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Isolation and identification of lactic acid bacteria from boza, and their microbial activity against several reporter strains

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Abstract: A total of 45 lactic acid bacteria were isolated from 10 different boza samples consumed in Turkey. The isolates were identified with respect to colonial, cellular morphology, and biochemical characters based on API CHL50 (Biomérieux) kit and also automated riboprinting was used to identify of the isolates. These isolates were tested for inhibitory activity against food-borne bacterial pathogens. Antimicrobial effects of these lactic acid bacteria were determined by the agar spot test and also agar diffusion method. As a result of the RiboPrinter® Microbial Characterization System (DuPont Qualicon) lactic acid bacteria isolates having antimicrobial activity (45 isolates) were identified as *Lactococcus lactis* subsp. *lactis* (2 isolates), *Leuconostoc citreum* (5 isolates), *Lactobacillus brevis* (4 isolates), *Lactobacillus plantarum* (24 isolates), *Lactobacillus paraplantarum* (1 isolate), *Enterococcus faecium* (1 isolate), *Lactobacillus graminis* (4 isolates), *Pediococcus species* (1 isolate), and *Lactobacillus paracasei* subsp. *paracasei* (3 isolates). In addition, the amount of lactic acid produced by lactic acid bacteria was minimum 0.16 mg/mL with Y-12 isolate and maximum 7.79 mg/mL with KB13 isolate, hydrogen peroxide minimum 0.69 µg/mL with Z-12 isolate and maximum 2.18 µg/mL with X-13 isolate, and proteolytic activity 0.04 mg/mL with Y-12 isolate and maximum 2.57 mg/mL with KB13 isolate.

Key words: Antimicrobial activity, API ZYM, boza, lactic acid bacteria, proteolytic activity, riboprinter

Bozadan izole edilen laktik asit bakterilerinin izolasyonu, identifikasyonu ve bazı test mikroorganizmalarına karşı mikrobiyal aktivitesi

Özet: Bu çalışmada Türkiye'deki boza örneklerinden toplam 45 adet laktik asit bakterisi izole edilmiştir. Bu izolatlar koloni yapıları, hücre morfolojileri ve API CHL50 (Biomérieux) kit sistemine dayalı olarak tanımlanmışlardır. Ayrıca otomatik RiboPrinter® Mikrobiyal Karakterizasyon Sistemi (DuPont Qualicon) de identifikasyon için kullanılmıştır. Bu izolatlar gıda kaynaklı bakteriyel patojenlere karşı inhibitor aktivitesi bakımından test edilmiştir. Laktik asit bakterilerinin antimikrobiyal etkisi agar spot test ve agar difüzyon metodu ile belirlenmiştir. Antimikrobiyal aktiviteye sahip izolatlar (45 izolat) RiboPrinter® Mikrobiyal Karakterizasyon Sistemi (DuPont Qualicon)'ne göre *Lactococcus lactis* subsp. *lactis* (2 izolat), *Leuconostoc citreum* (5 izolat), *Lactobacillus brevis* (4 izolat), *Lactobacillus plantarum* (24 izolat), *Lactobacillus paraplantarum* (1 izolat), *Enterococcus faecium* (1 izolat), *Lactobacillus graminis* (4 izolat), *Pediococcus species* (1 izolat) ve *Lactobacillus paracasei* subsp. *paracasei* (3 izolat) olarak tanımlanmışlardır. Ayrıca laktik asit bakterileri tarafından üretilen laktik asit miktarı Y-12 izolatı ile elde edilen minimum 0.16 mg/mL ve maksimum KB13 izolatı ile elde edilen 7.79 mg/mL'dir. Hidrojen peroksit miktarları Z12 izolatı ile elde edilen minimum 0.69 µg/mL ve maksimum X-13 izolatı ile elde edilen 2.18 µg/mL'dir. Proteolitik aktivite miktarları ise minimum Y-12 izolatı ile elde edilen 0.04 mg/mL ve maksimum KB13 izolatı ile elde edilen 2.57 mg/mL'dir.

