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CHARACTERIZATION OF THE BACTERIOCIN-PRODUCING STRAIN *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI* BGUB9

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Abstract – The strain *Lactobacillus paracasei* subsp. *paracasei* BGUB9 that was isolated from traditionally homemade hard cheese produces bacteriocin designated as BacUB9, with an approximate molecular mass of 4 kDa. Biochemical characterization and the antimicrobial activity test of BacUB9 were performed. The onset of BacUB9 biosynthesis was observed at the end of an exponential phase of growth. Bacteriocin UB9 retained the antimicrobial activity within the pH range from 1 to 10 and after treatment at 100ºC for 30 min. The bacteriocin is susceptible to the activity of proteolytic enzymes. Bacteriocin BacUB9 has a very narrow antimicrobial spectrum, limited to several strains that belong to closely related species. The effect of BGUB9 on the growth of the strain *Lactobacillus paracasei* subsp. *paracasei* BGHN14 in a mixed culture was monitored. The mode of action of BacUB9 on the strain BGHN14 was identified as bacteriostatic. Plasmid curing results indicated that a plasmid, designated as pUB9, seemed to be responsible for both bacteriocin BacUB9 production and host immunity.

Key words: Lactobacillus, bacteriocin, antimicrobial activity

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INTRODUCTION

Bacterial strains with antimicrobial activity play an important role in the food industry, agriculture and pharmaceutical industry. Many bacterial species inhabit the ecological niches with a limited amount of nutrients. Because of this, many bacterial species produce a variety of antimicrobial substances, such as lactic acid, acetic acid, diacetyl, hydrogen peroxide and the other substances including enzymes, defective phages and lytic agents with potential importance for food fermentation and biopreservation. (Lindgren and Dobrogosz, 1990). Apart from metabolic end products, some LAB strains also secrete antimicrobial substances termed bacteriocins, originally defined as the proteinaceous compounds that kill closely related bacteria (Tagg et al., 1976). Bacteriocin production seems to be aimed to compete against other bacteria which are present in the same ecological niche (Barefoot et al., 1993; Dykes, 1995; Riley, 1998). Some of these inhibitory substances are active against food borne pathogens and they become the focus of research interest concerning their potential role as food preservatives. Use of either bacteriocin-producing LAB strains, which are generally regarded as safe (GRAS), or their bacteriocins in food production could have a positive effect on food preservation and safety. Bacteriocins produced by LAB have been classified on the basis of their size, chemical properties, mode of action and mechanism of export. There are two main classes of LAB AMPs: the lanthionine-containing bacteriocins (class I) and the unmodified, heat stable bacteriocins (class II) (Cotter et al., 2005). Class II bacteriocins are heterogeneous and may be further divided into four subgroups: (i) pediocin-like bacteriocins, (ii) two-peptide bacteriocins, (iii) cyclic peptides, and (iv) non-pediocin one-peptide linear bacteriocins (Cotter et al., 2005). Most of the genetically characterized class II bacteriocin gene clusters are composed of three gene modules: a module that includes the structural and immunity genes, a transport gene module, and a regulatory gene module. The struc-

tural gene for the bacteriocin is cotranscribed with the corresponding immunity gene located downstream, although there are exceptions to this genetic organization (Franz et al. 1999; Franz et al., 2000). Class III bacteriocins include large, heat-labile proteins with a molecular mass of 30 kDa and higher. Many bacteriocins are capable of resisting inactivation at the high temperatures used in food processing and can remain functional within a broad pH range. Bacteriocins are usually inactivated by proteolytic enzymes in the human digestive tract and would be digested just like any other protein in the diet.

According to our knowledge, only two bacteriocins from *L. paracasei* subsp. *paracasei* isolates have been purified and their primary structure characterized, since one of them has also been characterized genetically (Kojić et al., 2010). The purpose of this study was to analyze the bacteriocin production by the natural isolate of *L. paracasei* subsp. *paracasei* BGUB9. This strain produces bacteriocin, designated as BacUB9, and we have tested its antimicrobial activity, especially against spoilage microorganisms and food-borne pathogens.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Lactobacillus paracasei subsp. *paracasei* BGUB9 was isolated from homemade hard cheese traditionally manufactured in the village Ubli, Montenegro. *L. paracasei* subsp. *paracasei* BGUB9 was grown in MRS broth (Merck GmbH, Darmstadt, Germany) at 30°C and it was stored in the same medium containing 15% (w/v) glycerol (Sigma Chemie GmbH, Deisenhofen, Germany) at -80°C. Agar plates were prepared by the addition of agar (1.5%, w/v) to the MRS broth.

