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CHARACTERIZATION OF LACTOCOCCI ISOLATED FROM HOMEMADE KEFIR

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Abstract – Five bacteriocin-producing lactococci isolates from traditionally prepared kefir were determined as *Lactococcus lactis* subsp. *lactis*. The analyzed isolates showed different plasmid profiles and no cross inhibition between them was detected. Moreover, natural isolate BGKF26 was resistant to the antimicrobial activity of nisin producing strain NP45. Plasmid curing experiments revealed that the genes encoding bacteriocin and proteinase production are located on separate genetic elements, except in BGKF26. Production of the tested bacteriocins depends on the concentration of casitone or triptone in the medium. Higher concentrations of casitone or triptone induce bacteriocin activity. Our DNA-DNA hybridization analyses suggest that the analyzed antimicrobial compounds probably are lactococcin-like bacteriocins.

Key words: Kefir, lactococci, bacteriocin, proteinase

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INTRODUCTION

Food fermentation processes are dependent on the biological activity of microorganisms for production of a range of metabolites that can suppress the growth and survival of an undesirable microflora in foods (R o s s et al., 2002). *Lactococcus* strains have been used for centuries as starters for the manufacture of cheeses and other fermented dairy products. There is a growing demand for new and improved strains in order to replace and complement the commercial starter cultures (particularly for *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) currently used in industrial fermentation. On the other hand, food producers face a major challenge in an environment in which consumers demand safe foods with a long shelf-life, but also express a preference for minimally processed products that do not contain chemical preservatives. Bacteriocins present an attractive option that could become at least part of the solution. They are produced by food-grade organisms, are usually heat stable, and have the ability to inhibit many primary pathogenic and spoilage microorganisms.

Lactic acid bacteria (LAB) can produce a wide range of substances with antimicrobial activity. These include proteinaceous compounds called bacteriocins. Bacteriocins are peptides or proteins with antimicrobial activity directed against related species (T a g g et al., 1976; J a c k et al., 1995). Producer organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins. Although bacteriocins are found in numerous gram-positive and gram-negative bacteria, those produced by LAB have received particular attention due to their potential application in the food industry as natural preservatives (C l e v e l a n d et al., 2001). In addition to typical LAB bacteriocins, with a narrow antibacterial spectrum, LAB also produce bacteriocins with a wider antibacterial spectrum have been also described (J a c k et al., 1995; K l a e n h a m m e r, 1988).

On the basis of their chemical, structural and functional properties bacteriocins can be classified into three main groups (N e s et al., 1996; N i s s e n-M e y e r and N e s, 1997). Moreover, the proposed revised classification scheme divides bacteriocins into two distinct categories: the lanthionine-containing lantibiotics (class I) and the non-lanthionine-containing bacteriocins (class II), while placing the large, heat-labile murein hydrolases (formerly class III bacteriocins) in a separate category called 'bacteriolysins' (C o t t e r et al., 2005).

Class II bacteriocins are small (<10 kDa) heat-stable peptides, but unlike lantibiotics they are not subject to

extensive post-translational modification. The majority of class II bacteriocins act by inducing membrane permeabilization and causing subsequent leakage of molecules from target bacteria.

In addition to initially added starter bacteria, the secondary developed microflora composed of non-starter lactic acid bacteria (NSLAB) also has an impact on flavor and texture development of fermented food products. These bacteria are mainly present in traditionally homemade fermented products manufactured at specific ecological localities. Such bacteria represent the local, geographically specific microflora, and it is believed that differences between qualities of fermented products arise from the presence of NSLAB (P e t e r s o n et al., 1990; C o g a n et al., 1997; B e r e s f o r d et al., 2001). The precise role of NSLAB strains in flavor development is still unclear. However, whether exerting a positive or negative effect, they certainly contribute unpredictably to the quality of fermented products. We have isolated LAB from different traditionally homemade fermented products created without the addition of any known starter cultures.