Anahtar sözcükler: Antimikrobiyal aktivite, API ZYM, boza, laktik asit bakterileri, proteolitik aktivite, riboprinter

Introduction

Boza is a Turkish traditional drink based on cereals widely consumed by people of all ages in Turkey. It is also consumed in some areas in Bulgaria, Albania, and Romania (1). Boza is a viscous non-alcoholic beverage, produced from different cereals and slightly fermented. These kinds of product have many advantages. They are superior in digestibility and nutritive value compared to their unfermented counterparts. Fermentation improves organoleptic qualities of the products. Natural mixtures of yeast and lactic acid bacteria cause fermentation. Little information is available about its microflora (2,3). Boza has a mixed microflora of lactic acid bacteria and yeasts. The most common fermenting bacteria are *Pediococcus cerevisia, Leuconostoc paramesenteroides,* and *Lactobacillus plantarum* (3-5). Several studies conducted on boza and bacteriocin-producing lactic acid bacteria with possible probiotic properties have been described (4,5). Probiotic strains have to survive harsh conditions in the gastrointestinal tract and adhere to intestinal epithelial cells. Probiotic strains also have some defence mechanisms against pathogenic microorganisms and in case of some stress conditions, the number of lactic acid bacteria decreases and, therefore, pathogenic bacteria cause some clinical diseases (6-8).

Increasing consumer demand for natural and 'additive-free' products has led to greater interest in the application of natural inhibitory substances as food preservatives, which could replace or reduce the use of chemical additives (9). The antimicrobial compounds produced by lactic acid bacteria are natural preservatives as such and could be used as preparations for increasing the shelf-life and safety of minimally processed foods. Bacteriocins of lactic acid bacteria are biologically active proteins or protein complexes that act as bactericidal against gram-positive bacteria usually closely related to the producer strain.

In this work, the antagonistic activity of lactic acid strains isolated from boza was investigated and determined for their spectrum of activity against spoilage and food-borne pathogens. These isolates having antimicrobial activity were identified based on phenotypic and genotypic characteristics. In addition, their ability to produce lactic acids, hydrogen peroxide, proteolytic activities, and enzyme profiles was investigated.

Materials and methods

Bacterial strains, media, and cultivation conditions

Test bacteria were obtained from the USDA Agriculture Research Service, IL, USA and our laboratory culture collection. These are *L. monocytogenes* (our laboratory culture), *B. cereus* NRRL B-3711, *B. subtilis* NRRL NRS-744, *S. aureus* NRRL B-767, *Y. enterocolitica* (our laboratory culture), *E. faecalis* (our laboratory culture), *E. coli* NRRL B-3704, *P. vulgaris* NRRL B-123, *P. aeruginosa* NRRL B-23, *S. typhimurium* NRRL B-4420, *K. pneumoniae* (our laboratory culture), and *A. hydrophila* (our laboratory culture). Cultures were maintained as frozen stocks at –80 °C in 20% glycerol.

Isolation and identification of lactic acid bacteria from boza

Ten different boza samples purchased from different markets. Each 25 g boza sample was aseptically weighed and homogenized by adding 225 mL of physiological saline solution. Further decimal dilutions were prepared from this homogenized mixture. It was spread-plated onto Lactobacillus Agar acc. to DE MAN, ROGOSA and SHARPE (MRS) agar (Oxoid). The plates were placed in an anaerobic flask (Oxoid) in the presence of a gas-generating kit (Anaerobic System BR0038B, Oxoid) and incubated at 30 °C for 2 days. About 10-12 colonies of bacteria having different appearances were randomly subcultured from MRS agar plates and plating on MRS agar purified isolates. All isolates were examined for Gram reaction, production of catalase, and oxidase activity. Gram-positive and catalase- and oxidase-negative isolates were stored for further analyses.

Isolates were identified using the following tests: ammonia production from arginine, CO_2 production from glucose, and growth at different temperatures (4, 8, 10, 15 and 45 °C), growth at different pH values, and growth at different NaCl concentrations (10). Carbohydrate fermentation tests were carried out by using the API 50 CHL kit according to the manufacturer's instruction (Biomérieux, France).

Ribotyping was performed with a RiboPrinter® Microbial Characterization System (DuPont Qualicon) and the standard *Eco*RI DNA preparation kit as described in the manufacturer's operations and analytical guides. Pure culture samples were obtained from MRS plates incubated for 24-48 h at 30 °C by using appropriate colony picks. The microbial samples were subsequently analysed following the indications of the producer. The ribotype profiles of the isolates were compared with the reference DuPont identification database DUP2003. The identification of each isolate was obtained when the corresponding pattern matched one of the patterns of the DuPont Identification Library with a similarity ≥ 0.85. The isolates were automatically grouped in ribogroups by the RiboPrinter™ based on the similarity of the respective ribotype patterns.