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* and *Leuconostoc* strains were grown in MRS (Merck, Darmstadt, Germany) at 30°C and 37°C. *Lactococcus lactis, Streptococcus, Pediococcus* and *Enterococcus* strains were grown in M17 broth (Merck GmbH, Darmstadt, Germany) containing 0.5% glucose (GM17 broth) and incubated at 30°C or 37°C. The other indicator strains were cultivated in the following media: *Staphylococcus aureus* on Baird parker agar (Torlak, Belgrade, Serbia), *Salmonella typhi* on Wilson-Blair agar (Torlak), *Bacillus subtilis* on Columbia agar with the addition of 5% of horse blood (Torlak), *Streptococcus faecalis* on blood agar with tryptone-peptone (15 g l^{-1}), extract of bovine heart (3 g l^{-1}), and NaCl (5g l^{-1}) with the addition of 7% of sheep blood (Torlak). *Listeria innocua* was grown in a Brain Heart Infusion (BHI) medium (Oxoid Ltd., Basingstoke, England) at 30°C. *Clostridium* sp. was grown in Reinforced Clostridium Medium (RCM) (Oxoid Ltd., Basingstoke, England) at 37 °C in anaerobic conditions. *E. coli* and *Pseudomonas aeruginosa* strains were grown in Luria-Bertani (LB) medium (Miller, 1972) at 37°C. Other pathogenic strains were cultured on Mueller-Hinton agar (Torlak). To each medium agar (1.5%, w/v) (Torlak) was added when used as a solid medium. The plates were incubated overnight at 30°C or 37°C depending on the strain.

Molecular determination of the isolate BGUB9

Preliminary strain determination was done according to its fermentation ability by using API 50CHL (Api System S.A., Bio-Merieux, Marcy l'Etoile, France) and other classical microbiological techniques, which classified the strain BGUB9 as *Lactobacillus paracasei.* A final species identification of the natural isolate BGUB9 as *Lactobacillus paracasei* subsp. *paracasei* was performed by rep-PCR and 16S rDNA sequencing. Total DNA from pure cultures was extracted by the modified method described by Hopwood and coauthors (1985).

For rep-PCR analysis, products were generated with primers $(GTG)_5$ (5'-GTG GTG GTG GTG GTG -3') for all isolates. Samples were amplified in *GeneAmp PCR System 2700* (Applied Biosystems) programmed as follows: initial denaturation of DNA for 7 min at 95°C, 32 cycles of denaturation of 1 min at 94°C, annealing for 1 min at 40°C, polymerization for 8 min at 65°C; and extension of incomplete products for 16 min at 65°C. The PCR products were separated by electrophoresis on a

1.5% (wt/vol) agarose gel containing 0.5 μg ml^{-1} ethidium bromide in 1 x TAE buffer (2 mol l^{-1} Tris base, 1 mol l^{-1} glacial acetic acid, 0.05 mol l^{-1} EDTA [pH 8.0]), at a constant voltage 60 V,and temperature of 4°C for 20 h (Versalovic et al., 1994). The electrophoresis was performed in the horizontal gel electrophoresis system (Bethesda Research Laboratories, Gaithersburg, Maryland) and DNA fragments were visualized by CCD camera Biometra BDR2/5/6 (Bio Doc Analyze).

For 16S rDNA sequencing, PCR amplifications with primers UNI16SFW (5'-GAG AGT TTG ATC CTG GC-3') and UNI16REW (5'-AGG AGG TGA TCC AGC CG-3') were performed with a *Taq* DNA polymerase kit (Fermentas UAB, Vilnius, Lithuania). Reaction mixtures contained 20 mmol l^{-1} Tris-HCl (pH 8.4), 50 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} each of the four deoxynucleotide triphosphates (dNTP), 1 U of *Taq* polymerase, 5 pmol l^{-1} of each primer, and 0.1 μg of template DNA in a final volume of 50 μl. The PCR products were analyzed by electrophoresis on 1% agarose gel and purified with QIAquick PCR Purification KIT (Qiagen, Hilden, Germany). Purified PCR amplicons were sequenced by the Macrogen sequencing service in Seoul, Korea. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to determine the most related DNA sequence relatives in the NCBI nucleotide sequence database.

Test for bacteriocin production

For the detection of bacteriocin activity, an agar well diffusion assay was used (Tagg and McGiven, 1971). Soft MRS agar (0.7%, w/v), containing the indicator strain, *L. paracasei* subsp. *paracasei* BGHN14, was overlaid onto MRS plates. The wells were made in the lawn of hardened soft agar, to which aliquots (50μl) of the supernatant of an overnight culture (16h) were poured. To confirm the production of a bacteriocin-like substance, a crystal of pronase E was placed close to the edge of the bacteriocin-containing well. The plates were incubated overnight at 30°C. The appearance of a clear zone of inhibition around the well, but not in the vicinity of the pronase E crystal, was taken as a positive signal for bacteriocin production. To eliminate the possible inhibitory effect of either hydrogen peroxide or lactic acid on the indicator strain growth, pH-neutralized, a catalase-treated supernatant of the overnight cultures (16 h) was used.

