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EXAMINATION OF ANTIMICROBIAL POTENTIAL IN NATURAL ISOLATES OF *LACTOBACILLUS CASEI/PARACASEI* GROUP

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The aim of this study was to investigate the antimicrobial potential of 52 natural isolates of *Lactobacillus casei/paracasei*. The incidence of relevant genes encoding BacSJ (*bacSJ2-8/bacSJ2-8i* gene cluster), acidocin 8912 (*acdT*), ABC-transporter (*abcT*) and accessory protein (*acc*) was also studied. These genes were found to be widespread amongst the analyzed *L. casei/paracasei* strains. The *bacSJ2-8/bacSJ2-8i* gene cluster was present in 49 (94.23%) and *acdT* in 41 (78.85%) of the 52 tested strains. Forty of these strains (76.92%) harbored both analyzed genes. Interestingly, only 17 strains (32.69%) with the *bacSJ2-8/bacSJ2-8i* gene cluster and/or the *acdT*

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gene showed bacteriocin production. Strain *L. paracasei* BGNK1-62 contained the *bacSJ2-8/bacSJ2-8i* gene cluster, but did not produce bacteriocin BacSJ possibly due to absence of the *abcT* and *acc* genes. Hence, these genes were introduced into BGNK1-62 by transformation with constructed plasmid pA2A, after which BacSJ was produced. In addition, it was found that *L. paracasei* BGGR2-66 produced new bacteriocin designated as BacGR that was biochemically characterized and its N-terminal sequence was determined.

Key words: Lactobacillus casei/paracasei / BacSJ / acidocin 8912 / ABC-transporter / accessory protein / BacGR

INTRODUCTION

Bacteriocins from lactic acid bacteria (LAB) have attracted increasing interest of the food industry as natural preservatives, since they can inhibit the growth of closely related bacteria and in some cases food-spoilage and food-borne pathogenic bacteria (FIELD *et al*., 2007). The ability of certain strains of LAB to produce multiple bacteriocins with narrow but different inhibitory spectra plays an important role in their survival in the same ecological environment. Moreover, an additional role for bacteriocins as a mediator in quorum sensing was proposed (MILLER and BASSLER, 2001).

At least four genes are required for the production of class II bacteriocins: (i) a structural bacteriocin gene encoding a prebacteriocin; (ii) an immunity gene encoding an immunity protein that protects the producer from its own bacteriocin; (iii) a gene encoding an ABC transporter (ATP-binding cassette) necessary for secretion; and (iv) a gene encoding an accessory protein of unknown function (NISSEN-MEYER, *et al*., 2009). The genes necessary for bacteriocin production, secretion and immunity are generally localized as a cluster. It was shown that genes for pediocin AcH, immunity and the secretion system in *Pediococcus parvulus* ATO77, *Pediococcus pentosaceus* S34 and *Lactobacillus plantarum* WHE92 co-reside on single plasmids in each strain (MILLER, 2005). However, in the case of *Carnobacterium piscicola* LV17B the genes encoding the carnobacteriocin BM1 and its immunity protein are located on the chromosome, whereas the genes involved in secretion and regulation are present on a 61 kbp plasmid (QUADRI *et al.,* 1997).

The presence of bacteriocin related genes in bacteriocin non-producing strains of LAB has been reported previously. It was observed that only some of the genes responsible for bacteriocin production were present in such LAB strains or that the occurrence of point mutations within bacteriocin genes resulted in inactivity (QUADRI *et al*., 1997; TETTELIN *et al*., 2001; MORETRO *et al.*, 2005; DIEP *et al.,* 2006). On the other hand, the genes responsible for processing and transport of lactococcin A from *Lactococcus lactis* 9B4 were found on the chromosome of a lactococcin A non-producing strain, *Lactococcus lactis* IL1403, and immunity genes have been observed in various bacteriocin non-producing LAB (VENEMA *et al.,* 1996). Although seven putative bacteriocin genes were annotated in the *Streptococcus pneumoniae* TIGR 4 genome sequence (SP0042, SP0109, SP0531, SP0532, SP0533,

SP0539, and SP0541), no bacteriocin activity has been detected in that strain so far (TETTELIN et al., 2001).

Analysis of the respective phylogenetic trees indicated that the genes involved in bacteriocin production are often transferred horizontally and show great differences in operons organization encoding them, even in closely related genomes (MAKAROVA *et al*., 2006). Hence, transfer of plasmids or transposons by conjugation (BROADBENT *et al.,* 1995) could explain the presence of these genes at different locations in the genome of bacteriocin-producing and non-producing strains. It seems likely that localization of bacteriocin genes on mobilizable genetic elements assists intra- as well as inter-species dissemination of bacteriocins among LAB. Thus, the molecular systems responsible for the production of bacteriocins probably reflect the long-term existence of *Lactobacillales* in complex microbial communities (MAKAROVA *et al*., 2006; MAKAROVA *et al.,* 2007).