All strains were stocked in 15% glycerol and stored at –80 °C. Working cultures were stored at 5 °C and transferred periodically.

Detection of antagonistic activity

Antagonistic activity screening was investigated against test bacteria by agar spot test and well diffusion assay as described by Schillinger and Lüche (10). Agar spot test experiments were conducted by spotting 10 mL of an overnight lactic acid bacterial culture onto the surface of a MRS agar plate and incubating at 30 °C until good growth was evident (24 h). These plates were then overlaid with 8 mL of soft agar (0.75% agar) seeded with 8 mL of a test bacteria culture (approximately 10^7 stationary-phase cells). After overnight incubation at 37 °C, the plates were examined for zones of inhibition in the test bacteria.

Antagonistic activity was estimated by an agar well diffusion assay. Molten agar (48 °C) was first seeded with the indicator organism (200 mL of overnight culture per 25 mL of agar, 2×10^6 cells/mL). The inoculated medium was rapidly dispensed in sterile petri dishes and, after solidification, dried for 30 min under a laminar flow hood. Wells of uniform diameter (6 mm) were cork bored in the agar and sealed with 15 mL of tempered soft agar. The extracts of lactic cultures obtained from overnight cultures were applied to 4 different processes. (I): centrifuged at 2500 × *g* for 5 min. (II): neutralized by addition of 5 N NaOH to exclude the effects of organic acids. (III): treated with catalase (in 10 mM phosphate buffer, pH 7.0 to a final concentration of 1 mg/mL) for 30 min at 25 °C to exclude inhibitory effects of hydrogen peroxide and controls included phosphate buffer with and without catalase. (IV): added Proteinase K enzyme in 0.5 μg/mL final concentration and incubated for 4 h at 30 °C together with and without samples which including enzyme.

Each of the solutions was sterilized with Millipore membrane filter (0.22 mm pore diameter) before loading to wells. Aliquots (80 mL) of these solutions were dispensed in the wells, and plates were incubated overnight at 30 °C. Antagonistic activity was expressed as the area of inhibition surrounding each agar well. The antagonistic activities of samples were determined for each isolate by the well diffusion assay for persistence of the inhibition zone.

In addition, 80 mL of filter sterilized lactic acid solutions adjusted to the same pH as (I) was spotted as control tests. The plates were incubated 14-16 h of 37 °C and checked for inhibition zones.

pH and lactic acid determination

The pH of the each supernatant obtained by centrifuged after incubation was determined by using pH meter (Corning pH/ion analyser 350). Acid production was assessed in sterilized skim milk at the appropriate temperature having been inoculated in the rate of 2 mL/100 mL with active strains of lactic acid bacteria. Measurements of titratable acidity, expressed as grams lactic acid/mL, were made at 42 h (11).

Proteolytic activity

Protease activities were assessed in sterilized skim milk (SSM; pH 6.50) (Oxoid) at 37 °C for lactobacilli and 30 °C for lactococci using standardized inoculate. The cultures were revitalized in MRS broth, harvested at 4000 × *g* for 10 min, washed twice with sterile distilled water, and re-suspended in 8.5 g/L NaCl (w/v). Aliquots of 0.5 mL of the cell suspensions were inoculated in triplicate into 9.5 mL of SSM; these gave optical densities of 1.25 at 620 nm after a 1:10 dilution and they contained about 10^9 cfu/mL. After 24-48 h of incubation, 1.5-mL aliquots were removed from each SSM culture and used for proteolysis assessment. The proteolytic activities of cultures were determined spectrophotometrically. This method

detects the free tyrosine and tryptophan liberated in the reaction mixture. In the present study proteolytic activity was measured in triplicate (12). The results were calculated from a calibration curve obtained from dilution of tyrosine in distilled water and were expressed as mg/mL tyrosine.

Hydrogen peroxide detection

Hydrogen peroxide was determined spectrophotometrically according to Patrick and Wagner (13). Measurements were obtained after 24 h incubation period in skim milk, and the production was monitored at OD_{350}. H_2O_2 was quantified by using a H_2O_2 standard curve, performed with concentrations ranging from 1 µg/mL to 10 µg/mL.

Enzyme profiles

Enzyme activities were determined with the API ZYM kit (Biomérieux) and the manufacturer's instructions were followed throughout. The API ZYM kit is a standardized semiquantitative micromethod able to detect 19 different types of enzymes.

