, we decided to check if its nisin genes also hold any unique features.

Materials and Methods

Bacterial strain and Culture Conditions

The nisin-producing strain *Lactococcus lactis subsp. lactis* M78, an isolate from raw milk (Samelis et al., 2009), was kindly provided by J. Samelis ((National Agricultural Research Foundation, Dairy Research Institute, Ioannina, Greece). The strain was routinely grown under static conditions in M17 lactose broth at 30 °C (Terzaghi and Sandine, 1975).

DNA Extraction

Chromosomal DNA was extracted from 1 mL of overnight culture using Maxwell™ 16 Cell DNA Purification Kit and Maxwell™ 16 Instrument (Promega, WI 53711-5399 Madison, USA) according to manufacturer's instructions.

Sequencing Method

Based on known nisin genes sequences (GenBank/EMBL accession numbers X68307, L16226, X76884 and U17255), specific oligonucleotide primers were designed (LightCycler probe design software, v1.0; Roche Applied Science, Mannheim, Germany) and synthesized (Eurogentec, Seraing, Belgium) to produce approx. 900-bp amplicons in PCR reactions using

genomic DNA. The produced amplicons covered a 15 kb-long DNA fragment while each section of the sequence was present in at least two amplicons. Both amplicon strands were sequenced. Sequencing was performed by Microsynth (Switzerland) using the ABI 3730 XL capillary electrophoresis sequencer (Sanger et al., 1977). Assembly of sequences was performed using DNA Baser version 2.7x from HeracleSoftware (Germany), and all of the in-silico translations were performed by Expert Protein Analysis System (ExPASy) from the Swiss Institute of Informatics (Gasteiger et al., 2003).

Results and Discussion

Location of individual nisin A genes

The 15,016 bp of all eleven genes including flanking regions were sequenced and deposited in GenBank under accession number HM219853 (Figure 1). Between positions 828 and 1001 was a sequence identical to that reported by Buchman et al. (1988) that represents gene encoding a structural protein of nisin. Mulders et al. (1991) reports that the presence of cytosine (C), identical to the one located at position 975 in our sequence, translates to histidine residue (H) at position 50 (27 in the active nisin) and is a specific feature of the nisin A (*nisA*) variant of the bacteriocin. According to Steen et al. (1991), a membrane-associated dehydratase gene (*nisB*) is located after the *nisA* gene, and a corresponding sequence was identified in our results between positions 1109 and 4090. Gene *nisC* encodes the second modifying enzyme that catalyzes the cyclization of dehydrated amino acids. Between *nisB* and *nisC* is located a gene *nisT* that encodes an ABC transporter involved in the export of modified pre-nisin outside the cell (Engelke et al., 1992). Identical sequences for *nisT* and *nisC* were identified at positions 4101-5903 and 5896-7140, respectively. Outside the cell, a serine protease (NisP) cleaves the leader peptide off the pre-nisin, releasing the active peptide (de Ruyter et al., 1996). Kuipers et al. (1993) identified this protease gene (*nisP*) and next to it the immunity gene, *nisI*. We identified similar sequences, located at positions 7876-9924 and 7137-7874, that corresponded to genes *nisP* and *nisI*, respectively. Engelke et al. (1994) reported about regulatory genes *nisR* and *nisK* that encode for a two-component signal transduction system. Sequences corresponding to *nisR* and *nisK* were located at positions 9993-10679 and 10672-12015, respectively. Siegers and Entian (1995) reported that a second immunity system involving another ABC transporter is encoded by three genes (*nisF*, *nisE* and *nisG*) located at the end of the nisin gene cluster. In our results, three identical sequences were located between 12114-12791, 12793-13521 and

13508-14152, respectively. After the nisin A gene cluster (14669-15016) a partial sequence of *isxB* was identified, oriented in the opposite direction to the nisin A genes. The sequence of *isxB* is supposed to follow that of *isxA*, both showing homology with the lactococcal insertion sequence *IS981* (Siegers and Entian, 1995). *IS981*, together with sucrose operon, forms a large nisin-sucrose conjugative transposon *Tn5276* (Rauch and de Vos, 1992).

Location of regulatory sequence structures

Between genes *nisA* and *nisB* (1049-1093) was a sequence with an inverted repeat present. De Ruyter et al. (1996) reported in their results that this sequence forms a stem-loop structure that is supposed to facilitate a limited read-through from *nisA* promoter. Two additional stem-loop structures were found inside the structural gene *nisB* (3868-3921 and 3921-3933). The sequence/ upstream of the *nisF* transcription start site included a TCT direct repeat with an 8-bp spacer region similar to the TCT-N_8-TCT motif present at the same position (-39 to -26) upstream of the transcription start site of *nisA*. Chandrapati and O'Sullivan (2002) reported that this motif is supposed to be involved in a co-operative binding of the NisR response regulator of the NisRK two-component regulatory system. They also report a second TCT-N_8-TCT motif present upstream of *nisA* at positions -107 to -94, which was also shown in our results. This TCT repeat, together with the first one, is involved in the optimal binding of NisR and also in the induction by a Leloir pathway of galactose metabolism. Chandrapati and O'Sullivan also indicated that this could be the reason for the stronger induction capacity of promoter located in front of *nisA* compared to promoter in front of *nisF*. Engelke et al. (1994) report that *nisRK* regulatory genes are followed by a stem-loop structure identical to the one identified in our results (12001 to 12048). The entire gene cluster of nisin A finishes with the *nisG* gene, after which an inverted repeat sequence is present between 14485 and 14531. This

inverted repeat forms a large stem-loop structure that probably acts as a transcription terminator.