We have demonstrated previously that *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 produces two bacteriocins (BacSJ and acidocin 8912). The genes encoding these bacteriocins as well as the ABC-transporter and accessory protein are located on the plasmid pSJ2-8 (KOJIC *et al*., 2010). Considering these findings, this study was focused on an examination of the antimicrobial potential of 52 *Lactobacillus casei/paracasei* isolates from different ecological niches, followed by a PCR screening of the bacterial genomes in order to determine the distribution of the genes for BacSJ (*bacSJ2-8/bacSJ2-8i*), acidocin 8912 (a*cdT*), the ABC-transporter (*abcT*) and accessory protein (*acc*). In addition, we examined the correlation between bacteriocins BacSJ and acidocin 8912 production and the presence of genes coding for ABC-transporter and accessory protein. This approach enabled the identification of putative bacteriocin genes regardless of whether the analyzed bacterial strains produced bacteriocin or not. As a result of this analysis, we isolated and characterized new bacteriocin BacGR produced by strain *L. paracasei* subsp. *paracasei* BGGR2-66.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The natural isolates of *Lactobacillus casei/paracasei* analyzed in this study are listed in Table 1a and 1b. Other bacterial strains and plasmids used are presented in Table 2. *Lactobacillus* strains were routinely grown in MRS medium (Merck GmbH, Darmstadt, Germany) at 30 °C. Stocks of cultures were kept at – 80 °C in MRS broth containing 15% (v/v) glycerol. *Lactococcus* strains were grown in M17 broth (Merck GmbH, Darmstadt, Germany) supplemented with glucose (0.5% w/v) (GM17). *Escherichia coli* DH5α strain used for cloning and propagation of constructs was grown in Luria-Bertani broth (LB) (MILLER, 1972) aerobically at 37 °C. Agar plates were made by adding 1.5% (w/v) agar (Torlak, Belgrade, Serbia) to the liquid media. *E. coli* transformants were selected on LB plates containing 250 µg/ml of erythromycin (Serva GmbH, Heidelberg, Germany) or 100 µg/mL of ampicillin (Sigma, Deisenhofen, Germany). For blue/white selection in *E. coli*

DH5α, 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added at the standard concentration of 20 μg/ml.

Table 1a. Bacteriocin activity of L. casei/paracasei strains and distribution of the relevant genes.

Class	Strains	16S rDNA	Source	Plasmids	Bac	*SJ2-8/SJ2-8i*	*acdT*	*abcT*	*acc*
P1	BGSJ2-8	LP	cheese	+	+	+	+	+	+
	BGUB9	LP	cheese	+	+	+	+	+	+
	BGKP19	LP	cheese	+	+	+	+	+	+
	BGBUK2-19	LP	cheese	+	+	+	+	+	+
P2	BGNJ1-61	LC/LP	cheese	+	+	+	+	+	-
P3	BGGR2-64	LR/LP	cheese	+	+	+	+	-	+
	BGGR2-66	LP	cheese	+	+	+	+	-	+
	BGCGK4	LP	kajmak	+	+	+	+	-	+
	BGDA17b	LP	cheese	+	+	+	+	-	+
	BGBUK2-16	LP	cheese	+	+	+	+	-	+
	BGZLS10-6	LP	cheese	+	+	+	+	-	+
P4	BGGR2-68	LP	cheese	+	+	+	+	-	-
	BGNJ1-24	LP	cheese	+	+	+	+	-	-
	BGNJ1-35	LC/LP	cheese	+	+	+	+	-	-
P5	BGNJ1-77	LP	cheese	+	+	+	-	+	-
P6	BGNJ1-64	LP	cheese	+	+	+	-	-	-
P7	BGJB9-123	LC/LP	cheese	-	+	-	+	-	-

16S rDNA – determination of strains by 16S rDNA sequencing; LP – *L. paracasei*; LC – *L. casei*; LR – *L. rennanqily*; Presence (+) or absence (-) of plasmids, bacteriocin production (Bac) and the appropriate genes (*bacSJ2-8/bacSJ2-8i, acdT, abcT* and *acc* encoding bacteriocin BacSJ/immunity to it, bacteriocin acidocin 8912, ABC-transporter and accessory protein, respectively).

For the detection of bacteriocin-producing *Lactobacillus casei/paracasei* strains, an agar-well diffusion method was used as described previously (LOZO *et al*., 2004).