Results and discussion

The 45 isolates obtained from boza samples were identified according to phenotypic and genotypic characters. According to carbohydrate fermentation reactions and physiological and morphological tests, they were members of the species *Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus graminis, Leuconostoc citreum, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum*, and *Enterococcus faecium.* Similarities and differences were observed when these results were compared with those obtained by Hancıoğlu and Karapınar (3) using Turkish boza. They reported *Leuconostoc paramesenteroides*, which was predominant (26%), *Lactobacillus sanfrancisco*, *Lb. coryniformis*, *Lb. fermentum*, *Lb. confusus*, *Ln. mesenteroides* subsp. *mesenteroides*, *Ln. mesenteroides* subsp. *dextranicum*, and *Ln. oenos*. In our work *Lb. plantarum* was found to be the most common species in our isolates. This strain was also detected as predominant in Nigerian ogi, a spontaneously fermented maize product (14) and Bulgarian cereal-based fermented beverage boza (1). In contrast with previous reports (1,4,5,15), *L. acidophilus*, *L. coprophilus*, *L. coryniformis*, *L. sanfrancisco*, *L. lactis* subsp. *lactis*, *L. mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. raffinolactis*, *P. pentosaceus*, and *L. oeni* were not isolated.

Additionally, RiboPrinter® Microbial Characterization System (DuPont Qualicon) results are shown in the Figure. *Leuconostoc citreum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus paraplantarum, Lactococcus lactis* subsp. *lactis, Lactobacillus paracasei* subsp. *paracasei, Enterococcus faecium, and Pediococcus* sp. were detected with RiboPrinter® system. As seen in the Figure, most of the isolates belong to *Lactobacillus plantarum* (Y-3, Y-7, Y-11, Y-12, X-1, X-3, X-4, X-8, X-9, X-10, X-13, X-14, X-11, X-15, Q-1, Q-5, Q-6, Q-7, Q-8, Z-2, Z-4, Z-7, Z-9, KB13). The smallest group was *Enterococcus faecium* and *Pediococcus species* represented with one member KB4 and An 1.4A respectively. Although these genotypic identification results were approximately found to be compatible with those of API CHL50 results, RiboPrinter has more reliable identification results than API CHL50.

Ribotyping with the Riboprinter is an automated process that requires little preparation and has a rapid turnaround time. Ribotyping is a useful, consistent method that allows easy comparisons and precise recognitions of the isolates. The reference DuPont identification database enlists all the profiles of the reference strains for each single species and is used for the comparative identification of the isolates. Conversely, the riboprofiles of all analysed isolates that have been studied with the customer's RiboPrinter unit and the ribogroup library are regularly updated as new isolates are examined, progressively extending the crucial information stored in the resident database. When the system compares a sample pattern to the variety of strains within the database it is looking for matches that have a 0.85 or greater similarity index (based on pattern homology, which factors in band positioning, band number, and band intensity) in order to assign a genus species match. Therefore, an exact strain match is not the objective of the identification database screen, but rather a similar match (species level). Any match at or above the 0.85 similarity index is considered a high confidence automated ID and thus



KB2, *Leuconostoc citreum*
KB12 (1), *Lactobacillus brevis*
KB8 (1), *Lactobacillus brevis*
KB8 (2), *Lactobacillus brevis*
Y7, KB13, *Lactobacillus plantarum*
Y8, *Lactobacillus paraplantarum*
Y11, Z2, X1, X3, *Lactobacillus plantarum*
X4, X13, Y12, *Lactobacillus plantarum*
Z10, Z12, *Lactococcus lactis* subsp. *lactis*
KB4, *Enterococcus faecium*
Z4, Z7, Z9, X8, X9, X14, Y3, *Lactobacillus plantarum*
Q5, *Lactobacillus plantarum*
KB3, *Lactobacillus brevis*
Q1, Q6, Q7, Q8, *Lactobacillus plantarum*
X10, X11, X15, *Lactobacillus plantarum*
An 4.1, An 2.6, *Leuconostoc citreum*
X7, KB9, Q2, *Lactobacillus graminis*
An 30.5.2A, *Lactobacillus paracasei* subsp. *paracasei*
An 1.4A, *Pediococcus* species
An Mrs 4.1, An Mrs 2.6, *Leucocnostoc citreum*
30.5.2, *Lactobacillus paracasei* subsp. *paracasei*
An 30.1.2, *Lactobacillus graminis*
30.5.3B, *Lactobacillus paracasei* subsp. *paracasei*
Lactobacillus plantarum DUP 16541
E.coli DUP 3039
Lactobacillus paracasei subsp. *paracasei* DUP 18181
Lactobacillus brevis DUP 14534
Enterococcus faecium DUP 16233
Leuconostoc citreum DUP 13515
Lactobacillus lactis subsp. *lactis* DUP 12759
Lactobacillus species DUP 13458

