Turkish Journal of Biology

Volume 35 | Number 1 Article 3

1-1-2011

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Turk J Biol
35 (2011) 21-33

doi:10.3906/biy-0906-48

Characterization of lactococcin BZ produced by *Lactococcus lactis* subsp. *lactis* BZ isolated from boza

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Received: 17.06.2009

Abstract: A bacterium isolated from boza produced in Turkey was identified, and the physico-chemical and microbiological properties of its inhibitory compound were characterized. The isolate was identified as *Lactococcus lactis* subsp. *lactis*, based on morphology, physiology, carbohydrate fermentation, the fatty acid profile, and 16S rDNA gene sequence homology. The antimicrobial compound produced by the microorganism, lactococcin BZ, was sensitive to papain, pepsin, trypsin, and β-mercaptoethanol, but was resistant to catalase, amylase, lipase, organic solvents (methanol, chloroform, etc.), detergents (SDS, urea, Tween-80, Triton X-100), and EDTA. Lactococcin BZ was active against several gram-positive and gram-negative foodborne pathogens and food spoilage bacteria. Lactococcin BZ maintained its activity after high-heat treatment (90 °C for 30 min), at acidic and neutral pHs (2.0-7.0), and after storage at –20 to –80 °C for 3 months in lyophilized form. Lactococcin BZ was produced at the maximum level in MRS broth with an inoculum volume of 0.1%, an initial pH of 7.0, and an incubation temperature of 25 °C. Bacteriocin production began during the logarithmic phase and reached the maximum level during the early stationary phase. Its mode of action against *Listeria monocytogenes* was bactericidal and its molecular weight was about 5500 Da, as determined using tricine SDS-PAGE. *Lactococcus lactis* subsp. *lactis* BZ or its bacteriocin, which has a wide inhibitory spectrum, has the potential for use as a biopreservative in food products.

Key words: Bacteriocin, boza, *Lactococcus lactis* subsp. *lactis*, lactococcin BZ, antimicrobial compound

Bozadan izole edilen *Lactococcus lactis* subsp. *lactis* BZ tarafından üretilen laktokoksin BZ'nin karakterizasyonu

Özet: Bu çalışmada, Türkiye'de üretilen bozadan izole edilen bir bakteri tanımlanmış ve üretmiş olduğu antimikrobiyal bileşiğin fiziko-kimyasal ve mikrobiyolojik özellikleri karakterize edilmiştir. İzole edilen bakteri morfolojik, fizyolojik, karbonhidrat fermantasyon, yağ asidi profili ve 16S rDNA gen dizi homolojisi analiz sonuçları doğrultusunda *Lactococcus lactis* subsp. *lactis* olarak tanımlanmıştır. Laktokoksin BZ olarak adlandırılan antimikrobiyal bileşiğin papain, pepsin, tiripsin ve β-merkaptoetanol'e karşı duyarlı, ancak katalaz, amilaz lipaz, organik çözücülere (metanol, kloroform vb.), detejanlara (SDS, üre, Tween X-100) ve EDTA'ya karşı dayanıklı olduğu belirlenmiştir. Laktokoksin BZ'nin inhibitör spektrumunun geniş olduğu gözlenmiştir, çünkü birçok Gram-pozitif ve Gram-negatif gıda kaynaklı patojen ve bozuma etmeni bakterilere karşı aktivite gösterdiği belirlenmiştir. Laktokoksin BZ yüksek ısıl işleme (90 °C/30 dk), asidik ve nötral pH (2,0-7,0)'ya dayanıklı olduğu ve liyofilize formda (-20)-(-80) °C'de 3 ay depolama koşullarında aktivitesini koruduğu saptanmıştır. MRS sıvı besiyerinde inokulum miktarı %0,1, başlangıç pH'sı 7,0 ve inkübasyon sıcaklığı 25 °C tutulduğunda laktokoksin BZ'nin maksimum düzeyde üretildiği bulunmuştur. Bakteriyosin üretiminin logaritmik fazda başladığı ve erken durgun fazda maksimum düzeye ulaştığı tespit edilmiştir. *Listeria monocytogenes*'e karşı etki mekanizmasının bakterisidal, molekül ağırlığının ise yaklaşık 5500 Da olduğu trisin SDS-PAGE kullanılarak bulunmuştur. *Lactococcus lactis* subsp. *lactis* BZ veya geniş antimikrobiyal spektruma sahip olan bakteriyosini gıdalarda biyokoruyucu olarak potansiyel kullanıma sahiptir.

Anahtar sözcükler: Bakteriyosin, boza, *Lactococcus lactis* subsp. *lactis*, laktokoksin BZ, antimikrobiyal bileşik

Introduction

Boza is a traditional cereal-based fermented cold drink with a slightly acidic sweet flavor that is popular in Turkey. Boza is made primarily from hulled millet, as well as rice, maize, or wheat. To produce boza the cereal is first cooked, and then strained to remove most of the solids when it is cool. Next, water and sugar are added to the mixture. The sludge is fermented at 30 °C for 24 h, and then cooled and kept refrigerated for 3-5 days. Fermentation is caused by a natural mixture of yeast and lactic acid bacteria (LAB) (1-3). Based on the literature, the microbiota of boza is composed of LAB (*Weissella paramesenteroides*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactobacillus sanfransisco*, *Lactobacillus fermentum, Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus rhamnosus, Lactococcus lactis* subsp. *lactis,* and *Pediococcus pentosaceus*) and yeasts (*Saccharomyces uvarum*, *Saccharomyces cerevisiae*, *Candida* spp., and *Geotricum* spp.) (4-8).