Table 1b. Bacteriocin activity of L. casei/paracasei strains and distribution of the relevant genes.

Class	Strains	16S rDNA	Source	Plasmids	Bac	*SJ2-8/SJ2-8i*	*acdT*	*abcT*	***acc***
N1	BGNJ1-14	LP/ LC	cheese	+	-	+	+	+	**+**
	BGNK1-77	LP	cheese	-	-	+	+	+	**+**
	BGBK1-67	LC/LP	cheese	-	-	+	+	+	**+**
N2	BGLI18	LP	cheese	+	-	+	+	+	**-**
	BGNK1-99	LC/LP	cheese	+	-	+	+	+	**-**
	BGGR2-20	LC/LP	cheese	+	-	+	+	+	**-**
	BGNJ1-48	LC/LP	cheese	+	-	+	+	+	**-**
	BGNJ1-53	LC/LP	cheese	+	-	+	+	+	**-**
	BGNK1-50	LC/LP	cheese	-	-	+	+	+	**-**
	BGHN14	LP	cheese	-	-	+	+	+	**-**
	BGGR2-59	LC/LP	cheese	-	-	+	+	+	**-**
N3	B-4560[a]	LP	milk	+	-	+	+	-	**+**
	BGBUK2-8	LP	cheese	+	-	+	+	-	**+**
	BGNJ1-83	LP/LC	cheese	-	-	+	+	-	**+**
	LMG10774[b]	LP	csf*	-	-	+	+	-	**+**
N4	LMG19719[b]	LP	blood	+	-	+	+	-	**-**
	B-4564[a]	LP	saliva	+	-	+	+	-	**-**
	BGNJ1-27	LP	cheese	+	-	+	+	-	**-**
	BGGR2-18	LC/LP	cheese	+	-	+	+	-	**-**
	BGGR2-94	LC/LP	cheese	+	-	+	+	-	**-**
	BGNJ1-3	LC/LP	cheese	+	-	+	+	-	**-**
	BGNJ1-93	LC/LP	cheese	+	-	+	+	-	**-**
	BGNK1-31	LC/LP	cheese	+	-	+	+	-	**-**
	BGKP20	LP	cheese	-	-	+	+	-	**-**
	BGNK1-11	LP/LC/LR	cheese	-	-	+	+	-	**-**
	LMG13552[b]	LP	cheese	-	-	+	+	-	**-**
N5	BGNJ1-38	LC/LP	cheese	+	-	+	-	+	**-**
	BGNK1-70	LC/LP	cheese	+	-	+	-	+	**-**
N6	BGAR75	LP	cheese	+	-	+	-	-	**+**
	LMG11459[b]	LP	caries	-	-	+	-	-	**+**
N7	BGJB9-124	LC/LP	cheese	+	-	+	-	-	**-**
	BGNK1-62	LC/LP	cheese	+	-	+	-	-	**-**
	BGNK1-80	LC/LP	cheese	+	-	+	-	-	**-**
N8	BGLI15	LP	cheese	+	-	-	-	-	**-**
	BGGR1-15	LP	cheese	+	-	-	-	-	**-**

16S rDNA – determination of strains by 16S rDNA sequencing; LP – *L. paracasei*; LC – *L. casei*; LR – *L. rennanqily*; Presence (+) or absence (-) of plasmids, bacteriocin production (Bac) and the appropriate genes (*bacSJ2-8/bacSJ2-8i, acdT, abcT* and *acc* encoding bacteriocin BacSJ/immunity to it, bacteriocin acidocin 8912, ABC-transporter and accessory protein, respectively). [a]Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection; [b]Agricultural Research Service Culture Collection (NRRL), USA; Bac - bacteriocin activity; *csf – cerebro-spinal fluid.

Table 2. Bacterial strains and plasmids used in this study.

Bacterial strain and plasmids	Phenotype	Source or reference
E. coli DH5α	supE44, □lacU169, (u80lacZDM15), hsdR17, recA1, endA1 gyrA96 thi-1, relA1	(WOODCOCK *et al*., 1989)
Lactococcus lactis subsp. *lactis* BGMN1-596	plasmid free derivative of BGMN1-5 Bac$^-$, Bac^s, Prt$^-$	(GAJIC *et al*., 1999)
Lactococcus lactis subsp. *lactis* MG1363	plasmid-free derivative of NCDO 712 Bac$^-$, Bac^s, Prt$^-$, Lac$^-$	(GASSON, 1983)
Lactococcus lactis subsp. *lactis* IL1403	plasmid-free derivative of IL594, Bac^S, Prt$^-$	(CHOPIN *et al*., 1984)
Plasmids		
pGEM-T Easy Vector	3015 bp, Amp^r, bacterial, nonviral, transient, constitutive, high expression level	Promega
pA13	4600 bp, lactobacilli/lactococci/*E. coli* shuttle cloning vector; Em^r	(KOJIC *et al*., 2010)
pG1	6485 bp, derivative of pGEM-T Easy Vector carrying *Pst*I-*Sph*I fragment with the *abcT* and *acc* genes (3470 bp)	This study
pA2A	8071 bp derivative of pA13 plasmid carrying the *Pst*I-*Sph*I fragment from pG1 vector	This study