Figure. Ribotyping profiles of isolates and some standards were shown with DUP numbers (first layer represents riboprinter marker).

a genus species name is automatically assigned. If no matching patterns (≥0.85 similarity) are found within the ID database, the system will bring up the most similar patterns (below this 0.85 similarity threshold) within the ID database so that the user may manually ID these patterns to the genus or species level. All IDs that are assigned as a result of matches that is below this 0.85 similarity threshold are indicated by the presence of {brackets}. This functionality was built into the software to allow the user the ability to identify strains that show a bit more variation than those strains within the supplied DuPont database but that still show certain conserved bands, which are demonstrated across a species or a genus.

Lactic acid bacteria used in the present study screened for antimicrobial activity in the deferred agar spot assay and well diffusion assay. A total of 45 lactic acid bacteria were isolated from the boza samples and all of these isolates showed antimicrobial activity against one or more of test microorganisms under the agar spot test (not shown). As can be seen in Table 1, under the well diffusion test, there are bacteria strains within the lactic acid bacteria isolates that exhibit significant inhibitory activity against test bacteria. However, the well diffusion tests indicated that 33 isolates had inhibitory activity against tested one or more of test bacteria.

Previously conducted studies have also shown that different strains of *L. plantarum* possess inhibitory activity against *S. aureus* (16). The well diffusion test conducted by Kıvanç (17) showed that *L. plantarum* had no inhibitory effect on *S. aureus*.

Z-7, Z-10, and Z-12 isolates had inhibitor activity against all bacteria with agar spot test, but no activity against *S. aureus* with well diffusion test (Table 1). The findings of this study are consistent with the results of Schillingger and Lücke (10) in which some of the LAB isolates show antimicrobial activity on spot agar antagonism test, but none on well diffusion test.

The antimicrobial activity of cell-free filtrate from lactic cultures was generally greater against the gram positive than the gram negative organisms tested. In deferred antagonism on agar, producer colonies may either generate more bacteriocin or continuously excrete bacteriocin so as to replenish the inhibitor over the entire course of the antagonism test.

We obtained 33 LAB isolates from boza having antimicrobial activity against most of the tested bacteria *L. monocytogenes*, *B. cereus*, *B. subtilis*, *S. aureus*, *Y. Enterocolitica, E. faecalis*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, *S. typhimurium*, *K. pneumoniae*, and *A. hydrophila*. Although inhibitory activity was detected for all isolates in a well diffusion assay, only 8 isolates, namely *Lactobacillus plantarum* Y-11, Y-12, X-8, X-9, X-13, X-15, Q-5 and *Lactococcus lactis* Z-12, maintained its activity in the neutralized supernatant against all of the tested bacteria except *S. aureus*. Of these, 3 isolates, *Lactobacillus plantarum* Y-11, X-13, and O-5, have antimicrobial effect for *S. aureus*. The inhibitory activity of the other 25 isolates most probably was due to organic acids. Similar antimicrobial results to those reported here, were obtained by Aslim et al. (18) when testing 19 cell-free supernatants of LAB isolated from Turkish dairy products. Among the *Lactobacillus* spp. cultures assayed, it was observed that inhibitory activity against strains of *L. monocytogenes*, *S. aureus*, and *Y. enterocolitica* was lost in 15 cell-free supernatants, including *L. plantarum* culture-free supernatants, when the pH was neutralized. In another study, performed by Gómez et al. (19), the inhibitory spectrum of lactic acid bacteria strains isolated from lettuce juice was detected. None of the tested strains displayed inhibitory activity against *E. cloacae*, *A. hydrophila*, and *E. coli* and the inhibitory spectrum varied among the isolates. Some of the isolates have inhibitory activity against *Lactococcus lactis*, *Listeria monocytogenes*, *Stapyhlococcus aureus*, *Erwinia carotovora*, *Salmonella thyphimurium*, and *Citrobacter freundii* by using the agar cell-free supernatant diffusion test.