LAB are known for their production of antimicrobial compounds, such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, antifungal peptides, and bacteriocins (9-11). Although many gram-positive and gram-negative bacteria produce bacteriocins, LAB bacteriocins have received particular attention in recent years due to their potential application in the food industry as natural preservatives. Bacteriocins produced by LAB are small, ribosomally synthesized antimicrobial peptides or proteins that possess activity towards closely related gram-positive bacteria, whereas producer cells are immune to their own bacteriocins. In many bacteriocins the cellular membrane is a target (12,13). Interest in bacteriocins produced by some LAB has been stimulated by the fact that they are active against gram-positive foodborne pathogens, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*. In addition, it was recently reported that some bacteriocins are active against certain gram-negative bacteria (14-16). Because of their activity against foodborne pathogens and consumer demand for more natural preservatives, bacteriocins have been suggested for use as biopreservatives in foods, and their use in food or in model food studies has received much attention in recent years (17-20).

A number of bacteriocins produced by *Lactococcus* spp. have been described. Nisin, bacteriocin 164, MM19, and MC38, lactococcin 140, 972, R, and MMFII, lacticin 481 and 3147 from *L. lactis* subsp. *lactis* and diplococcin, lactococcin A, B, G, and M from *L. lactis* subsp. *cremoris* have been identified (21-31).

Recently, some researchers focused on bacteriocins produced by LAB isolated from boza (14,32-37). The bacteriocins produced by boza isolates that are active against some gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) are bozacin 14, and bacteriocins ST194BZ, ST414BZ, ST664BZ, ST712BZ, ST461BZ, ST462BZ, ST242BZ, ST284BZ, and JW15BZ (14,15,36,37).

The principle aim of the present study was to identify the bacterium isolated from boza and to characterize the chemical, physical, and microbiological properties of the inhibitory substance produced by this isolate.

Materials and methods

Bacterial strains and growth media

Boza products used in this study were obtained from a retail market in Tokat, Turkey. Ten grams of each boza sample were re-suspended in 90 mL of peptone water, and then serially diluted and plated onto de Mann-Rogosa-Sharpe (MRS) agar (Merck, Germany). All samples were incubated at 30 °C for 24 h. The sandwich overlay method was used to determine which colonies had inhibitory activity on the indicator bacteria (38). The indicator microorganisms tested were *L. plantarum, E. coli, S. aureus, Bacillus cereus, L. monocytogenes*, and *Enterococcus faecalis*. Ten colonies exhibited antimicrobial activity against different indicator bacteria. Interestingly, one colony had antimicrobial activity against the gram-negative bacterium *E. coli*, in addition to activity against gram-positive bacteria; therefore, we focused on this colony. The isolate's pure culture was prepared and maintained frozen at –75 °C in MRS broth containing 20% (v/v) glycerol. LAB and other bacteria (indicator organisms) were grown in MRS and brain-heart infusion (BHI) (Merck, Germany) broth, respectively.

Identification of the isolated strain

The isolate was subjected to morphological, physiological, and biochemical tests, including Gram and endospore staining, motility, catalase, hemolysis, indole, and Voges-Proskauer tests, as well as growth in milk, growth under different NaCl concentrations, at different pHs, and at different temperatures. The strain was further identified using Step-API 20, API 50 CH (Biomérieux, Marcy-l'Etiole, France), and the fatty acid profile (Sherlock Microbial Identification System, MIDI, Inc.). Ultimate identification of the strain was based on 16S rDNA homology using a pair of bacteria-specific universal primers: LLF 5'-AGA GTT TGA TCC TGG CTC AG-3' and LLR 5'-CCG TCA ATT CCT TTG AGT TT-3' (39). PCR products were purified and sequenced using the dideoxy chain-termination method (40). Sequence similarities were determined via GenBank BLAST analysis (41).

Bacteriocin bioassay

Bacteriocin activity tests were performed on cell-free supernatants of 24-h-old cultures using the spot-on-lawn technique. Cell-free supernatants were adjusted to pH 7.0 with sterile 1 M NaOH and filter sterilized using a membrane filter (0.45 μm). For activity testing 20 μL of sample was placed onto the agar surface. Antimicrobial activity was expressed as arbitrary units (AU) per mL. The arbitrary unit was defined as the reciprocal of the highest serial 2-fold dilution showing a clear zone (at least 2 mm) of growth inhibition of the indicator strain (42).

Detection of the inhibitory spectrum of the bacteriocin

To detect the inhibitory spectrum against food spoilage bacteria and foodborne pathogens, as indicator organisms, the agar spot test was used; 20 μL of the cell-free supernatant culture was spotted onto the surface of agar plates (MRS or BHI) that had been overlaid with indicator organisms in 5 mL of soft MRS or BHI agar. These plates were incubated at 30 °C or 37 °C, depending on the indicator strains. After incubation for 24 h the plates were checked for inhibitory zones. Target microorganisms used in the sensitivity tests are listed in Table 1.

The effect of heat, pH, enzymes, detergents, and organic solvents on bacteriocin activity

To evaluate the heat stability of the bacteriocin neutralized cell-free supernatant fluids were heated at 121 °C or 110 °C for 15 min, at 80 °C or 90 °C for 15 or 30 min, and at 60 °C or 70 °C for 30 min. After cooling to room temperature the samples were tested for residual activity using the agar spot test.

The effect of pH on the activity of the bacteriocin was tested by adjusting each of the cell-free supernatants from pH 2.0-12.0 (at increments of 1.0 pH unit) with sterile 1-5 N NaOH or phosphoric acid. After 2 h of incubation at room temperature the samples were readjusted to pH 6.5 with sterile 1-5 N NaOH or phosphoric acid, and then tested for antimicrobial activity.