Comparing the obtained sequence with previously published sequences

Comparing the nucleic acid sequence HM219853 and translated protein sequence with previously published sequences, several differences can be observed (Table 1). The nucleotide sequence of *nisA* determined in this study is identical to the earlier published sequences (J04057, M27277, M65089, and L16226). At position 995, one nucleotide was different compared to sequences M24527 and X68307 (C vs. T). This difference does not affect the amino acid composition (encoded Ser residue at position 56) of the protein. The nucleotide sequence of *nisB* has G instead of C at position 3746 (L16226), which results in Glu to Gln substitution at position 880. Besides this difference, there are also differences at positions 1164 (C vs. G), 3791 (C vs. A) and 3074 (G vs. A), from sequence X68307. This corresponds to substitutions at positions 19 (Ser to Cys), 895 (Pro to Thr) and 656 (Glu to Lys), respectively. The sequence of *nisT* has 100% identity with the sequence L16226 while there are two nucleotide differences in X68307 at positions 4140 (A to T) and 4552 (T to A), resulting in Asn-14-Tyr and Val-151-Glu substitutions, respectively. For *nisC*, there is the same A to T difference at position 6423 present in both L16226 and X68307 sequences. Amino acid substitution (Lys to Asn) is located at position 176. The *nisI* sequence is identical to the L16226 and X76884 sequences. Compared to sequences X76884 and L11061, a difference in one nucleotide can be observed in the sequence of *nisP*. The difference at position 8328 (T vs. A) results in Asn to Lys substitution at position 151. Gene *nisR* differs from sequences Z22813, L11061 and X76884 in its nucleotide sequence at positions 10081 (T vs. C) and 10589 (A vs. G). The difference at position 10081 results in Met-30-Thr substitution while the second substitution preserves the Glu residue. The sequence of *nisK* is

identical to the *nisK* sequence from X76884. Compared to nisin Z sequence (Z22813), there are six differences present in *nisK* sequence, of which only the differences present at positions 10896 (T vs. G) and 11570 (C vs. G) result in amino acid differences (Ile-75-Met and Thr-300-Arg, respectively). Gene *nisF* has one difference in a nucleotide present at position 12723 (A vs. G), compared to sequence U17255. The difference results in Asn-Asp substitution at position 204. The sequences of *nisE* and *nisG* are 100% identical to the sequence U17255.

Biro (2006) in his work analyzed 20 AAs based on their size, charge and hydrophobicity. He calculated and arranged all 20 AA into compatibility tables, based on these three features. The most important compatibility feature is hydrophobicity, based on which the most profound difference was the Val-151-Glu substitution observed in NisT (CAA48382). NisT is a membrane-bonded protein, and a switch from hydrophilic AA to hydrophobic can potentially affect the integration process and overall activity of the protein. The second difference with a little less hydrophobicity incompatibility was the Thr-300-Arg difference of NisK (CAA80467). This difference can also have some charge incompatibility that can affect the attraction forces between AAs and also affect the folding and interactive features of this regulatory protein. Guzzo (1965) analyzed the distribution of AAs in helical and non-helical sections of proteins, and reports that glutamic acid is rarely and prolin is basically never present inside a helical structure of the protein. In our results we again found the Val-151-Glu substitution observed in NisT (CAA48382), but the difference Pro-895-Thr (CAA48381) observed in NisB could be the one with the most probable effect on structure and function. The presence of prolin would mean that a helical structure potentially present at this part of the protein would not be present. Such difference in the structure of NisB could mean that the interaction of this protein with NisA is not the same, which means that the dehydration

process of AA in NisA is also not the same. What this and other differences in the AA sequence might have is hard to say. Do these potential differences have a positive or negative effect on synthesis and activity, also? To resolve all of these questions, a study would have to be conducted that would involve each protein separately. The features of each protein would have to be compared between different strains from which the nucleic acid and AA sequences that were compared in this study were derived.