Kinetics of bacteriocin production

In order to determine the kinetics of bacteriocin BacUB9 production, a culture of BGUB9 containing approximately 10^5 cells per ml was made. The cells of the overnight culture of *L. paracasei* subsp. *paracasei* BGUB9 were washed two times with MRS broth (1ml) in order to remove previously synthesized bacteriocin. The culture was incubated at 30°C and samples were taken every hour to determine bacteriocin production (AU/ml) and CFU/ml. In order to quantify the yield of bacteriocin, cell-free supernatants were serially diluted in MRS broth before loading 5 μl of each dilution onto the indicator strain *L. paracasei* subsp. *paracasei* BGHN14. One arbitrary unit (AU) of bacteriocin was defined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn (Mayr-Harting et al., 1972). Determination of the kinetics of bacteriocin production was done in duplicate; the variation of AU values was less than 5%.

Effect of enzymes on bacteriocin activity

The test of the enzymes' effect on bacteriocin UB9 activity was done as described previously (Kojić et al., 1991). The following enzymes (final concentration 1 mg/ml) and buffers were used: pronase E (Sigma) and proteinase K (Sigma) in 10 mM Tris–HCl (pH 8); trypsin (Calbiochem) and α-chymotrypsin (Sigma) in 50 mM Tris–HCl (pH 8); pepsin (Calbiochem) in 20 mM HCl (pH 2); catalase (Sigma), and lipase (Sigma). The reaction mixtures were incubated at 37°C for 1 h. The remaining bacteriocin activity was tested by the agar well diffusion assay. Enzyme-free buffers and supernatants with buffers, incubated at 37°C for 1 h, were used as controls.

Thermal and pH stability of bacteriocin

To study the thermostability of the bacteriocin, aliquots of 50 μl of cell–free supernatant of the

overnight culture of a bacteriocin producer *L. paracasei* subsp. *paracasei* BGUB9 were incubated for 15 min at temperatures ranging from 40°C to 100°C with 10 degrees increments, and also autoclaved (121°C; 15 min). After the heating treatment, the samples were cooled to room temperature and the remaining bacteriocin activities were determined.

To determine the effect of pH on bacteriocin activity, the pH of the bacteriocin samples was adjusted stepwise from 1 to 12, in steps of one pH unit using either 1M HCl or 1M NaOH. The samples were incubated for 1 h at 30°C and bacteriocin activity was determined by the agar well diffusion assay. Supernatants of the bacteriocin UB9 non-producing derivatives BGUB9-96, Bac^- was pre-treated on the same way as bacteriocin samples and used as controls to eliminate the effect of pH alone on the growth of indicator strain.

Partial purification of bacteriocin BacUB9

Purification of bacteriocin was performed by ammonium sulfate fractionation. A 5% transfer from an overnight culture (16h) was made into 1l of MRS broth. The cells were grown at 30°C for 16 h, and the bacteria were removed by centrifugation at 4,500xg for 20 min. Ammonium sulfate was added to 30% saturation and the precipitate thus formed was removed by centrifugation, followed by a further addition of salt up to 60% saturation; the supernatant was stirred overnight at 4°C. The mixture was centrifuged (8,000xg, 4°C, 60 min) and the obtained precipitate was collected and dissolved in 10 ml 10mM sodium phosphate buffer (pH 7). The active solution was treated with chloroform (in ratio 1:1, v/v). The mixture was centrifuged (13,000xg, 4°C, 40 min) and the interphase fraction was collected and dried in a Speed Vac. The pellet was resuspended in 10 ml 10mM sodium phosphate buffer (pH 7). This active fraction was separated on a reverse-phase C18 column. Elution was done at room temperature with a step gradient from 30% to 100% isopropanol. Fractions were collected and concentrated in Speed Vac. Eluted fractions were dried and resuspended in 5 ml of 10 mM sodium phosphate buffer pH 7. After each step of this isolation and purification procedure the antimicrobial activity was measured.