To determine the effect of enzymes on bacteriocin activity the cell-free supernatant (pH 6.5) was incubated for 1 h at 37 °C in the presence of 100 and/or 300 mg mL^{-1} of lipase (EC 3.1.1.3, Sigma), catalase (EC 1.11.1.6, Sigma), α-amylase (EC 3.2.1.1, Sigma), papain (EC 3.4.22.2, Merck), pepsin (EC 3.4.23.1, Merck), pancreatin (EC 232.468.9, Sigma), and trypsin (EC 3.4.21.4, Sigma) respectively, and then analyzed for antimicrobial activity. Untreated bacteriocin and enzyme solutions without bacteriocin were used as controls.

The sensitivity of the antimicrobial compound to detergents was assayed by incubating cell-free supernatant with 1% (m/v) sodium dodecyl sulfate (SDS), Tween 20, Tween 80, urea, and Triton X-100. Ethylenediamine tetraacetic acid (EDTA) was added to make final concentrations of 0.1 mM, 2.0 mM, 5.0 mM, and 50 mM. All samples were incubated at 37 °C for 5 h, and then tested for antimicrobial activity using the agar spot test method. Untreated bacteriocin-containing cell-free supernatant suspended in sterile distilled water and detergents without bacteriocin served as positive and negative controls, respectively (43). In addition, the bacteriocin supernatant with β-mercaptoethanol (10%) was heated at 90 °C for 5 min, and then tested for residual bacteriocin activity after cooling to room temperature. Heated and unheated bacteriocin supernatants, and heated β-mercaptoethanol were used as controls.

Table 1. Target strains and growth media (n = 4).

Indicator Strains	Growth Medium	Growth Temperature (°C)	Antimicrobial Activity
Lactobacillus plantarum RSKK	MRS	30	+++
Lactobacillus plantarum AU	MRS	30	+++
Enterococcus faecium ATCC 9097	MRS	30	++
Enterococcus feacalis ATCC 8043	MRS	30	++
Enterococcus faecium RSKK	MRS	30	+++
Enterococcus dissduens AU	MRS	30	++
Lactococcus cremoris AU	MRS	30	+++
Leuconostoc mesentereoides RSSK	MRS	30	+++
Listeria monocytogenes AU	BHI	37	++
Listeria ivonovii RSKK	BHI	37	+
Bacillus cereus RSKK	BHI	37	+
Bacillus subtilis RSKK	BHI	37	++
Bacillus treurgesis spp. *plasteni* AU	BHI	37	++
Enterobacter cloaceae RSSK	BHI	37	++
Escherichia coli Tip I AU	BHI	37	+
Escherichia coli RSSK	BHI	37	++
Rhodococcus equi RSKK	BHI	37	++
Salmonella spp. AU	BHI	37	+
Salmonella Enteritidis AU	BHI	37	+
Yersinia enterocolitica O:9 AU	BHI	37	+
Citrobacter freundi RSSK	BHI	37	+
Staphylococcus aureus CAMP AU	BHI	37	-
Staphylococcus aureus RSKK	BHI	37	-
Listeria ivonovii RSSK	BHI	37	-
Escherichia coli O157:H7 RSSK	BHI	37	-
Proteus mirabilis RSSK	BHI	37	-
Yersinia enterocolitica O:3 RSSK	BHI	37	-
Enterobacter aerogenes RSSK	BHI	37	-
Camphylobacter jejuni RSSK	BHI	37	-

AU: Ankara University; RSKK: Refik Saydam Hıfzısıhha Culture Collection, Ankara/Turkey
– =no antimicrobial activity; + = inhibition zone < 10 mm (the smallest inhibition zone was 4 mm); ++ = inhibition zone > 11 mm; +++ = inhibition zone > 20 mm.

In a separate experiment, crude bacteriocin was dissolved in various organic solvents, including formaldehyde (10%), chloroform (10%), acetone (10%), 2-propanol (10%), ethyl alcohol (25%), hexane (25%), and ethyl ether (25%), and then incubated at 25 °C for 1 h. After incubation the antimicrobial activity of the samples was determined. Organic solvents without bacteriocin and bacteriocin without organic solvents were used as negative and positive controls, respectively.

For storage stability the bacteriocin supernatants and freeze-dried bacteriocin were kept at 4, 25, –20, and –70 °C, and the samples were then withdrawn periodically and assayed for antimicrobial activity.

The effect of the volume of inoculum and initial growth pH on bacteriocin production

The effect of different volumes of inoculum on the production of the bacteriocin was evaluated by inoculating 0.05%, 0.1%, 0.5%, 1.0%, 1.5%, 2.0%, and 2.5% boza isolate into MRS broth. All samples were incubated at 25 °C for 24 h. Bacterial growth (OD at 600 nm, PerkinElmer UV/VIS spectrophotometer, USA), changes in culture pH, and production of bacteriocin (AU/mL) were determined during the incubation period.

To determine the effect of the initial pH of the medium on the production of bacteriocin, MRS broth (150 mL) was adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with 5 M HCl or 5 M NaOH, and then autoclaved. Each flask was inoculated with 0.1% (v/v) of a 24-h-old culture of boza isolate and incubated at 25 °C for 24 h under unbuffered conditions. Changes in bacterial growth (OD at 600 nm), culture pH, and production of bacteriocin were determined at different time intervals.

The effect of growth phase and growth temperature on bacteriocin production

The kinetics of bacteriocin production at different growth temperatures was assayed in MRS broth at 25, 32, and 37 °C for 72 h. A 24-h-old culture of boza isolate was inoculated (0.1%, v/v) into MRS broth. Samples were taken at certain time intervals and examined for bacterial growth (OD at 600 nm), changes in culture pH, and production of the bacteriocin (AU/mL).