The purpose of the present study was to analyze bacteriocin production in non-starter lactococci isolated from kefir in order to determine their spectrum of action and genes responsible for production and immunity. Another goal of this work was to investigate the correlation between the plasmid content and phenotype characteristics such as bacteriocin production and synthesis of cell envelope-associated proteinases in lactococcal natural isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The bacterial stains used in this study are listed in Table 1. Lactococci were isolated from kefir using standard microbiological procedures and identified with the API 50 CHS system (Api System S.A., Bio-Merieux, Montelieu-Vercieu, France). *Lactococcus* strains were grown in M17 medium (T e r z a g h i and S a n d i n e, 1975) supplemented with glucose (0.5% w/v) (GM17 broth). Agar plates were prepared by the addition of agar (1.5%, w/v) (Torlak, Belgrade) to GM17 broth. For the test of proteinase production, cells were induced by growing on milk-citrate agar (MCA) plates containing 4.4% reconstituted skim milk, 0.8% Na-citrate, 0.1% yeast extract, 0.5% glucose, and 1.5% agar (w/v). In addition, chemically defined medium (CDM) was prepared [solution A: 2 g $(NH_4)_2SO_4$, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.011 g Na_2SO_4, 1 g NaCl, dissolved in 200 ml of water; solution B: 0.2 g $MgCl_2$, 0.01 g $CaCl_2$, 0.0006 g $FeCl_3x7H_2O$, dissolved in 800 ml water; solutions A and B were subsequently mixed after autoclaving by pouring A into B and adding glucose (5 gl^{-1}), vitamins, sodium acetate (2 gl^{-1}), and asparagine (80 mgl^{-1}); the final concentrations of the added vitamins were 0.1 mgl^{-1} (for biotin), 1 mgl^{-1} (for folic acid, riboflavin, nicotinic acid, thiamine, and pantothenic acid), and 2 mgl^{-1} (for pyridoxal) (D i c k e l y et al., 1995)]. For testing the induction of bacteriocin production of lactococcal strains, casamino acids from CDM were replaced with different concentrations of casitone or triptone (modified CDM medium).

Test for bacteriocin production. For detection of bacteriocin production, an agar well diffusion method was performed. First of all, GM17 soft agar (0.7% w/v) containing sensitive strain *L. lactis* subsp. *lactis* IL1403 was overlaid onto GM17 plates. Wells were made in the lawn of solidified soft agar onto which aliquots (50 µl) of filtered supernatant of overnight culture (16 h) were poured. The plates were then incubated overnight at 30°C. The appearance of a clear zone representing growth inhibition of a sensitive strain around the well was taken as a positive signal indicating the production of inhibitory molecules. To test the protein nature of inhibitory molecules, pronase E was used in bacteriocin assays. A clear zone of inhibition around the well, except in the area close to pronase E crystals, was taken as a positive signal that the inhibitory molecule is a bacteriocin. Filtered supernatant of a overnight culture of bacteriocin non-producing strain, *L. lactis* subsp. *lactis* IL1403, was used as a negative control.

Assay for bacteriocin activity in SDS-polyacrilamide gels. Lactococcal strains were grown on GM17 plates for 48 h at 30°C prior to cell collection. Cell films (1 g) were collected from the surface of GM17 plates with a glass spreader, resuspended in 1 ml of buffer (100 mM Na-phosphate, pH 7.2), and vigorously agitated. Cell suspensions were incubated for 15 min at room temperature and cells pelleted by centrifugation (5 min at 13000 rpm). The clear supernatant was mixed with the same volume of doubly concentrated sample buffer (125 mM Tris-HCl buffer, pH 6.8, 10 mM EDTA, 0.1% SDS, 25% glycerol, and 0.07% Brom phenol blue). Samples pre-

Table 1. Bacterial strains and plasmids. Prt^+ - proteolytically active; Prt^- - proteolytically inactive; Bac^+ - bacteriocin producer; Bac^- - bacteriocin non-producer; Bac^S - sensitivity to bacteriocin; Bac^R - immunity to bacteriocin; Rif^r - rifampicin resistance; Em^r - erythromycin resistance; LcnA – lactococcin A; LcnB - lactococcin B; LcnM/N – lactococcin M/N.