Amp^r – resistance to ampicillin; Em^r - resistance to erythromycin; Bac$^-$ - bacteriocin non-producer; Bac^s- sensitive to bacteriocin; Prt$^-$- proteolytically inactive; Lac - lactose-fermenting ability.

Specific primers and DNA amplification

A set of specific primers (Table 3) was synthesized by Metabion (Metabion International AG, Martinsried, Germany). Template chromosomal DNA was isolated from lactobacilli according to the procedure of Hopwood and co-authors (1985), with modification of cell lysis (40 mM NH_4-acetate pH 8.0, 20 mM Mg-acetate, 0.5 M saccharose) by supplement with lysozyme (7 mg/ml) and incubation at 37 °C for 15 min. Kapa Taq DNA Polymerase (Kapa Biosystems, Inc., Boston, MA, USA) was used to amplify PCR with a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR reactions were performed with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, different primer annealing temperatures (Table 3) for 1 min and 72 °C extension for 30 s, followed by a final extension step at 72 °C for 7 min. PCR

products were separated by electrophoresis on 1% (w/v) agarose gels run at 8 V/cm. All obtained PCR fragments selected for sequencing were purified with a 'QIAquick PCR Purification kit' (Qiagen, GmbH, Hilden, Germany) and sequenced by Macrogen (Sequencing Service Macrogen, Seoul, Korea). The sequences were analyzed in the NCBI database using BLAST, the standard nucleotide-nucleotide homology search (http://www.ncbi.nlm.nih.gov/BLAST).

Table 3. Specific primers used for PCR amplification.

Primer	Sequence	Annealing temperature
Primers for 16S rDNA		50°C
UNI16SF	5'-GAGAGTTTGATCCTGGC-3'	
UNI16SR	5'-AGGAGGTGATCCAGCCG-3'	
Primers for *bacSJ2-8/bacSJ2-8i* gene cluster		50°C
F0 BacSJ	5'-GGGGTGTGACTTTTTTGGC-3'	
R4 BacSJ	5'-GATACCGTTCCAGAGATTC-3'	
Primers for *acdT* gene		39°C
F1 acdT	5'-TATGAAAACTAGACTAT-3'	
R1 acdT	5'-AATACGAAGGCTTTCCCAGA-3'	
Primers for *abcT* gene		43°C
PRBamN	5'-CGGGATTCAAACGGCTAG-3'	
PF	5'-CCATTACTGAGCGC-3'	
Primers for *acc* gene		45°C
PR	5'-CTTAGCAGATGGTCG-3'	
PFPst	5'-AACTGCAGGCTAGCACTAACA-3'	
Primers for fragment carrying *abcT* and *acc* genes		55°C
PRBamN	5'-CGGGATTCAAACGGCTAG-3'	
PFPst	5'-AACTGCAGGCTAGCACTAACA-3'	

DNA manipulations

The QIAprep Spin Miniprep Kit (Qiagen, GmbH, Hilden, Germany) was used for plasmid isolation from *E. coli*. Plasmids were isolated from lactobacilli employing the same kit but with the following modification. Complete lysis of cells was achieved by resuspending the cell pellets in solution E1 (50 mM Tris-HCl pH

8.0, 10 mM EDTA, RNase 100 µg/ml) supplemented with lysozyme (4 mg/ml) followed by incubation at 37 °C for 30 min. Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas, Vilnius, Lithuania). Standard DNA cloning techniques were performed as described by SAMBROOK and co-authors (1989). DNA fragments were purified using a QIAquick Gel Extraction Kit as described by the manufacturer (Qiagen, GmbH, Hilden, Germany).