The effect of lactococcin BZ on the growth of *Listeria monocytogenes*

Actively growing *L. monocytogenes* cells were added to BHI broth to reach an OD value of 0.25, and then incubated for 1 h at 37 °C for adaptation of the bacteria to the medium. Then, filter-sterilized (0.45 μm Sartorius, Germany) bacteriocin preparations of 0 and 1250 AU/mL were added. The samples were periodically withdrawn to determine the OD value at 600 nm.

Partial purification of the bacteriocin

The bacteriocin from the boza isolate was partially purified according to the method of Morena et al. (44). Briefly, the strain was grown for 18 h at 25 °C in MRS broth. Cells were removed by centrifugation (8500 × *g* at 4 °C for 20 min) and the pH of the cell-free culture supernatant was adjusted to 6.5 by the addition of 10 N NaOH, and then passed through a sterile filter (0.45 μm). The supernatant was brought to a final concentration of 50% saturation of ammonium sulfate via slow addition and overnight stirring at 4 °C. The mixture was centrifuged (8500 ×*g* at 4 °C for 30 min), and the surface pellicles and bottom pellets were harvested and resuspended in 10 mL of sodium phosphate buffer (5 $mmol^{-1}$, pH 6.5). To the resuspended ammonium sulfate precipitate was added 15 volumes of a methanol/chloroform mixture (1:2, v/v), and then incubated at 4 °C for 1 h. The sample was centrifuged (8000 × *g* at 4 °C for 30 min), and the pellet was subsequently resuspended in 10 mL of sterile distilled water and was then freeze-dried. This partially purified bacteriocin preparation was stored at –70 °C. For each step of the purification the antimicrobial activity (AU/mL) was determined.

Molecular size of the bacteriocin

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Bhunia and Johnson (45), and Schagger and von Jagov (46) in 16% tricine-SDS-PAGE gradient gel. Low molecular weight markers, ranging from 1.423 to 26.625 kDa (BioRad), were used. After electrophoresis the gel was sliced into 2 pieces. One half of the gel was immersed in a fixation solution (400 mL of methanol, 100 mL of glacial acetic acid, and 500 mL of distilled water) for 15 min, stained with Coomassie blue G-250 for 1 h, and then destained until the background stain disappeared. The other half of the gel was immersed in a fixation solution for 15 min, stained for 30 min, and then destained for 1.5 h. After the gel was equilibrated in changing distilled water for 3 h it was overlaid with *L. plantarum* embedded in MRS agar at 30 °C for 24 h. The position of the bacteriocin was visualized by the inhibition zone around the active protein.

Statistical analysis

Data were analyzed using Minitab v.12.1 (Minitab Inc., 1998). All experiments were performed 4 times. Variance analysis was performed for the volume of inoculum, initial pH test, and storage stability. Differences were considered significant at $P < 0.05$.

Results and discussion

Identification of the bacteriocin-producing strain

Screening for the bacteriocin-producing strain from traditionally produced boza was performed using the sandwich overlay method against *L. plantarum, E. coli, S. aureus, B. cereus, L. monocytogenes*, and *E. faecalis*. Colonies with inhibitory activity towards *L. plantarum*, *B. cereus*, *L. monocytogenes*, and *E. faecalis* were collected, and

their pure cultures were prepared for further study. We observed that one of the isolates had inhibitory activity against some gram-negative bacteria. The isolated strain was gram-positive coccus, non-motile, non-pigmented, non-spore forming, and catalase-, gelatinase-, indole-, and Voges-Proskauer-negative, hemolysis-negative, and able to grow in the presence of 3.0%-4.0% NaCl and at pH 4.4-9.6 and at 10-45 °C (Table 2). The carbohydrate fermentation (API 20 STREP and API 50 CH) pattern of the isolate is shown in Table 3. The isolate had the characteristic properties of *L. lactis* subsp. *lactis*, with respect to morphology and physiology, as well as carbohydrate fermentation (47-49). Comparison of these carbohydrate fermentation reactions to the API Strep 20 and 50 CHL databank (99.9%), and the fatty acid profile (MIS) (0.805 sim index) revealed homology to *L. lactis* subsp. *lactis*. Additionally, the sequence pattern of the 900-bp PCR amplification product of the 16 rRNA gene (Figure 1) showed 100% homology with the *L. lactis* subsp. *lactis* genome. This result confirmed the biochemical identification. Therefore, the isolate was named *L. lactis* subsp. *lactis* BZ and its antimicrobial compound was designated as lactococcin BZ.

Table 2. Morphological and biochemical characteristics of the boza isolate (n = 4).

Test	Boza isolate
Gram Morphology	Coccus
Catalase	-
Endospor	-
Hemolysis (sheep blood)	-
Motility	-
Indol test	-
Voges Proskauer	-
Gelatine hydrolysis	-
Growth at different pH ranges	
pH 3.0-4.0	-
pH 4.4-9.6	+
Growth in different NaCl concentrations	
3.0%-4.0% NaCl	+
6.5%-10.0% NaCl	-
Growth at different temperatures	
10-45 °C	+
50 °C	-
Acid production in milk (24 h) at 30 °C	pH 5.28
Acid production in milk (7 days) at 30 °C	pH 4.58
Final pH in glucose broth	pH 4.05

All tests were done in MRS broth as a grown medium, except when indicated differently.

Table 3. Carbohydrate fermentation pattern of boza isolate (n = 4).