Bacterial strain	Description	Source or references
Lactococcus lactis subsp. *lactis*		
BGKF8	Natural isolate from kefir, Bac^+, Bac^{Im}, Prt^-	This work
BGKF17	Natural isolate from kefir, Bac^+, Bac^{Im}, Prt^-	This work
BGKF17-6	plasmid cured BGKF17 derivative, Bac^-, Bac^s, Prt^-	This work
BGKF26	Natural isolate from kefir, Bac^+, Bac^{Im}, Prt^+	This work
BGKF26-5	plasmid cured BGKF26 derivative, Bac^-, Bac^s, Prt^-	This work
BGKF49	Natural isolate from kefir, Bac^+, Bac^{Im}, Prt^-	This work
BGKF49-21	plasmid-cured BGKF49 derivative, Bac^-, Bac^s, Prt^-	This work
BGKF49-31	plasmid-cured BGKF49 derivative, Bac^-, Bac^s, Prt^-	This work
BGKF55	Natural isolate from kefir, Bac^+, Bac^{Im}, Prt^+	This work
BGMN1-501	Derivative of BGMN1-5 producing bacteriocin 501, Prt^+, Agg^+	G a j i ć et al., (1999)
BGMN1-513	Derivative of BGMN1-5 producing bacteriocin 513, Prt^-, Agg^-	G a j i ć et al., (1999)
BGMN1-596	Plasmid-free derivative of BGMN1-5, Prt^-, Bac^S, Agg^-	G a j i ć et al., (1999)
BGIS29	Natural isolate from soft home-made cheese, Bac^+, Bac^{Im}, Prt^+	M i l a d i n o v et al., (2001)
NP45	Nisin producer	Laboratory collection
IL1403	Plasmid-free derivative of IL594, Prt^-, Bac^S,	C h o p i n et al., (1984)
IL1403/pMB553	Em^r, specifying lactococcin A	v a n B e l k u m et al., (1989)
IL1403/pMB580	Em^r, specifying lactococcin B	v a n B e l k u m et al., (1992)
IL1403/pMB225	Em^r, specifying lactococcin M/N	v a n B e l k u m et al., (1989)
SM19		Laboratory collection
Lactococcus lactis subsp. *lactis* bv. *diacetylactis*		
S50	Prt^+, $Bac^+_{(LcnA)}$, Bac^R, Rif^R	K o j i ć et al., (1991b)
Lactococcus lactis subsp. *cremoris*		
NS1		K o j i ć et al., (1991b)
Wg2		Haandrikman (1990)

pared without boiling were subjected to SDS (15%) polyacrylamide gel electrophoresis according to L a e m m l i (1970). Rainbow™ (Amersham International, Buckinghamshire, UK) was used as the protein standard. After electrophoresis, the gel was divided into two parts. One part was stained with Coomassie brilliant blue G250 and distained in a methanol (20%) and acetic acid (7%) mixture to determine molecular size. To detect bacteriocin activity, the other part of the gel was pre-treated for 2 h with 200 ml of 20% isopropanol and 10% acetic acid mixture, subsequently washed with Tween 80 (0.5%), and left to soak in water for 24 h, as described previously (B h u n i a and J o h n s o n, 1992). After washing, the gel was placed on a sterile Petri dish and overlaid with GM17 soft agar (0.7%, w/v) containing 100 ml of 10^{-2} diluted overnight culture of the indicator strain *L. lactis* subsp. *lactis* IL1403. The plate was incubated overnight at 30°C and the appearance of inhibition zones was examined.

Assay of proteinase activity. Detection of caseinolytic activity after induction by growth of lactococcal cells on MCA plates was performed using a modified version of the procedure of H i l l and G a s s o n (1986) as

described previously (K o j i ć et al., 1991a). Cells were collected from the plates and resuspended in ammonium acetate buffer (100 mM, pH 6.5) to an approximate density of about 10^{10} cells per×ml. The cell suspension was mixed with the same volume of b-casein solution in ammonium acetate buffer (100 mM, pH 6.5) (5 mg ml^{-1}) (Sigma Chemie GmbH, Deisenhofen, Germany). The suspensions of cells and β-casein were incubated for 3 h at 30°C and then centrifuged to remove the cells. Degradation of b-casein in the samples was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Supernatants were taken and mixed with the sample loading buffer (125 mM TrisHCl, pH 6.8, 10 mM EDTA, 4% SDS, 25% glycerol, 5% 2-mercaptoethanol and 0.07% bromophenolblue) in a 1:1 volume ratio. Prior to loading, samples were heated at 100°C for 3 min and analyzed by SDS-PAGE on 15% gels (w/v).

Plasmid isolation and curing from lactococci. For plasmid isolation from lactococci, the method described by O' S u l l i v a n and K l a e n h a m m e r (1993) was used.