The partially purified bacteriocin (10 µl) was mixed with 10 µl of sample buffer containing β-mercaptoethanol, boiled for 5 min at 70°C and run on 16.5% acrylamide gel for tricine-SDS-PAGE on a vertical electrophoresis system, together with a polypeptide standard (Bio-Rad, Hercules, California, USA). Gels were stained with Coomassie blue R250 (SERVA, Heidelberg, Germany), according to the protocol supplied with the Bio-Rad polypeptide standard, and destained in a methanol (20%) and acetic acid (7%) mixture to determine molecular size. To detect the bacteriocin activity, the other part of the gel was pre-treated with an isopropanol (20%) and acetic acid (10%) mixture and subsequently washed, first with Tween 80 (0.5%) and then in water for 24 h, as described by Bhunia and Johnson (1992). After washing, the gel was placed on a 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* BGHN14. The plate was incubated overnight at 30°C and the appearance of inhibition zones was examined.

The effect of Lactobacillus paracasei subsp. paracasei BGUB9 on Lactobacillus paracasei subsp. paracasei BGHN14 growth in mixed cultures

To test the effect of *Lactobacillus paracasei* subsp. *paracasei* BGUB9 on *Lactobacillus paracasei* subsp. *paracasei* BGHN14 growth in a mixed culture, the MRS broth was initially inoculated with *L. paracasei* subsp. *paracasei* BGHN14 (10^6 cfu ml^{-1}) transformed with pA13 (Em^r) and *L. paracasei* subsp. *paracasei* BGUB9 10^6 cfu ml^{-1}. As a control, the samples of MRS broth inoculated with each strain separately were used. The cultures were incubated at 30°C. Samples were taken every three hours in order to determine cfu ml^{-1} for each strain. The number of CFU was determined on a selective medium (MRS Erythromycin 5 µl/ml) for *L. paracasei* subsp. *paracasei* BGHN14 by the agar plate count method. The plates were incubated for

48 h at 30°C, and the CFU of *L. paracasei* subsp. *paracasei* BGHN14 was determined.

Mode of action of bacteriocin

To determine the antimicrobial effect of bacteriocin UB9 on *L. paracasei* subsp. *paracasei* BGHN14, the loss of cell viability was monitored. For this purpose, 3 ml of filtered 10x concentrated supernatant that was buffered to pH7 was added to a culture of *L. paracasei* subsp. *paracasei* BGHN14 and incubated at 30°C. The initial inoculums of *L. paracasei* subsp. *paracasei* BGHN14 were 10^6 cfu ml^{-1}. As a control, a sample without bacteriocin (filtered 10x concentrated supernatant of bacteriocin non-producer) was used. Samples were taken every 30 minutes and the number of cfu per milliliter was determined by plating 0.1 ml of appropriate dilutions on *L. paracasei* subsp. *paracasei* BGHN14 selective medium agar plates. The plates were incubated for 48 h at 30°C, and the numbers of survived cells were determined.

Plasmid isolation

For plasmid isolation from *E. coli* "JETstar Plasmid Miniprep Kit" (Genomed, GmbH) were used. Analytical amounts of plasmid isolation from the lactobacilli was performed as described previously (O'Sullivan and Klaenhammer, 1993).

Plasmid curing

Plasmid curing was achieved, as described previously, by growing the cells in the presence of novobiocin at a sublethal temperature (Kojić et al., 2005). Pre-warmed MRS broth (41°C) containing novobiocin (8 μg/ml) was inoculated with 10^3 cells per ml. After 2 h incubation, the cells were collected by centrifugation and resuspended in the same volume of pre-warmed novobiocin containing MRS broth to avoid a bacteriocin-killing effect on the cured (Bac^-, Bac^s) cells. The same procedure was repeated five times, and the aliquots (0.1 ml) were then plated onto MRS agar plates that were incubated at 30°C for 48 h. To detect Bac^- Bac^s derivatives, master plates were made in duplicate. One of the plates was overlaid with a reconstituted MRS soft agar, containing indicator cells (strain BGHN14), and incubated overnight at 30°C. Colonies that lost the ability to inhibit the indicator strain were taken from the original master plate and used as indicator strains for rechecking their Bac^- Bac^s phenotype.

RESULTS

Characterization of the strain Lactobacillus paracasei subsp. paracasei BGUB9 and determination of its antimicrobial spectrum

A collection of autochthonous lactic acid bacteria (LAB) was isolated from homemade cheeses produced in the village Ubli, Montenegro. The manufacturing of the cheese was performed in a traditional way, without the addition of any commercial starter cultures. The strain *L. paracasei* subsp. *paracasei* BGUB9 was isolated by using standard microbiological procedures for the detection and isolation of LAB. The isolate was identified on the basis of the sugar fermentation pattern, according to the API50 CHL system (bioMerieux, Marcy l'Etoile, France), and other classical microbiological techniques. The identity of the isolate was confirmed by $(GTG)_5$-PCR and 16S rDNA sequencing (data not shown). According to these results, the isolate BGUB9 was identified at the subspecies level as *L. paracasei* subsp. *paracasei.* The antimicrobial spectrum of the strain BGUB9 is presented in Table 1. Beside an inhibitory effect on the growth of several strains of the same species (*L. paracasei*) and *L. rhamnosus,* the strain BGUB9 also inhibited the growth of many pathogenic bacteria, such as *Streptococcus pneumoniae, Streptococcus pyogenes, Shigela flexneri, Shigela dysenteriae, Listeria innocua* and other.