Plasmid construction

To obtain a DNA fragment containing the *abcT* and *acc* genes, PCR reaction with PRBamN/PFPst primers (Table 3) and total plasmid DNA isolated from *L. paracasei* subsp. *paracasei* BGSJ2-8 was carried out. The obtained fragment was ligated into pGEM-T Easy (Promega, Madison, Wis, USA) giving the construct designated pG1 (Table 2). DNA was ligated with T4 DNA ligase (New England, BioLabs, USA) according to the manufacturer's instructions. The *Pst*I/*Sph*I fragment from pG1 was re-cloned into pA13 vector (KOJIC et al., 2010), resulting in the construct designated pA2A (Table 2). Restriction enzyme digestion and sequencing of the construct was performed to confirm that the anticipated plasmid construct had been obtained. *E. coli* DH5α competent cells were transformed with the obtained construct using the standard heat-shock transformation procedure (SAMBROOK *et al.,* 1989). The construct pA2A was re-isolated from E*. coil* and then transferred into *L. casei* subsp. *paracasei* BGNK1-62 by electroporation (WALKER *et al.,* 1996) using an Eppendorf Electroporator (Eppendorf, Hamburg, Germany). The obtained Em^r transformants were tested for bacteriocin production and sensitivity to it.

Partial purification of bacteriocin BacGR

Active bacteriocin fraction obtained from resuspended bacterial cells collected from MRS Petri dishes in 5 ml of 0.1 M Na-phosphate buffer pH 7.0 was mixed with chloroform (in ratio 1:1, v/v) and intensively mixed in a vortex 30 sec. Phases were separated by centrifugation on 5000 rpm (Eppendorf 5804R, Hamburg, Germany). After complete removal of the upper and lower phases and drying in "Speed-Vac", interphase was resuspended in 0.1 M Na-phosphate buffer pH 7.0.

To determine molecular size of bacteriocin the partially purified bacteriocin was analyzed by Tricine SDS-PAGE on 16% acrylamide gel. One part of gel were stained with Coomassie blue R250 (SERVA, Heidelberg, Germany) and the other part of gel was pre-treated with isopropanol (20%) and acetic acid (10%) mixture and subsequently washed first with Tween 80 (0.5%) and then in water for 24 h, to detect the bacteriocin activity (BHUNIA and JOHNSON, 1992). After washing, the gel was placed on sterile Petri plate and overlaid with MRS soft agar (0.7%, w/v) containing 100 µl of 10^{-2} diluted overnight culture of the indicator strain *L. paracasei* subsp. *paracasei* BGKP20. The plate was incubated overnight at 30 °C and the appearance of inhibition zones was examined.

For N-terminal sequencing, protein band corresponding to the active sample was extracted from the gel, and after that separated on discontinuous gel system consisting of 16% and 10% acrylamide gel, by using a modified method described

by COHEN and CHAIT (1997). Gel with the protein band was chopped and soaked with a solution which consisted of 0.09% TFA, 80% acetonitrile in MilliQ water with intensive mixing on shaker for 48 h at 8 °C. The active bacteriocin sample was obtained and it was run again on the 16% Tricine-SDS-PAGE. Afterwards, the sample from gel was transferred to a PVDF membrane. Membrane was stained with 0.1 % (m/v) Coomassie Blue R-250, 1 % (v/v) acetic acid, and 40% methanol. Membrane was distained with 50% methanol. N-terminal amino acid sequence of purified bacteriocin BacGR was obtained by Edman degradation method and the "Pulsed liquid-Blot" method. Sequence analysis of a PVDF membrane-bound sample was performed in Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia. The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number B3EWP5.

Biochemical characterization of bacteriocin BacGR

In order to determine kinetics of bacteriocin BacGR production, effect of different enzymes, temperature and pH on bacteriocin activity methods described previously by LOZO and co-authors (2004) was performed. For the analysis of biochemical characteristics of bacteriocin BacGR, we used a partially purified bacteriocin fraction, obtained by chloroform extraction.

RESULTS AND DISCUSSION

Detection of antimicrobial activity of strains from *Lactobacillus casei/paracasei* group

The ability of bacteriocin production is an important characteristic of *Lactobacillus* strains because of their possible applications in food preservation (BURITI *et al*., 2007; ANAS *et al.,* 2008) or as probiotic (GHOSH *et al*., 2004) a numerous studies have focused on the studying the genetic potential of natural isolates. All 52 *L. casei/paracasei* strains isolated from artisanal cheeses and kajmak produced at different geographical locations were screened for antimicrobial activity using *Lactobacillus* and *Lactococcus* indicator strains. Among them, 17 of 52 strains (32.69%) showed antimicrobial activity against at least one of the 23 indicator strains (Table 4). In addition, all 17 strains were screened for antimicrobial activity against each other. Thirteen of them exhibited inhibitory activity against at least three bacteriocin-producing indicator strains when tested by agar-well diffusion method. The bacteriocin producer strains were divided into five groups (A-E) based on the results of antimicrobial activity spectrum. The group A includes seven strains that have a similar antimicrobial spectrum as previously characterized bacteriocin producer strain BGSJ2-8. In addition, strains of group A showed the widest spectrum of activity compared to the other tested groups of *L. casei/paracasei* strains. The group B consists of only two strains, characterized by the same spectrum of antimicrobial activity. In the group C, there were BGGR2-64 and BGGR2-66 strains, which show the same inhibitory spectrum, and the strain BGBUK2-16, which additionally inhibits two lactococcal strains. A probable explanation could be that the