Carbohydrate	Isolate	Carbohydrate	Isolate
Glycerol	-	Lactose	+
Erythritol	-	Mellibiose	-
D-arabinose	-	Sucrose	-
L-arabinose	-	Threhalose	+
Ribose	+	Inuline	-
D-xylose	+	Melezitose	-
L-xylose	-	D-raffinose	-
Adonitol	-	Amidon	+
ß-methyl-D-xyloside	-	Glycogen	-
Galactose	+	Xylitol	-
D-glucose	+	β-gentiobiose	-
D-fructose	+	D-turanose	-
D- Mannose	+	D-lyxose	-
L-sorbose	-	D-tagatose	-
Rhamnose	-	D-fucose	-
Dulcitol	-	L-fucose	-
Inositol	-	D-arabitol	-
Mannitol	+	L-arabitol	-
Sorbitol	-	Gluconate	-
a-methyl-mannoside	-	2 ceto-gluconate	-
a-methyl-glucoside	+	5 ceto-gluconate	-
N-acetyl-glucosamin	+	Hippurate hydrolysis	-
Amygdalin	+	Pyrrolidonylarylamidase	-
Esculin	+	β-galactosidase	+
Salicine	+	a-galactosidase	-
Cellobiose	+	β-glucuronidase	-
Arbutine	+	Leucine arylamidase	+
Starch	-	Arginine dehydrolysis	+
Maltose	+	Alkaline phosphatase	-

+ : Positive; - : Negative; ND: Not determined

Antimicrobial spectrum of lactococcin BZ

The cell-free culture supernatant of *L. lactis* subsp. *lactis* BZ was tested against the gram-positive and gram-negative bacteria listed in Table 1. Lactococcin BZ inhibited the growth of some *Lactobacillus, Enterococcus, Leuconostocs, Listeria, Bacillus, Enterobacter, Escherichia*, *Rhodococcus*, *Salmonella*, *Yersinia*, and *Citrobacter* spp., but did not exhibit inhibitory activity towards *S. aureus* CAMP, *E. coli* O157:H7, *Proteus mirabilis*, *Y. enterocolitica* O:3, *Camphylobacter jejuni*, or *Enterobacter aerogenes*.

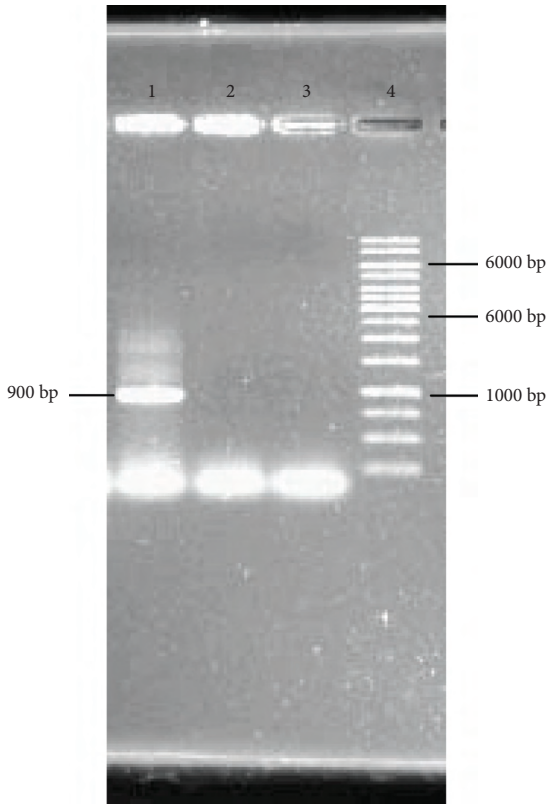

Figure 1. 16S region of *L. lactis* subsp. *lactis* BZ amplified with primers LLF and LLR. 1: 900 bp fragment. 2-3: Negative control (no DNA). 4. 1 kb marker (Fermentas).

The antimicrobial activity of lactococcin BZ was strain-specific and its spectrum was wide, as it exhibited inhibitor activity against 13 different genera. The recorded activity of lactococcin BZ against gram-negative bacteria is of particular interest, because activity against gram-negative bacteria is an unusual phenomenon and to date has only been reported for a few bacteriocins, such as bozacin, enterocin AS-48, thermophylin 81, plantaricin 35 d, lacticin NK24, and bacteriocin HV219 (14-16,33,35-37,49-53). Lactococcin BZ is different from these bacteriocins in that it inhibits some species of *Salmonella, Enterobacter, Yersinia*, and *Citrobacter*, in addition to *E. coli* (Table 3).

Physico-chemical stability of lactococcin BZ

Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant with trypsin, papain, and pepsin (Table 4). Treatment with papain and pepsin

Table 4. Effect of enzymes, pH, temperature, detergents, EDTA, β-mercaptoethanol, organic solvents, and storage conditions on the activity of lactococcin BZ (n = 4).

Treatment	Residual activity (%)[a]
Untreated bacteriocin	100
Enzymes	
Lipase (100 and 300 mg mL^{-1})	100
Catalase (100 and 300 mg mL^{-1})	100
α-amylase (100 and 300 mg mL^{-1})	100
Pepsin (100 and 300 mg mL^{-1})	0
Papain (100 and 300 mg mL^{-1})	0
Trypsin (100 mg mL^{-1})	25
Trypsin (300 mg mL^{-1})	50
Panctreatin (100 and 300 mg mL^{-1})	100
pH	
2.0-7.0	100
8.0-9.0	50
10	25
11	6.25
12	3.12
Heat treatment	
60 °C and 70 °C for 30 min	100
80 °C and 90 °C for 30 min	100
110 °C for 15 min	0
121 °C for 15 min	0
Surfactants	
SDS	100
Tween 20	100
Tween 80	100
Urea	100
Triton X-100	100
Protease inhibitor (0.1-50.0 mM)	
EDTA	100
β-mercaptoethanol	50
Organic solvents	
Formaldehyde (10%)	100
Chloroform (10%)	100
Acetone (10%)	100
2-propanol (10%)	100
Ethyl alcohol (25%)	100
Hexane (25%)	100
Ethyl ether (25%)	100
Lyophilization	100
Storage of freeze dried bacteriocin	
4 °C for 3 months	50
–20 °C for 3 months	100
–80 °C for 3 months	100
Storage of unfreeze dried bacteriocin	
4 °C for 3 months	18.5
–20 °C for 3 months	37.5
–80 °C for 3 months	50