Table 1. Antimicrobial activity of *L. paracasei* subsp. *paracasei* BGUB9. Bac$^+$-bacteriocin producer; + bacteriocin BacUB9 inhibitory effect on indicator strain; - immunity to bacteriocin BacUB9.

Indicator species	Strain	Source	Activity
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	BUK2-16	Lab. colection	+
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	SJ2-8 Agg$^+$	Lab. colection	-
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	BGPT4-82	Lab. colection	+
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	ZLS10-1	Lab. colection	+
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	ZLS10-6	Lab. colection	-
Lactobacillus paracasei subsp. *paracasei* Bac$^+$	ZLM1-2	Lab. colection	+
Lactobacillus paracasei subsp. *paracasei*	BGHN14	Kojic *et al.*, 1991	+
Lactobacillus paracasei	BGLI17	Lab. colection	+
Lactobacillus paracasei	BGLI18	Lab. colection	+
Lactobacillus casei	BGHN40	Lab. colection	-
Lactobacillus casei	BGCG35	Lab. collection	-
Lactobacillus casei	BGCG37	Lab. collection	-
Lactobacillus casei subsp. *casei*	1157^T	JCM	+
Lactobacillus plantarum	NSDO1193	NSDO	-
Lactobacillus plantarum	ATCC14917	ATCC	+
Lactobacillus plantarum	A112	Vujcic and Topisirovic, 1993	+
Lactobacillus plantarum	BGAN8	Lab. collection	+
Lactobacillus acidophilus	V74	Visby's strain	-
Lactobacillus acidophilus	CH2	Chr. Hansen's strain	-
Lactobacillus acidophilus	C7	Lab. collection	-
Lactobacillus rahmnosus	BGEN1	Lab. collection	+
Lactobacillus divergens	BG742	Kojić *et al.*, 1995	-
Lactobacillus delbrueckii subsp. *bulgaricus*	BGPF1	Fira at al., 2001	-
Bifidobacterium bifidum	TC1	Lab. collection	-
*Lactococcus lactis,*Bac$^+$	BGIS29	Miladinov *et al.*, 2001	+
Lactococcus lactis subsp. *lactis,*Bac$^+$	NP45	Lab. collection	-
Lactococcus lactis subsp. *lactis*	NSDO712	NSDO	-
Lactococcus lactis subsp. *lactis*	ATCC11454	ATCC	-
Lactococcus lactis subsp. *cremoris*	NS1	Kojić *et al.*,1991	-
Lactococcus lactis subsp. *cremoris*	Wg2	Haandrikman, 1990	-
Lactococcus lactis subsp. *lactis* bv. diacetylactis,Bac$^+$	S50	Kojic at al., 1991	-
Leuconostoc mesenteroides subsp. *mesenteroides*	M1	Lab. collection	-
Leuconostoc mesenteroides subsp. *mesenteroides*	R1	Lab. collection	-
Leuconostoc mesenteroides subsp. *mesenteroides*	Ø	Lab. collection	-
Leuconostoc mesenteroides subsp. *dextranicum*	A1	Lab. collection	-
Leuconostoc mesenteroides subsp. *dextranicum*	K10	Lab. collection	-
Leuconostoc mesenteroides subsp. *dextranicum*	Rz2	Lab. collection	-
Streptococcus pyogenes	B080	Lab. collection	-
Streptococcus pyogenes	A443	Lab. collection	+
Streptococcus pyogenes	A337	Lab. collection	+
Streptococcus pneumoniae	25	Lab. collection	+
Clostridium tetani	ATCC10779	ATCC	-
Staphilococcus aureus	ATCC25923	ATCC	+
Bacillus subtilis	ATCC6633	ATCC	+
Bacillus coagulans	2257^T	JCM	-
Micrococcus flavus	ATCC10240	ATCC	+
Micrococcus luteus	12780	IFO	-
Enterococcus faecalis	ATCC29212	ATCC	+
Enterococcus faecalis	5803^T	JCM	+
E. coli	ATCC25922	ATCC	+
Pseudomonas aeruginosa	ATCC27853	ATCC	+
Proteus mirabilis	971	Lab. collection	-
Proteus vulgaris	246	Lab. collection	+
Salmonella typhi	1802	Lab. collection	-
Klebsiela sp.	NCIB9111	NCIB	+
Shigella flexneri		Lab. collection	+
Shigella dysenteriae		Lab. collection	+
Shigella sonnei		Lab. collection	+
Listeria innocua	ATCC33090^T	ATCC	+