Acknowledgements

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Figure 1
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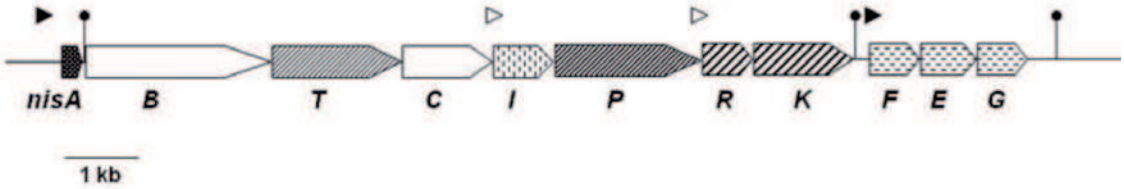

Figure 1. Organization of genes involved in nisin production, regulation and immunity. Black and white triangles indicate nisin-inducible and constitutive promoters, respectively. Hairpins indicate putative transcription terminator loops. *nisA*, nisin structural gene; *nisB* and *nisC*, post-translational modification; *nisT*, translocation; *nisP*, activation; *nisR* and *nisK*, regulation; *nisI* and *nisFEG*, immunity (Trmčić et al., 2011).

Table 1. Comparison of nucleotide and amino acid sequences of HM219853 with previously published nisin sequences

	Nucleotide[1]	Nucleotide Position[2]	Nucleotide Difference[3]	Amino Acid[4]	Amino Acid Position[5]	Amino Acid Difference[6]
NisA	/	/	/ (J04057)	/	/	/ (AAA88606)
	/	/	/ (M27277)	/	/	/ (AAA25188)
	/	/	/ (M65089)	/	/	/ (AAA73038)
	/	/	/ (L16226)	/	/	/ (AAA25189)
	C	995	T (M24527, X68307)	Ser	56	Ser (AAA26948, CAA48380)
	C	975	A (X61144)	His	50	Asn (CAA43440)
NisB	G	3746	C (L16226, X68307)	Glu	880	Gln (AAA25190, CAA48381)
	C	1164	G (X68307)	Ser	19	Cys (CAA48381)
	G	3074	A (X68307)	Glu	656	Lys (CAA48381)
	C	3791	A (X68307)	Pro	895	Thr (CAA48381)
NisT	/	/	/ (L16226)	/	/	/ (AAA25191)
	A	4140	T (X68307)	Asn	14	Thr (CAA48382)
	T	4552	A (X68307)	Val	151	Glu (CAA48382)
NisC	A	6423	T (L16226, X68307)	Lys	176	Asn (AAA25192, CAA48383)
NisI	/	/	/ (L16226, X76884)	/	/	/ (AAA25193, CAA54209)
NisP	T	8328	A (X76884, L11061)	Asn	151	Lys (CAA54210, AAA25200)
NisR	T	10081	A (X76884, L11061, Z22813)	Met	30	Thr (CAA54211, AAA25201, CAA80466)
	A	10589	G (X76884, L11061, Z22813)	Glu	199	Glu (CAA54211, AAA25201, CAA80466)
NisK	/	/	/ (X76884)	/	/	/ (CAA54212)
	T	10896	G (Z22813)	Ile	75	Met (CAA80467)
	T	11064	A (Z22813)	Pro	131	Pro (CAA80467)
	A	11349	G (Z22813)	Lys	226	Lys (CAA80467)
	G	11493	A (Z22813)	Leu	274	Leu (CAA80467)
	C	11570	G (Z22813)	Thr	300	Arg (CAA80467)
	C	11766	T (Z22813)	Val	365	Val (CAA80467)
NisF	A	12723	G (U17255)	Asn	204	Asp (AAC43327)
NisE	/	/	/ (U17255)	/	/	/ (AAC43328)
NisG	/	/	/ (U17255)	/	/	/ (AAC43329)

/ No difference in nucleic acid or protein sequence.

[1] The nucleotide present in HM219853 which differs from other published sequences.

[2] Position of the nucleotide in HM219853 which differs from other published sequences.

[3] The nucleotide present (sequence in brackets) that differs from nucleotide present in sequence HM219853.

[4] The amino acid encoded in sequence HM219853 at the point of nucleotide difference.

[5] Position of the amino acid encoded in sequence HM219853 at the point of nucleotide difference.

[6] The amino acid encoded from sequence (sequence in brackets) at the point of nucleotide difference.

View Letter

Date: 21-01-2011
To: "Aljosa Trmcic" aljosa.trmcic@bf.uni-lj.si
From: "Nam-Soo Kim" kimnamsu@kangwon.ac.kr
Subject: GENG: Your manuscript entitled Complete nisin A gene cluster from Lactococcus la

Ref.: Ms. No. GENG-D-10-00140R3
Complete nisin A gene cluster from Lactococcus lactis M78 (HM219853) - obtaining the nucleic acid sequence and comparing it to other published nisin sequences
Genes & Genomics

Dear Mr. Trmcic,

I am pleased to tell you that your work has now been accepted for publication in Genes & Genomics.

It was accepted on 21-01-2011.

Thank you for submitting your work to this journal.

With kind regards

Nam-Soo Kim
Editor-in-Chief
Genes & Genomics

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