Plasmid curing was achieved by cultivation of cells in the presence of novobiocin at sublethal temperature as described previously (K o j i ć et al., 2005). Prewarmed GM17 broth (41°C) containing novobiocin (8 μg ml^{-1}) was inoculated with 10^3 cells per×ml. After the incubation at 41°C for 2 h, cells were collected by centrifugation and resuspended in the same volume of fresh prewarmed GM17 broth containing novobiocin. Medium replacement was done every two hours in order to avoid the bacteriocin-killing effect towards cured cells (Bac^-, Bac^s) by newly synthesised bacteriocin. The same procedure was repeated six times after which cell-aliquots (0.1 ml) were plated onto GM17 agar plates that were incubated at 30°C for 48 h. The plates were then overlaid with GM17 soft agar containing the indicator strain *L. lactis* subsp. *lactis* IL1403 and incubated overnight at 30°C. Colonies that failed to inhibit growth of the indicator strain were purified and tested for the Bac^- phenotype. In addition, the Bac^- derivatives were used as indicator culture in bacteriocin tests to examine their sensitivity to bacteriocin.

DNA/DNA hybridization. The gene probes *lcnA*, *lcnB, lcnMN, and lcnD* (v a n B e l k u m et al., 1992) were used in hybridization experiments. The DNA probes were labelled using the BioNick Labelling System (GIBCO-BRL, Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD 20877, USA). Transfer of DNA was carried out in 10xSSC overnight. Hybridizations were performed as described previously (K o j i ć et al., 1991a) and carried out at 50°C.

Recombinant DNA techniques. Digestions with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, and SDS PAGE were performed as described by S a m b r o o k et al., (1989). For PCR analysis, total DNA from natural isolates *L. lactis* subsp. *lactis* BGKF17, BGKF26, and BGKF49 was isolated as described by H o p w o o d et al., (1985). The following primers were used for PCR analyses: HE13 5'-CGGGATCCCCCATCCTTCTGCCATTACACC-3' and HE36 5'-AAAACTGCAGTAAGGAGATTATTAT-GAAAAATCAATTAAA-5', both located in the 5'-end of *lcnB* gene. The *Taq* polymerase and buffers used were purchased from Perkin Elmer Cetus, and 50 mL reactions were performed on a thermal cycler of the Gene Amp^R PCR System 2700 type (Applied Biosystems, USA) with the following settings: 94°C for 5 min, 30 cycles (94°C for 30 s; 50°C for 30 s; 72°C for 1 min) and 72°C for 7 min. The reactions were analyzed by agarose gel electrophoresis. For final determination of natural isolates to the species level, rep-PCR genomic fingerprinting with $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') was used as described previously by V e r s a l o v i ć et al., (1994).

RESULTS

Isolation and identification of bacteriocin-producing lactococci from kefir. Bacteriocin-producing lactococcal strains (named BGKF8, BGKF17, BGKF26, BGKF49, and BGKF55; Fig. 1B) were isolated from kefir using standard microbiological procedures for the detection and isolation of lactic acid bacteria. The aforementioned five strains represented approximately 10% of all tested colonies. The analyzed kefir was produced in the way traditional in Serbia without the addition of any commercial starter cultures. On the basis of carbohydrate fermentation patterns (analyzed using the API50 CHS system), growth temperature (mesophilic cocci), and salt tolerance (growth in medium with 4%, but not with 6% NaCl), the isolates were identified as the species *Lactococcus lactis*. Identity of all isolates was confirmed by $(GTG)_5$-PCR, and they were identified at the subspecies level as *Lactococcus lactis* subsp. *lactis* (Fig. 2).