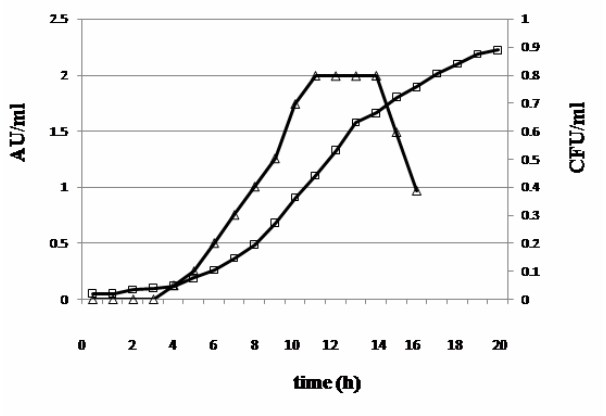

Fig. 1. Kinetics of bacteriocin bacUB9 production during the growth of *L. paracasei* subsp. *paracasei* BGUB9 in MRS broth at 30°C. Growth of the strain BGUB9 was followed by using the agar-plate count method (CFU/ml) (□). Bacteriocin concentration is expressed as arbitrary units per milliliter (AU/ml) (△). The indicator strain was *L. paracasei* subsp. *paracasei* BGHN14.

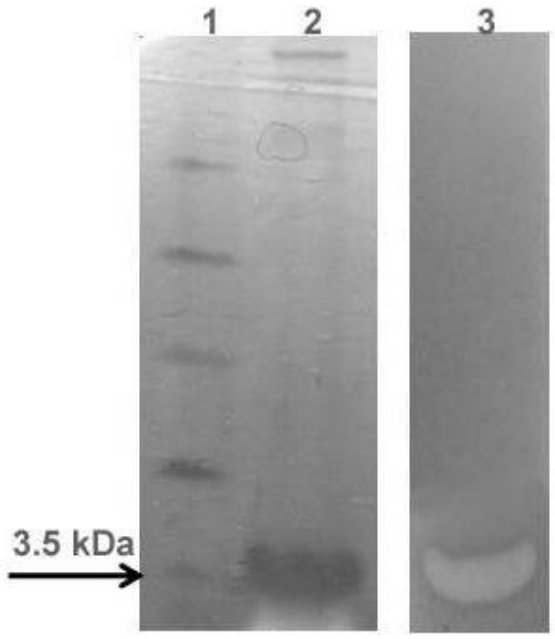

Fig. 2. Analysis of the activity of partially purified bacteriocin BacUB9.

SDS-PAGE of active fractions after preparative reversed phase chromatography on C18 column. Lane 1: Molecular mass marker; Lane 2: active fraction; Lane 3: Gel overlaid with soft agar containing cells of indicator strain *L. paracasei* subsp. *paracasei* BGHN14. The protein band that showed inhibitory activity is indicated by an arrow.

Bacteriocin production

The inhibitory substance produced by the isolate BGUB9 was designated bacteriocin BacUB9. It was established that the bacteriocin production of the strain BGUB9 in a MRS medium was dependent on the growth phase. It was not possible to detect the bacteriocin activity during the first 5 h of the growth of BGUB9 culture. The production of bacteriocin UB9 by BGUB9 reached a plateau after 12 h of incubation at 30ºC when the culture entered the early stationary phase, and production continued until 15 h of incubation (Fig. 1). Bacteriocin production in different growth media was also examined. When BGUB9 was grown in MRS broth, the production of bacteriocin UB9 was very moderate. However, a considerable increase in bacteriocin UB9 production, as judged by the size of the inhibition zone on the bacteriocin test, was observed when the strain was grown in a MRS medium containing 2 % of glucose.

Biochemical characterization of bacteriocin BacUB9

Bacteriocin BacUB9 is a relatively heat-stable molecule. The antimicrobial activity was not affected by treatment at 100ºC for 30 min. After heat treatment at 100ºC for 60 min their activity decreased; after heat treatment at 100ºC for 120 min, the activity of bacteriocin BacUB9 was completely abolished. The bacteriocin BacUB9, when present in a cell-free supernatant of the producer strain, retained its activity within the pH range from 1 to 10. Antimicrobial activity was lost at pH 11. After 30 min, when the pH of the assay suspension was adjusted to 11, the activity of bacteriocin cannot be restored upon returning the pH of the sample to 7. Treatment of bacteriocin UB9 with various proteolytic enzymes (pepsin, trypsin, α-chymotrypsin, pronase E and proteinase K) resulted in the loss of its antibacterial activity, thereby suggesting its protein nature. The same set of proteolytic enzymes