detected differences in inhibitory spectrum is actually a consequence of the growth conditions of producer strains as it was shown that propionicin T1 was produced under different growth conditions of the two *Propionibacterium thoenii* strains (FAYE *et al*., 2000). In addition, different environmental factors (HECHARD and SAHL, 2002) as well as presence of complex regulatory mechanisms involved in the regulation of bacteriocin synthesis (DIEP *et al.,* 2000; NES *et al*., 2007) could affect bacteriocins production. The group D consists of four strains that showed inhibitory activity solely against *L. paracasei* subsp. *paracasei* BGLI18 indicator strain (Table 4), while in the group E was only BGJB9-123 strain with a different antimicrobial activity than the strains from other groups. Interestingly, bacteriocin producing strains generally showed antimicrobial activity after 24 h, except for strain BGGR2-66 when the inhibition zone appear after 48 h of incubation.

Correlation between the presence of putative *bacSJ2-8* and *acdT* genes and the production of bacteriocins

On the basis of whether they produce bacteriocins, and whether they have or do not possess the analyzed bacteriocin genes, strains of *L. casei/paracasei* group were divided into two main groups: bacteriocin producer strains (P1-P7) and strains in which the bacteriocin activity was not detected (N1-N8). Strains BGSJ2-8, BGUB9, BGKP19 and BGBUK2-19 (Table 1a, P1) contained all four analyzed genes and showed similar antimicrobial spectra (Table 4). Two strains BGSJ2-8 and BGUB9 were resistant to all tested strains when used as indicators (Table 1a, P1 and Table 4). Most of the 17 bacteriocin-producing strains harbored both structural bacteriocin genes (14 of 17 strains; 82.35%), two strains contained only the *bacSJ2-8/bacSJ2-8i* gene cluster (BGNJ1-77 and BGNJ1-64; Table 1a, P5-P6) and one exclusively the *acdT* gene (BGJB9-123; Table 1a, P7). Further analysis revealed that variability existed among bacteriocin-producing strains regarding the presence of *abcT* and *acc*. Thus, four out of 17 bacteriocin-producing strains (23.53%) contained both genes (Table 1a, P1) six of them (35.29%) lacked only the *abcT* gene (Table 1a, P3) and in two (11.76%) only the *acc* gene was missing (Table 1a, P2, P5). Interestingly, five of 17 bacteriocin-producing strains (29.41%) did not contain either gene (Table 1a, P4, P6, and P7). Thus, it could be possible that different inhibitory spectrum of these strains depending on the presence or absence of *abcT* or *acc* genes. A common feature of strains from the P3 group, which distinguishes them from strains from the P1 group, was the absence *abcT* gene. It could be assumed that in the case of the strains from the P3 group, which showed a similar antimicrobial spectrum as that from the P1 group, most likely the role of bacteriocin ABC-transporter took over some other transporter whose primary function was not bacteriocin transport.

Table 4. Antimicrobial activity of bacteriocin-producing Lactobacillus casei/paracasei strain

Bac producer	BGSJ2-8	BGUB9	BGKP19	BGBUK2-19	BGCGK4	BGDA17b	BGZLS10-6	BGNJ1-61	BGNJ1-24	BGGR2-64	BGGR2-66	BGBUK2-16	BGGR2-68	BGNJ1-35	BGNJ1-77	BGNJ1-64	BGJB9-123
Indicator strain	**A**							**B**		**C**			**D**				**E**
BGSJ2-8	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BGUB9	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BGKP19	-	-	/	-	-	-	-	+	+	-	-	-	-	-	-	-	-
BGBUK2-19	-	-	-	/	-	-	-	+	+	-	-	-	-	-	-	-	-
BGCGK4	-	-	-	-	/	-	-	+	+	-	-	-	-	-	-	-	-
BGDA17b	-	+	-	-	-	/	-	+	+	-	-	-	-	-	-	-	+
BGZLS10-6	-	+	-	-	-	-	/	-	-	-	-	-	-	-	-	-	-
BGNJ1-61	+	+	+	+	+	+	+	/	-	-	-	-	-	-	-	-	-
BGNJ1-24	+	+	+	+	+	+	+	-	/	-	-	-	-	-	-	-	-
BGGR2-64	+	-	+	+	+	+	-	-	-	/	-	-	-	-	-	-	-
BGGR2-66	+	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	+
BGBUK2-16	+	+	+	-	+	+	+	-	-	-	-	/	-	-	-	-	+
BGGR2-68	+	+	+	+	+	+	+	-	-	+	+	+	/	-	-	-	-
BGNJ1-35	+	+	+	+	+	+	+	-	-	+	+	+	-	/	-	-	-
BGNJ1-77	+	+	+	+	+	+	+	-	-	+	+	+	-	-	/	-	-
BGNJ1-64	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	/	-
BGJB9-123	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	/
BGHN14	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-
BGLI18	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	-
BGKP20	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-
BGMN1-596	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-
MG1363	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
IL1403	+	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-