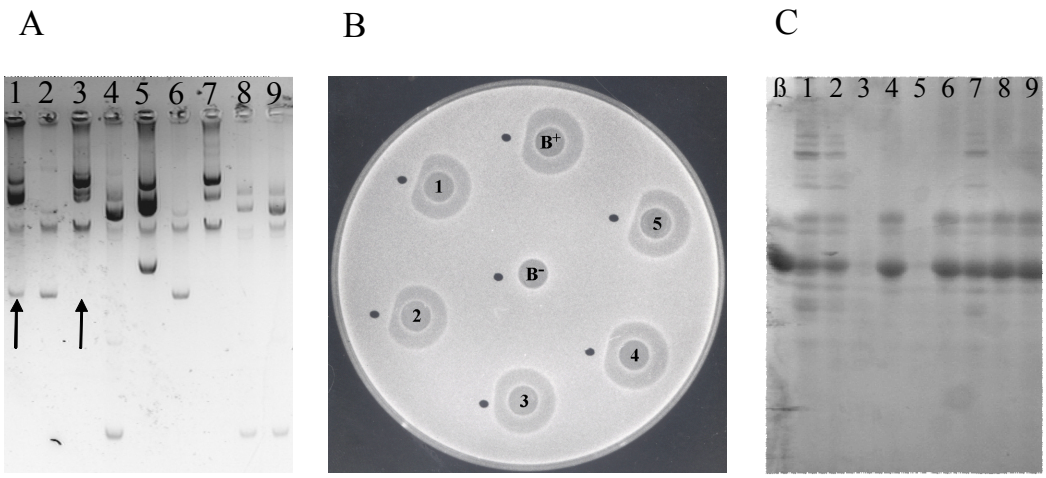

Fig. 1. Plasmid profiles (A), bacteriocin activity (B), and proteolytic activity (C) of *Lactococcus lactis* subsp. *lactis* BGKF natural isolates**.** 1. BGKF8; 2. BGKF17; 3. BGKF26; 4. BGKF49; 5. BGKF55; 6. BGKF17-6; 7. BGKF26-5; 8. BGKF49-21; 9. BGKF49-31. Black spot close to well- position of crystal of pronase E; B^+ - bacteriocin producing strain NP45; B^- - bacteriocin non-producing strain IL1403; b - starting substrate b-casein.

Characterization of bacteriocin-producing strains. Since all bacteriocin-producing isolates were identified as *L. lactis* subsp. *lactis,* further characterization of strains was performed in more detail. Plasmid profiles of the isolates were compared after electrophoresis on agarose gel. All Bac^+ isolates of the BGKF group contained a number of plasmids of different size, ranging from 2 to 50 kb (Fig. 1A). From the position on the gel, it was possible to conclude that all five isolates showed distinct plasmid profiles, although some plasmid bands appear to be common for different isolates (for example, the smallest plasmid in isolates BGKF8 and BGKF17, Fig. 1A, lanes 1 and 2). The most similar plasmid profiles were observed for isolates BGKF8 and BGKF26 (Fig. 1A, lanes 1 and 3). The only difference between these two profiles was in one plasmid band of about 20 kb that was present only in strain BGKF8 (indicated by the arrow).

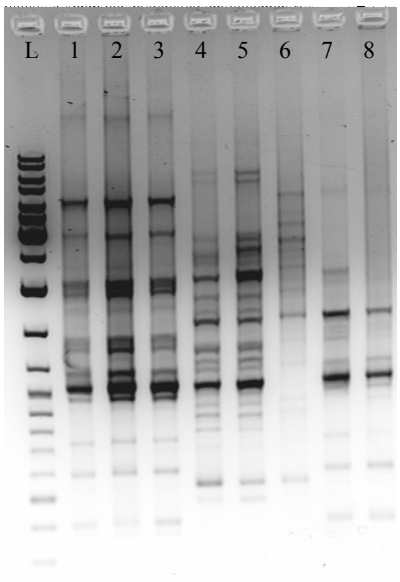

Fig. 2. $(GTG)_5$ rep-PCR profiles of *Lactococcus lactis* subsp. *lactis* BGKF natural isolates. 1. BGKF8; 2. BGKF17; 3. BGKF26; 4. BGKF49; 5. BGKF55; 6. *Lactococcus lactis* subsp. *cremoris* Wg2; 7. *Lactococcus lactis* subsp. *lactis* NS1; 8. *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* S50; 9. *Lactococcus lactis* subsp. *lactis* SM19. L – GeneRuler DNA Ladder Mix, Fermentas, Lithuania.

Antimicrobial spectrum and cross-immunity between bacteriocin-producing strains. The antimicrobial spectrum and cross-immunity among various bacteriocin producers were tested. Antimicrobial activity was detected against different lactococcal strains: *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* BGIS29 (lactococin B-like producer), *L. lactis* subsp. *lactis* BGMN501 (derivative of BGMN1-5, producing bacteriocin 501), *L. lactis* subsp. *lactis* BGMN513 (derivative of BGMN1-5, producing bacteriocin 513), *L. lactis* subsp. *lactis* BGMN1-596 (plasmid-free derivative of BGMN1-5), *L. lactis* subsp. *lactis* bv. *diacetylactis* S50 (lactococin A producer), *L. lactis* subsp. *lactis* NP45 (nisin producer), and *L. lactis* subsp. *cremoris* NS1. All isolates inhibited the growth of *L. lactis* subsp. *lactis* IL1403 harboring plasmids pMB553, pMB580, and pMB225, which specify production of lactococcin A, lactococcin B, and