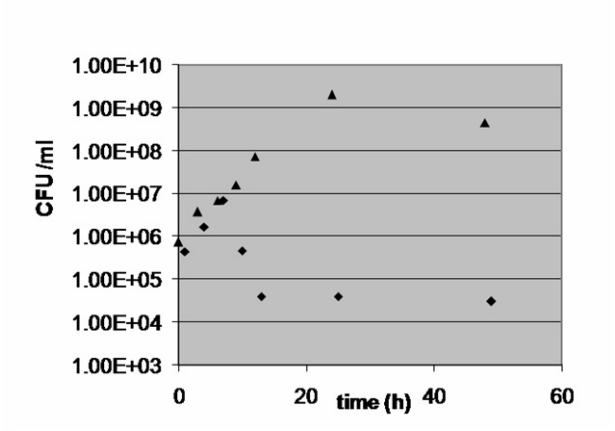

Fig. 3. The effect of *L. paracasei* subsp. *paracasei* BGUB9 on *L. paracasei* subsp. *paracasei* BGHN14 in a mixed culture. Both *Lactobacillus* strains were inoculated at 10^6cfu ml^{-1}; the growth of strain BGHN14 in pure (▲) and mixed (♦) cultures.

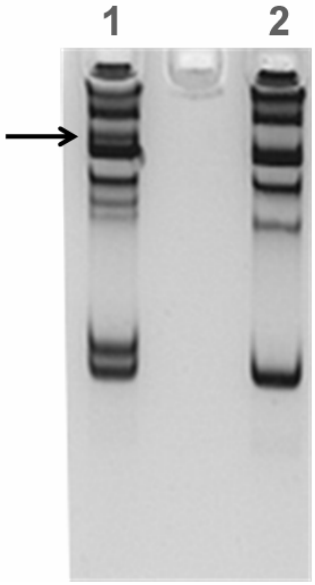

Fig. 4. Plasmid profile of DNA isolated from different derivatives of *L. paracasei* subsp. *paracasei* BGUB9. Lane 1: BGUB9 Bac^+; Lane 2: BGUB9-96 Bac^-. Arrow indicates the plasmid, which is lacking in the Bac^- derivatives..

inactivate bacteriocins such as nisin (Hurst, 1981), bacteriocin S50 (Kojić et al., 1991), bacteriocin 501 (Gajić et al., 1999) as well as many other bacteriocins. The inhibitory action on the growth of sensitive indicator cells was not affected by treatment with catalase, DNase I, RNase A or lysozyme. The results obtained by the biochemical characterization of bacteriocin UB9 showed that it is of a proteinaceous nature and strongly suggest that it belongs to class II bacteriocins defined as a group of small, heat-stable nonlantibiotics.

Estimation of molecular size of bacteriocin BacUB9

After ammonium sulphate precipitation and chloroform extraction, the solution containing the active substance was separated on a reverse phase C18 column. The fractions bound to the column were eluted by step gradient by using 30, 40, 50 and 60 % isopropanol. Elution fractions were concentrated by vacuum evaporation; pH values were adjusted to 7 and tested for antimicrobial activity by agar well diffusion assay. The active fraction that was eluted with the 50% isopropanol solution was then dried and resuspended in 5 ml of 10 mM sodium phosphate buffer, pH 7. The sample of partially purified bacteriocin was stored at 4°C.

Bacteriocin activity was visualized on gel after SDS-PAGE and one band corresponding to the zone of inhibition was detected on the gel. Its position agrees with the expected migration of a protein of approximately 4 kDa. (Fig. 2).

The effect of L. paracasei subsp. paracasei BGUB9 on L. paracasei subsp. paracasei BGHN14 in mixed culture

The effect of BGUB9 on the growth of the sensitive strain *L. paracasei* subsp. *paracasei* BGHN14 in a mixed culture was monitored. Results showed that even though the number of viable cells decreased significantly, complete inhibition of growth of the strain BGHN14 was not obtained. (Fig. 3).

Mode of bacteriocin BacUB9 action

In order to find whether bacteriocin BacUB9 has a bactericidal or bacteriostatic mode of action, the effects of the partially purified bacteriocin on the

viability of indicator cells were examined. Treatment of *L. paracasei* subsp. *paracasei* BGHN14 with bacteriocin BacUB9 led to a considerable decrease in the number of viable cells. When the initial number of *L. paracasei* subsp. *paracasei* BGHN14 cells was 10^6 cfu ml^{-1}, after 12 h of treatment the number of *L. paracasei* subsp. *paracasei* BGHN14 cells was significantly decreased to 3 x 10^4 cfu ml^{-1}. Therefore it could be inferred that BacUB9 possesses a bacteriostatic mode of action.