[a] *Lactobacillus plantarum* was used as sensitive strain

at 100 mg/mL decreased the activity of lactococcin BZ by 100%. Treatment with trypsin (300 mg/mL) reduced the activity by 50%, but only by 25% with 100 mg/mL, showing that the concentration is an important factor; however, lactococcin BZ was resistant to pancreatin (300 mg/mL) (Table 4). Protease sensitivity is a key criterion for the characterization of bacteriocin, as it confirms its proteinaceous nature. No change in activity was recorded when the cell-free supernatants were treated with catalase (Table 4), indicating that H_2O_2 was not responsible for the observed inhibition. Treatment with α-amylase and lipase did not change the antimicrobial activity of the bacteriocin (Table 4), suggesting that lipid and carbohydrate moieties were not responsible for the antimicrobial activity of lactococcin BZ. This has also been reported for other LAB bacteriocins (37,54).

The pH stability of the culture supernatant was studied from pH 2.0 to 12.0. The inhibitory activity withstood exposure to pH values of 2.0-7.0 for 2 h at room temperature (Table 4). Loss in its inhibitory activity was 50% at pH 8.0-9.0 and 75-95% at pH 10-12. Similar results were reported for many other bacteriocins produced by LAB (14,15,55,56). The high stability observed at low pH is an important technological factor, because it facilitates use of bacteriocins in fermented foods (57).

Lactococcin BZ remained stable after being heated at 90 °C for 30 min (Table 4); however, it was inactivated after heat treatment at 110 and 121 °C for 15 min. Lactocin NK24 (*L. lactis*), nisin (*L. lactis*), and bozacin B14 (*L. lactis*) lost 90%-100% of their activity after heat treatment at 121 °C for 15 min (14,21,52). Lactocin MMFII (*L. lactis*) maintained only 8.3% of its activity after 30 min at 110 °C (31). Bacteriocin H-559 (*L. lactis* spp. *lactis* H-559) retained its biological activity after being heated at 100 °C, but it was almost completely inactivated at 121 °C for 20 min (55).

Lactococcin BZ retained its biological activity when treated with Tween 20, Tween 80, Triton X-100, SDS, urea, and EDTA (Table 4). Similar results were reported for bozacin 14, and bacteriocins JW3BZ, JW6BZ, JW11BZ, and JW15BZ produced by LAB isolated from boza (14,37). After treatment with 10% (w/v) β-mercaptoethanol, lactococcin BZ activity decreased by 50% (Table 4). This result indicates that lactococcin BZ may have disulfide bonds in its active region, similar to class IIa bacteriocins (58,59). Lactococcin BZ was resistant to all the organic solvents (formaldehyde, chloroform, acetone, isopropanol, methanol, ethyl ether, hexane, and ethyl alcohol) tested (Table 4). These data confirm that lipid moiety was not responsible for the observed antimicrobial activity.

To evaluate storage stability freeze-dried and concentrated (10 times) supernatant samples were kept at 4, 25, –20 and –70 °C. Lactococcin BZ was stable under lyophilization conditions. Lyophilized lactococcin BZ was more stable than in concentrated form (Table 4). Freeze-dried samples retained their activity during storage at –20 and –80 °C for 3 months; however, the concentrated lactococcin BZ (10 times) lost 62.5% and 50% of its biological activity after 3 months of storage at –20 and –80 °C, respectively. Similar results were reported for many LAB bacteriocins (60,61).

The kinetics of growth and bacteriocin biosynthesis at different incubation temperatures

To determine the kinetics of growth and bacteriocin biosynthesis *L. lactis* subsp. *lactis* BZ was inoculated (0.1%, v/v) into MRS broth and incubated at different temperatures (25, 32, and 37 °C). *L. lactis* subsp. *lactis* BZ had better growth at 32 °C than at 25 and 37 °C (Figure 2). Lactococcin BZ was produced at the highest level at 25 °C, and retained its biological activity longer at 32 °C. Bacteriocin production was lowest at 37 °C. Lactococcin BZ activity was observed during the early logarithmic phase (6-7 h of growth), suggesting that the peptide is a primary metabolite (Figure 2). Bacteriocin production (400 AU/mL) was maximal at 18 h of incubation during the early stationary phase. During extended stationary phase incubation the activity of bacteriocin decreased considerably. Loss of activity has been ascribed to proteolytic degradation by endogenous extracellular proteases induced during this growth phase, protein aggregation, and adsorption to cell surfaces and feedback regulation (62,63). During 72 h of growth at 25 °C the pH of the MRS broth decreased from 6.44 to 4.20 (data not shown) and the optical density (600 nm) of the culture increased from 0.08 to approximately 1.75 (Figure 2). Similar results were reported for some LAB bacteriocins (15,24,37,64).