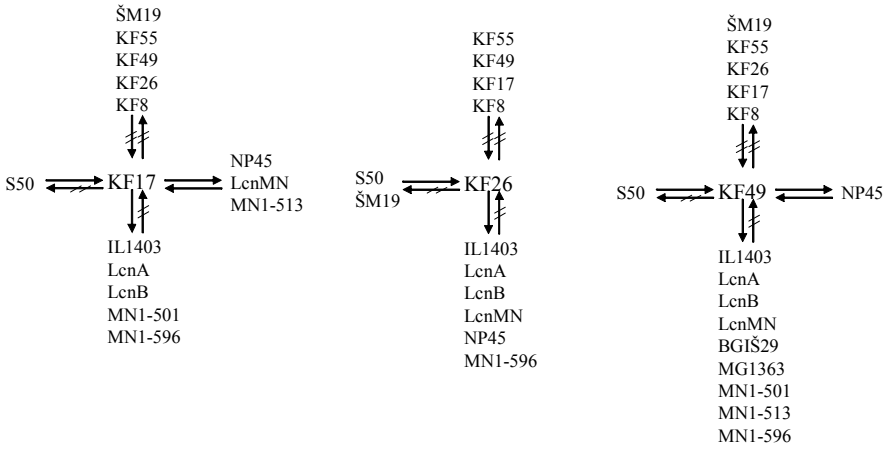

Fig. 3. Schematic presentation of cross-immunity between various bacteriocin producers. ——→ - inhibition; —//—→ - no inhibition.

lactococcin M/N, respectively. In addition, all isolates showed antimicrobial activity against BGMN501, BGMN513, and BGMN596. Reciprocal inhibitory activity of *L. lactis* subsp. *lactis* NP45 (nisin producer) was also observed, except in the case of BGKF26, which was resistant to activity of NP45, a nisin producing strain. In the case of *L. lactis* subsp. *lactis* bv. *diacetylactis* S50 (lactococin A producer), it was shown that this strain was active against all tested isolates, although they did not inhibit its growth. However, *L. lactis* subsp. *lactis* IL1403 harboring plasmids pMB553, pMB580, and pMB225 did not inhibit the growth of any BGKF bacteriocin-producing isolate, indicating that all BGKF isolates potentially produced lactococcin A, lactococcin B or lactococcin M/N. In cross-immunity experiments between BGKF isolates, no cross inhibition of BGKF growth was detected. From the results of cross-immunity experiments (Fig. 3), it can be concluded that all bacteriocin-producing

Table 2. Induction of bacteriocin production. Zones of inhibition are given in mm.

Media / Strain	BGKF17	BGKF26	BGKF49
GM17	5	4	5
CDM + 0.1% casitone	-	-	3
CDM + 0.5% casitone	6	4	4.5
CDM + 1.0% casitone	6.5	4	5
CDM + 2.0% casitone	6.5	4.5	5
CDM + 4.0% casitone	6.5	4.5	5
CDM + 0.5% triptone	5	5	6
CDM + 1.0% triptone	6	4.5	4.5
CDM + 2.0% triptone	6	4.5	5
CDM + 4.0% triptone	6	4	5.5

BGKF isolates belong to the group of lactococcin producers.

Estimation of molecular size of bacteriocins. In their plasmid profile and bacteriocin production, Bac^+ isolates BGKF17, BGKF26, and BGKF49 were the most interesting. Additionally, BGKF26 was also resistant to the activity of nisin-producing strain NP45. The activity of bacteriocins KF17, KF26, and KF49 was visualized on the gel after SDS-polyacrylamide gel electrophoresis; the zone of inhibition was positioned in the gel, which was in keeping with the expected migrations of approximately 5 kDa protein for bacteriocin BGKF17 and approximately 8 kDa protein for bacteriocins BGKF26 and BGKF49, judging from results obtained with the broad-range molecular mass marker used (data not shown).