Localization of the genes encoding the bacteriocin BacUB9 production

To examine a possible plasmid localization of the gene encoding for bacteriocin production and immunity to bacteriocin, plasmid curing of the producer strain BGUB9 was performed. In plasmid curing experiments, the derivatives of the strain BGUB9 that lost the immunity to bacteriocin and the ability of synthesis were obtained. The analysis of the obtained derivatives indicated that both of these features present in the parental strain BGUB9 are associated with a plasmid of approximately 25 kbp. Fig. 4 represents the plasmid profile of the producer strain BGUB9 compared with the profile of the Bac^- derivative BGUB9-96. The plasmid band present in the parental strain was absent in all tested Bac^- derivatives, strongly suggesting that the genes responsible for the production of bacteriocin BacUB9 are located on that plasmid.

DISCUSSION

The results obtained in this study showed that the strain *Lactobacillus paracasei* subsp. *paracasei* BGUB9, a natural isolate from homemade cheese, exhibits a very broad antimicrobial spectrum. Besides several *Lactobacillus* strains from different species, BGUB9 also inhibits the growth of various Gram-positive and Gram-negative pathogenic bacteria (*Streptococcus, Staphylococcus, Shigella, Listeria, Pseudomonas*). Lactobacilli are known to have a widespread application as the components of starter cultures in food fermentation (Abee et al. 1995; Nes and Holo 2000). On the other hand, the bacteria from the so-called *L. paracasei* group include the strains which represent the dominant non-starter LAB microflora in mature cheese (Fitzsimons et al., 1999), and many strains of *L. paracasei* have also been used as probiotics (Holzapfel et al., 2001). The production of bacteriocins by such strains can provide additional benefits concerning their application in the food industry. Because of that, the inhibitory activity of *L. paracasei* subsp. *paracasei* BGUB9 against different food-borne pathogens is a feature of interest for further investigation.

In this work, besides the determination of the antimicrobial spectrum of the strain *L. paracasei* subsp. *paracasei* BGUB9, we also presented the biochemical characterization and partial purification of the antimicrobial compound produced by that strain. The active compound, named BacUB9, was inactivated by proteolytic enzymes, suggesting its proteinaceous nature. Bacteriocin BacUB9 is a heat-stable, low-molecular-weight peptide, which remains active within a broad pH range. Taking these biochemical characteristics into consideration together, bacteriocin BacUB9 appears to belong to the class II bacteriocins, the non-lantibiotic, small, heat-stable inhibitory peptides (Nes et al., 1996). The mode of action of bacteriocin BacUB9 was identified as bacteriostatic. While the majority of bacteriocins from LAB exhibit a bactericidal effect on target cells, bacteriocins that are bacteriostatic have also been reported (Hale and Hinsdill, 1973). The purification of BacUB9 to homogeneity and N-terminal amino acid sequencing, as well as the mode of action of purified peptide and regulation of its biosynthesis, is currently under investigation.

The production of bacteriocin BacUB9 is most likely a plasmid-encoded function in the strain *Lb. paracasei* subsp. *paracasei* BGUB9. Based on the results obtained with the curing experiments, bacteriocin and its immunity function required the presence of a plasmid of approximately 25 kbp in size, encoding the genes responsible for biosynthesis and immunity for bacteriocin BacUB9 (Fig. 4). In separate experiments, it was shown that several other plasmids that are present in the

parental strain and absent in different plasmid curing derivatives were not involved in the production of bacteriocin BacUB9.

In order to confirm this, a library of all plasmids from the strain BGUB9 in the vector pA13 was prepared and used for the transformation of a plasmid free, bacteriocin non-producing strain *L. paracasei* subsp. *paracasei* BGHN14 (data not shown). The transformant of the strain BGHN14 that harbors the vector with a fragment of about 10 kbp showed the same Bac^+ phenotype as the parental strain, indicating that genes for the production and immunity to bacteriocin BacUB9 are located on this DNA fragment. Preliminary sequence analysis of the obtained fragment indicated the high level of homology between the structural gene for bacteriocin BacSJ from the strain *L. paracasei* subsp. *paracasei* BGSJ2-8 (Kojić et al., 2010) and the putative structural gene for bacteriocin BacUB9. However, unlike *L. paracasei* subsp. *paracasei* BGUB9, the strain BGSJ2-8 showed a very narrow antimicrobial spectrum, limited to several strains of closely related lactobacilli. With this in mind, studies concerning the regulation of bacteriocin production in both strains obviously merit further investigation.

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