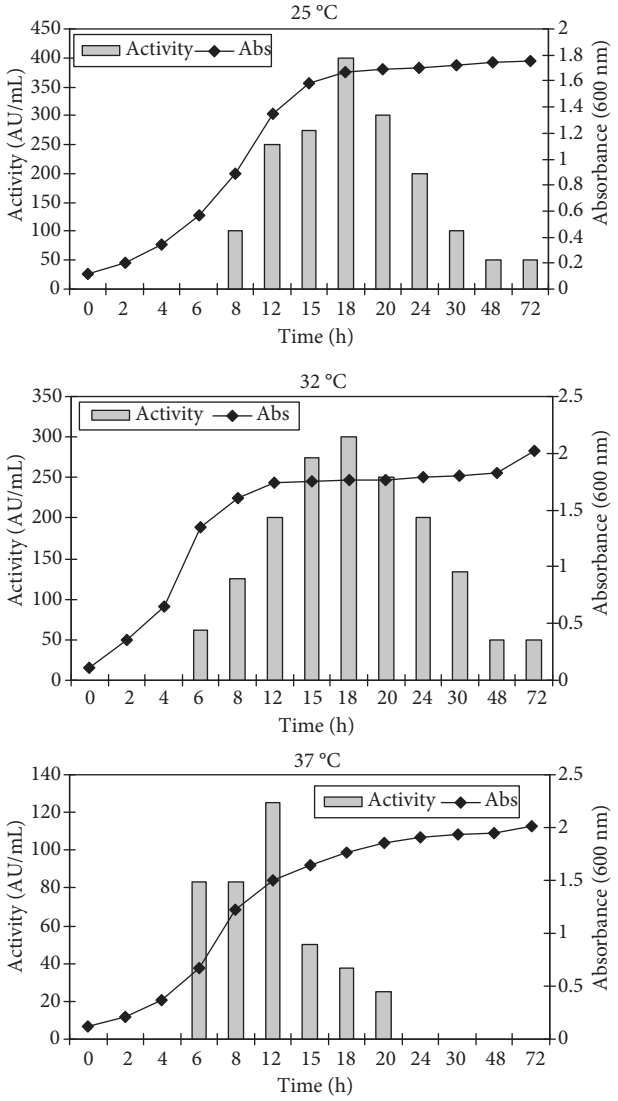

Figure 2. Effect of growth phase and growth temperature on the production of lactococcin BZ under unbuffered condition (n = 4).

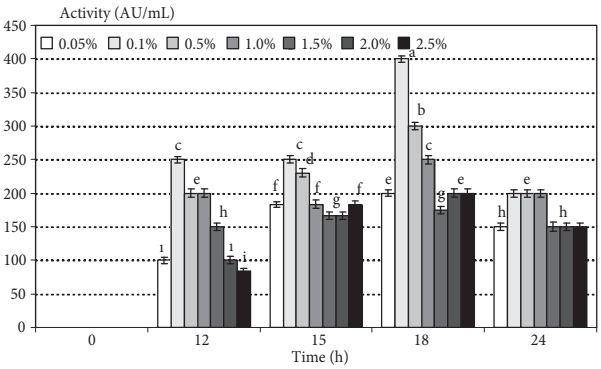

Figure 3. Effect of inoculum size on the production of lactoccocin BZ (n = 4). Any 2 means followed by the same letter are not significantly different (P > 0.05).

The effect of different volumes of inoculum and initial medium pH on lactococcin BZ production

The effects of inoculum volume (0.05%-2.5%, v/v) on cell growth and production of bacteriocin were investigated in MRS broth at 25 °C (Figure 3). During 18 h of growth the highest bacteriocin yield (400 AU/mL) was obtained by adding *L. lactis* subsp. *lactis* BZ at the level of 0.1% (v/v). The maximum absorbance value (600 nm) was 1.50-1.67 for all cultures, independent of inoculum volume (data not shown). It is well known that the biosynthesis of bacteriocin is often an inducible trait that depends on the cell density of the cell culture and concentration of the inducer (20,59,65).

The effect of initial medium pH on cell growth and lactococcin BZ production is shown in Figure 4. The highest lactococcin BZ level was recorded in MRS broth with an initial pH of 7.0 ($P < 0.05$). *L. lactis* subsp. *lactis* BZ grew to maximum density (data not shown) and produced the maximum level of bacteriocin in MRS broth adjusted to pH levels between 6.5 and 7.5; however, above pH 7.5 bacteriocin production appeared to decrease, even though the bacteriocin-producing bacterium grew well. This decrease in activity could be explained by the adsorption of the bacteriocin into the cell wall (60). Optical density values were the lowest for *L. lactis* subsp. *lactis* BZ grown in MRS broth adjusted to pH 5.0 and 5.5 (data not shown). Additionally, at these pH values no bacteriocin activity was detected (data not shown). It was reported that optimal bacteriocin production by *L. lactis* spp. occurred at pH 6.0-7.0 in MRS and M17 broth at 30 °C (24,64,66). As bacteriocin production is linked to cell growth, it also depends on factors that affect cell growth, such as pH and temperature (20).

Mode of action of lactococcin BZ on *Listeria monocytogenes*

Addition of lactococcin BZ (1280 AU/mL, pH 6.0) to a fresh culture of *L. monocytogenes* (OD_{600nm}= 0.25) caused a constant decrease in OD values during the incubation period (Figure 5), indicating a bactericidal

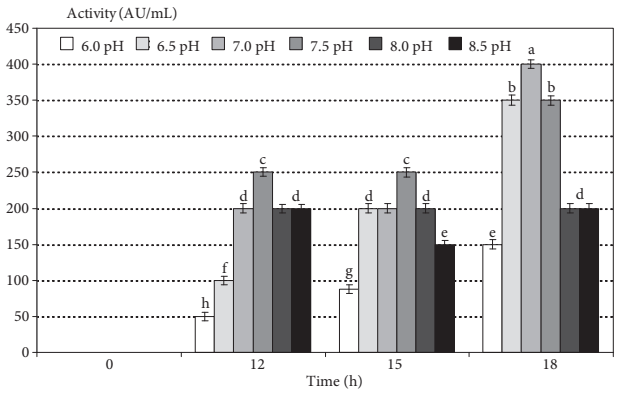

Figure 4. Effect of initial pH on the production of lactoccocin BZ (n = 4). Any 2 means followed by the same letter are not significantly different (P > 0.05).