Influence of growth medium on bacteriocin activity or production. Bacteriocin production in different growth media was also examined. For that purpose, BGKF17, BGKF26, and BGKF49 strains were grown in CDM containing casitone or triptone. When the tested strains were grown in GM17 broth, they produced bacteriocins KF17, KF26, and KF49. However, a considerable increase of bacteriocin production in all tested strains (as judged by the agar-well diffusion assay) was observed when the strains were grown in a chemically defined medium (CDM) containing casitone or triptone. The only exception was strain BGKF49, in which no induction of bacteriocin production was observed when grown in CDM containing casitone. It was found that the extent of bacteriocin production is directly dependent on the concentration of casitone or triptone present in CDM. Maximal bacteriocin activity was obtained in CDM containing 4% of casitone (C) or triptone (T) (Table 2).

Proteinase activity. The ability of BGKF isolates and their derivatives to hydrolyze b-casein was tested in ammonium acetate buffer, pH 6.5, after induction on MCA plates. It was revealed that two isolates (BGKF26 and BGKF55) produced proteinases with the ability to degrade only b-casein, while the other tested isolates were proteolytically inactive (Fig. 1C).

Determination of genes coding bacteriocin and proteinase production. To examine possible plasmid localization of the genes coding for bacteriocin and proteinase production, plasmid curing was performed. In order to establish correlation between plasmid profiles and phenotypes, different cured derivatives were selected; plasmid profiles of the parental strain and four derivatives are presented in Fig. 1A (lanes 6, 7, 8, and 9), and their phenotypes are listed in Table 1. From the plasmid profiles of cured derivatives of the BGKF17, BGKF26, and BGKF49 strains, it was possible to conclude that loss of bacteriocin production was associated of plasmid loss (Fig. 1A).

All derivatives except BGKF26-5 were proteolyt-

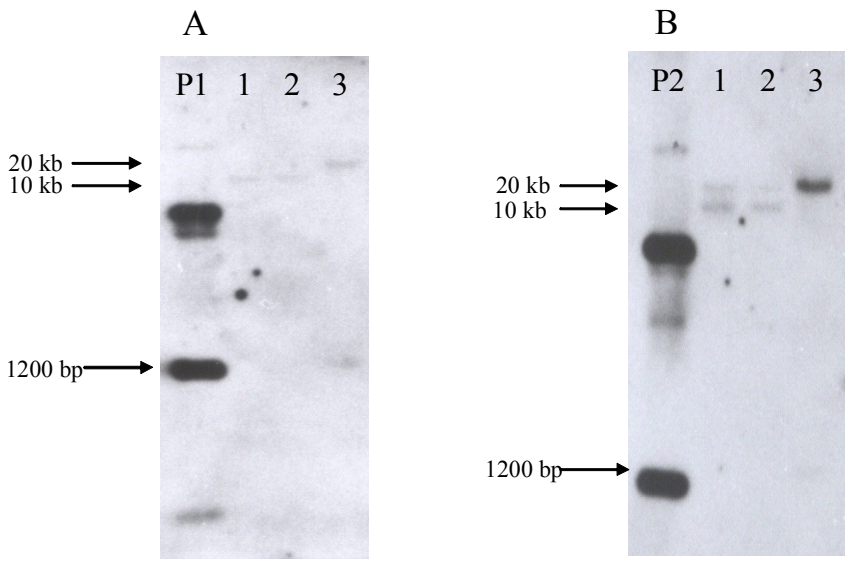

Fig. 4. Hybridiyation analysis of total DNA from BGKF17, BGKF26, and BGKF49 with DNA fragments originating from *lcnA* (A) and *lcnB* (B). 1. BGKF17; 2. BGKF26; 3. BGKF49. P1 – *lcnA* gene probe; P2 – *lcnB* gene probe. kb – kilobase, bp – base pairs.

cally inactive, indicating that the genes governing bacteriocin and proteinase production are located on different genetic elements. Derivative BGKF26-5 became proteolytically inactive, suggesting that the genes for bacteriocin and proteinase production are probably located on the same plasmid.

In order to confirm that BGKF bacteriocins belong to the lactococcin group, PCR and hybridization experiments were performed. The PCR amplification with primers originating from the *lcnB* gene (HE36 and HE13) was negative. We obtained PCR products only with positive control (plasmid pMB580 containing the *lcnB* gene), but not when total DNA from BGKF strains was used (data not shown). To confirm the results obtained by PCR amplification, analysis of hybridization of total DNA from BGKF17, BGKF26, and BGKF49 with DNA fragments originating from the *lcnA*, *lcnB, lcnM/N*, and *lcnD* genes was carried out. The obtained *Eco*RI digestion of total DNA of all three strains demonstrated that the *lcnA* and *lcnB* probes hybridized with fragments of 1200 and 10-20 kb (Fig. 4A and 4B). With the *lcnM/N* probe, a positive signal was obtained only with positive control (data not shown). After hybridization with the *lcnD* probe, positive results were obtained with total DNA from all three strains (data not shown). The obtained results showed that the bacteriocins most likely are lactococcin-like.