.= an inhibition zone sensitive to protease; ˜ = without a zone; A, B, C, D, E = Groups of *L. casei/paracasei* strains defined based on similarity of the antimicrobial spectra; Bacteriocin non-producing indicator strains *Lactobacillus paracasei* subsp. *paracasei* BGHN14, BGLI18 and BGKP20, and *Lactococcus lactis* subsp. *lactis* strains BGMN1-596, MG1363, and IL1403; Screening of *L. casei/paracasei* strains for antimicrobial activity using agar-well diffusion method was performed in triplicate.

In addition, we found that some strains harbored all four analyzed genes (3 of 35 strains, 8.57%; Table 1b, N1), but they did not produce bacteriocins. These strains were sensitive to bacteriocins produced by strains belonging to classes P1 and P3 when used as indicator strains. Finally, the same behavior was observed when strains lacking all four tested genes (BGLI15 and BGGR1-15, Table 1b, N8) were used in the test (data not shown). The absence of bacteriocin production in these strains may be due to mutation in the bacteriocin-encoding gene or the lack of some additional component involved in bacteriocin production or secretion in them. The existence of a large number of bacteriocin non-producing strains carrying putative bacteriocin genes, as well as bacteriocin producers with different antimicrobial spectra indicate that some isolates may have only part of the bacteriocin system (ABRIOUEL *et al*., 2006). The observation that bacteriocin-producing *Lactobacillus casei/paracasei* strains without the *acc* gene showed a narrower antimicrobial spectrum, regardless of the presence or absence of the *abcT* gene, than the strains possessing it, suggest a role for the *acc* gene in bacteriocin production. However, it is known that in the case of sakacin T and sakacin X the accessory protein encoded by the *acc* gene was not detected (VAUGHAN *et al.,* 2003). Nonetheless, the production of some still unidentified bacteriocin in tested strains might have an effect on the differences in their antimicrobial spectra.

Re-establishment of the ability to produce bacteriocin BacSJ

Analysis showed that three bacteriocin non-producing strains (BGJB9-124, BGNK1-62 and BGNK1-80; Table 1b, N7) harbored only the *bacSJ2-8/bacSJ2-8i* gene cluster. They are lacking *abcT* and *acc* genes. In all three strains the *bacSJ2-8/bacSJ2-8i* gene cluster had identical nucleotide sequences to the corresponding genes located on the plasmid pSJ2-8 (submitted to EMBL GenBank ID: FM246455) (KOJIC *et al.,* 2010). In order to examine whether the identified *bacSJ2-8/bacSJ2-8i* gene cluster can produce bacteriocin BacSJ, the expression of ABC-transporter and accessory protein *in trans* was performed. For this purpose, *L. paracasei* strain BGNK1-62 (Table 1b, N7) was selected and transformed with plasmid pA2A carrying the *abcT* and *acc* genes. The obtained transformants produced bacteriocin BacSJ confirming that *abcT* and *acc* genes present *in trans* could re-establish its production of BacSJ in the strain BGNK1-62. Thus, the finding that 96.15% of all analyzed strains had kept some of the bacteriocin structural genes suggests the possibility of their relatively quick response to changes in the environment they occupy, since in the presence of plasmids carrying the *abcT* and/or *acc* genes these strains could potentially become bacteriocin producers. This is consistent with the finding that genes involved in bacteriocin production are often transferred horizontally among bacteria (MAKAROVA *et al*., 2006).