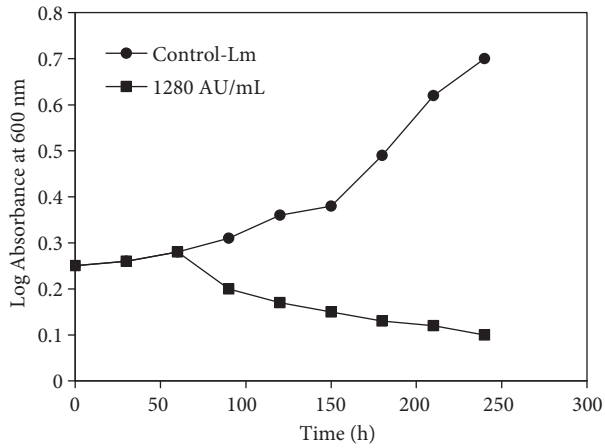

Figure 5. Effect of lactococcin BZ on the growth of *Listeria monocytogenes* in BHI broth at 37 °C. Bacteriocin was added at 60 min (n = 4).

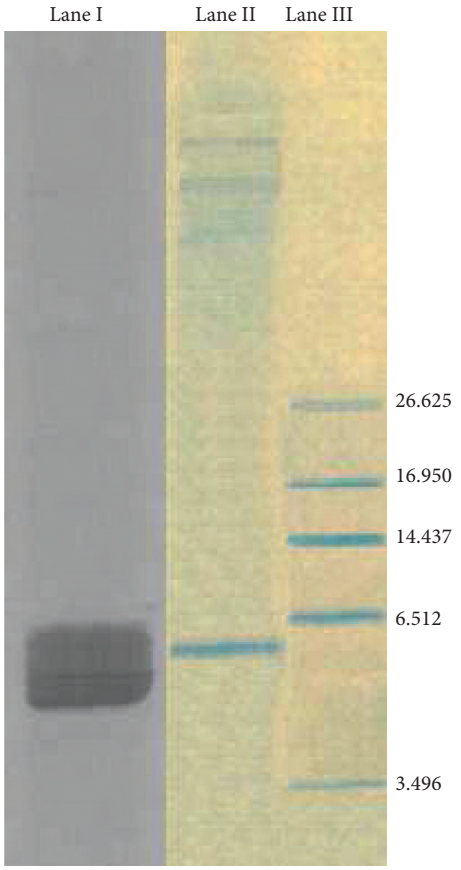

Figure 6. Molecular mass of lactococcin BZ. Lane I: Inhibition zone of lactococcin BZ (the gel was overlaid with a culture of *Lactobacillus plantarum* in MRS agar and incubated for 24 h at 30 °C), Lane II: partially purified lactococcin BZ; lane III: low molecular weight marker (BioRad).

mode of action. Similar results were reported for bacteriocin HV219 from *L. lactis* subsp. *lactis* B14, and bacteriocins JW3BZ and JW6BZ from *L. plantarum* JW6BZ isolated from boza (16, 37); however, bozacin B14 produced by *L. lactis* subsp. *lactis* B14 has a bacteriostatic mode of action (32).

Molecular size of lactococcin BZ

The molecular size of partially purified lactococcin BZ was analyzed using Tricine-SDS-PAGE. When 2× Tricine sample buffer (2 mL 4× Tris.Cl/SDS with pH 6.8, 2.4 mL of glycerol, 2 mL of β-mercaptoethanol, 0.8 g of SDS, and 2 mg of Coomassie blue G-250) was used for sample preparation, lactococcin BZ activity was not observed in the gel overlay assay. Omitting β-mercaptoethanol solved this problem. This result also shows that lactococcin BZ has intra- or inter-molecular disulfide bond(s) in its active region. After electrophoresis the gel overlaid with *L. plantarum* showed a clear zone of inhibition that was higher than the 3.5-kDa reference band (Figure 6). The molecular weight of this band was about 5.5 kDa, which is within the range of some bacteriocins reported for LAB. Bacteriocins produced by *L. lactis* subsp. *lactis* (21,24-26,52,67,68) have a molecular weight of 2.5-3.5 kDa, except bozacin 14 (6.2 kDa) (14) and bacteriocin MC38 (8.0 kDa) (31); however, it has not been previously reported that bacteriocins produced by boza isolates or *L. lactis* subsp. *lactis* are sensitive to β-mercaptoethanol, or their activity was lost during electrophoresis.

Conclusion

Lactococcin BZ produced by *L. lactis* subsp. *lactis* BZ is a new bacteriocin, as it is different from bacteriocins produced by *Lactococcus* species or bacteria isolated from boza, in terms of proteolytic enzyme, heat, pH, detergent, and chemical stability, and inhibitory activity and spectrum. Additionally, among these bacteriocins, lactococcin BZ is only sensitive to β-mercaptoethanol. Furthermore, its wide inhibitory spectrum, including many pathogenic gram-positive and gram-negative bacteria, is very important. Further research will be conducted to characterize lactococcin BZ in more detail using molecular technology, and to determine its suitability for use in the food industry.

Acknowledgement

This study was supported by the Turkish Republic Prime Ministry, State Planning Organization (project no. 2002K120270).

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