DISCUSSION

Lactococci are industrially important microorganisms that are used in a large variety of industrial food fermentations. Their fundamental contribution in these fermentation processes primary consists of the formation of lactic acid from the available carbon source, which results in rapid acidification of food, a critical parameter for the preservation of these food products. In this study, we analyzed natural isolates from kefir that are bacteriocin and proteinase producers.

Bacteriocins produced by BGKF17, BGKF26, and BGKF49 belong to the group of bacteriocins with a narrow antimicrobial spectrum, as they exclusively inhibit the growth of lactococci. Similar results were obtained in analyzis of bacteriocin 501, lactococcin A, lactocin S, or carnocin (H o l o et al., 1991; M ø r t v e d t et al., 1991; S t o f f e l s et al., 1992; G a j i ć et al., 1999). These bacteriocins are small peptides. The most interesting among those analyzed is bacteriocin KF26, since it was shown that strain BGKF26 is resistant to nisin. At the present time, only nisin and pediocin PA1/AcH have found widespread use in processes of food preservation.

It has been reported that the levels of lactococcin B and pediocin PA-1 production are dependent on the growth medium. The highest production of lactococcin B was found in double M17 medium containing glucose (V e n e m a et al., 1997). Analysis of growth medium-dependent bacteriocin BGKF17, BGKF26, and BGKF49 production revealed that significantly more bacteriocin was synthesized when natural isolates were grown in CDM with high casitone or tripton concentrations. Medium-dependent bacteriocin production has been shown previously (M i l a d i n o v et al., 2001). Taking all results into account, we are able to conclude that medium-dependent regulation of bacteriocin production exists. Strains producing bacteriocins and proteinases are of potential interest to the food industry because of their ability to inhibit food-related pathogens. In view of the role of proteolysis in cheese ripening, it would appear that proteinase-producing strains could be used as a source of proteinase for starter culture construction.

In this study, we found that novobiocin treatment applied together with sublethal temperature was a very efficient way of achieving plasmid curing in all of the tested natural isolates. In addition, it was previously demonstrated by us and other groups that novobiocin treatment is a very powerful method for plasmid curing in lactococci (K o j i ć et al., 2005; Y a r m u s et al., 2000). From the genetic point of view, it appears that genes encoding proteinase and bacteriocin production are located on separate genetic elements.

Hybridization experiments showed similarity with both the lactococcin A and the lactococccin B genes. It would seem that bacteriocins of natural lactococci isolates from kefir are different from the isolates originated from more common milk fermented products such as cheese. Although PCR products were not obtained with primers specific for the lactococcin B gene in hybridization experiments with both lactococcin A and lactococcin B specific gene probes positive signals were detected. It would seem that bacteriocins represent hybrids of both lactococcin A and lactococcin B.

In conclusion, our study involving isolation and characterization of natural lactococci isolates suggest they can have a potential impact on the creation of a pool of new industrially important strains that could be used as

starter cultures. In addition, isolation and characterization of strains such as lactococci that are used currently as traditional industrial strains, but with new specific characteristics, is very important for their future implementation in industrial processes.

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КАРАКТЕРИЗАЦИЈА ЛАКТОКОКА ИЗОЛОВАНИХ ИЗ КЕФИРА ПРИПРЕМЉЕНОГ У ДОМАЋИНСТВУ

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Пет изолата лактокока произвођача бактериоцина изолованих из кефира припремљеног на традиционалан начин детерминисано је као Lactococcus lactis subsp. lactis. Анализирани изолати поседују различите плазмидне профиле и међу њима није детектована узајамна антимикробна активност. Такође природни изолат BGKF26 је резистентан на активност соја NP45, произвођача низина. Експерименти чишћења плазмида показали су да се гени за синтезу бактериоцина и протеиназа налазе на различитим генетичким елементима, осим код изолата BGKF26. Синтеза анализираних бактериоцина зависи од концентрације казитона или триптона у медијуму. Више концентрације казитона или триптона индукују бактериоцинску активност. Хибридизациони експерименти су показали да су анализирани бактериоцини највероватније слични лактококцинима.