Purification and biochemical characterization of bacteriocin BacGR

The antimicrobial substance produced by isolate BGGR2-66 was designated as bacteriocin BacGR. Bacteriocin production in MRS medium was dependent on the growth phase. Bacteriocin activity in the culture of BGGR2-66 was detected after

6 h of the growth. It is important to note that the zones of inhibition of tested sample occur after the 48 h incubation at 30 °C. Isolation of bacteriocin BacGR was performed by chloroform extraction after cultivation of BGGR2-66 strain on solid MRS medium for 48 h. Bacteriocin BacGR is relatively thermostable molecule, which retains antimicrobial activity even after incubation for 30 min at 100 °C, while its inhibitory effect completely lost after autoclaving for 15 min at 121 °C.

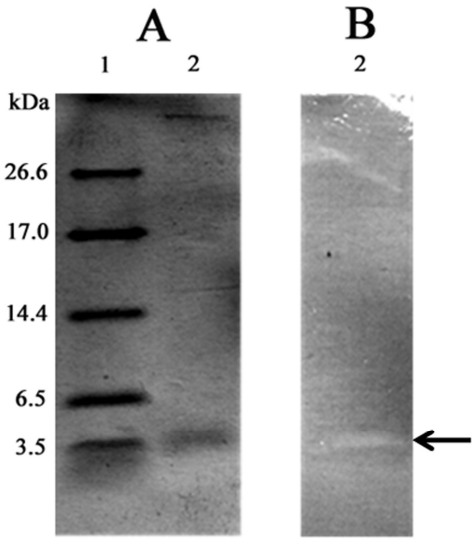

Figure 1. The activity of partially purified bacteriocin BacGR. A. Tricine-SDS-PAGE of active fractions after a chloroform extraction. Lane 1: Molecular mass marker; Lane 2: Protein band of bacteriocin BacGR; B. Gel overlaid with soft agar containing an indicator strain *Lb. paracasei* subsp. *paracasei* BGKP20. An arrow indicates the inhibition zone.

Bacteriocin BacGR was active in the pH range between 1 and 10. However, complete inactivation of tested bacteriocin occurred at pH 11.0. Bacteriocin was also inactivated with the proteolytic enzymes (protease type XIV, proteinase K, α-chymotrypsin, pepsin and trypsin). Bacteriocin activity was visualized on the gel after Tricine-SDS-PAGE and one band corresponding to the zone of inhibition was detected on the gel. Its position corresponded with the expected migration of a

protein of approximately 3.5 kDa (Fig. 1). The N-terminal amino acid sequencing showed that the first 10 amino acid sequence of bacteriocin BacGR was the G N V A E L T E V R. Searching the NCBI/BLAST database, a single protein possessing similar N-terminal domain was not found. Based on the molecular size, biochemical characterization and obtained N-terminal sequence bacteriocin BacGR probably belongs to the class II bacteriocins.

In summary, the results obtained in this work indicate the necessity of screening for bacteriocin genes in natural isolates of LAB in order to find potential producers of new bacteriocins that would otherwise could remain undiscovered. These results are significant, because in recent years, the use of bacteriocins as part of food bio-preservation technology has attracted great attention. The potential of new bacteriocin producing strains has the increasing importance for the more effective food preservation.

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IZUČAVANJE ANTIMIKROBNOG POTENCIJALA PRIRODNIH IZOLATA *LACTOBACILLUS CASEI/PARACASEI* GRUPE

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Cilj ove studije je izučavanje antimikrobnog potencijala 52 prirodna izolata vrste *L. casei/paracasei*. Učestalost gena koji kodiraju BacSJ (*bacSJ2-8/bacSJ2-8i* genski klaster), acidocin 8912 (*acdT*), ABC-transporter (*abcT*) i pomoćni protein (*acc*) su takođe izučavani. Genski klaster *bacSJ2-8/bacSJ2-8i* prisutan je kod 49 (94.23%), a *acdT* kod 41 (78.85%) od 52 testirana soja. Četrdeset sojeva (76.92%) poseduje oba analizirana gena. Interesantno je da samo 17 sojeva (32.69%) koji poseduju *bacSJ2-8/bacSJ2-8i* genski klaster i/ili *acdT* gen proizvode bakteriocine. Soj *L. paracasei* BGNK1-62 poseduje *bacSJ2-8/bacSJ2-8i* genski klaster, ali ne proizvodi bakteriocin BacSJ što je verovatno posledica nedostatka *abcT* i *acc* gena. Nakon transformacije soja BGNK1-62 konstruktom pA2A koji poseduje *abcT* i *acc* gene ostvarena je proizvodnja bakteriocina BacSJ. Osim toga, utvrđeno je da soj *L. paracasei* BGGR2-66 proizvodi nov bakteriocin označen kao BacGR, koji je biohemijski okarakterisan, a određena je i njegova N-terminalna sekvenca.

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