Our LAB isolates exhibited weak inhibitory activity against *S. typhimurium*. Among the cultures of LAB assayed, it was observed that neutralized culture supernatants of 7 *L. plantarum* and *Lactococcus lactis* isolates exhibited inhibitory activity against *S. typhimurium*. Similarly, Makras et al. (20) demonstrated that the inhibitory performances of some lactobacilli against *Salmonella enterica* serovar *typhimurium* could not be attributed to lactic acid alone but also to some as yet uncharacterized substances effective at pH values below 4.5 in the presence of lactic acid.

Table 1. Antagonistic activity of lactic acid bacteria against tested bacteria by the well diffusion assay (mm).

Isolates	*L. monocytogenes*	*B. cereus*	*B. subtilis*	*S. aureus*	*Y. enterocolitica*	*E. faecalis*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. typhimurium*	*K. pneumoniae*	*A. hydrophila*
Leuconostoc citreum												
KB2	++	+++	++	++	++	++	++	++	++	++	++	++
Lactobacillus brevis												
KB12 (1)	+++	+++	++	+++	+++	++	++	+++	+	+++	++	+
Lactobacillus plantarum												
Y-3	+++	-	+	-	++	++	+	++	++	+	-	+
Y-7	+++	++	+	+	+	++	++	++	++	+	-	+
Y-11	+++	++	++	++	++	++	+	++	++	+	++	++
Y-12	+++	-	+	-	++	++	++	++	+	+	-	+
Z-2	+++	-	-	+	+	++	++	+	+	+	-	+
Z-4	+++	-	++	-	++	++	++	++	++	++	+	++
Z-9	+++	+	-	-	+	++	++	++	++	+	+	+
X-1	++	-	+	-	+	++	++	++	+	+	-	+
X-3	+++	+	++	++	+	++	++	++	++	+	+	+
X-4	+++	+	-	++	+	++	++	++	++	+	++	-
X-8	+++	++	+	-	++	++	++	++	++	++	++	+
X-9	+++	+	++	+	++	++	++	++	++	+	++	-
X-10	++	+	++	+	+	++	++	++	+	+	+	+
X-11	++	-	-	+	+	++	++	++	-	+	-	-
X-13	+++	++	+	++	++	++	+	++	++	+	++	++
X-14	+++	+	++	-	+	++	+	++	++	++	-	++
X-15	++	+	-	-	+	++	++	++	+	+	+	+
KB13	++	+	+	++	+	++	++	++	+	+	+	+
Q-1	+++	+	-	++	++	++	++	++	++	+	++	+
Q-5	+++	+	++	-	++	++	+	++	++	+	+	-
Q-6	+	+	+	++	+	+	++	+	++	+	-	-
Q-7	+++	+	+	-	-	++	++	++	++	+	+	-
Q-8	+++	+	-	++	+	++	++	++	++	+	++	++
Z-7	+++	+	++	-	+	++	++	++	++	+	++	+
Lactococcus lactis												
Z-10	++	++	-	-	+	++	+	++	+	+	-	+
Z-12	+++	++	++	-	++	++	++	++	++	++	++	+
Lactobacillus graminis												
X-7	+++	++	++	-	+	++	+	++	++	+	+	+
KB9	+++	+	-	-	+	-	+	+	+	+	+	+
Q-2	+++	+	+	-	++	+	++	++	++	+	++	-
Enterococcus faecium												
KB4	++	+	++	-	++	+	++	++	++	+	++	+
Lactobacillus paraplantarum												
Y-8	+++	++	++	+	++	++	++	++	++	+	++	-

-: No effect, +: 6-11 mm, ++: 11.1-16, +++: 16.1-22 mm.

As indicated in Table 2, Y-11 and X-13 isolates have antimicrobial activity due to hydrogen peroxide produced by them since catalase addition to supernatant eliminated the antagonistic activity. Hydrogen peroxide is produced by a large number of LAB lacking the enzyme catalase in particular by *Lactobacillus* spp. and inhibits other microorganisms such as *Staphylococcus aureus* and *Listeria* spp. The potential of H_2O_2 produced by LAB for food preservation may be limited by the oxidizing nature of the molecule, and free radicals produced may have profound effects on the sensory quality, causing rancidity of fats and oils and discoloration reactions (21). The production of acids and hydrogen peroxide by LAB and inhibitory properties have been reported by several authors (22-24).

The antibacterial effect of Y-3, Z-4, Z-9, X-1, X-10, X-14, KB13, Q1, Q7, KB9, KB2, and KB12 isolates is caused by acidity produced by these isolates. Y-12, X-8, X-9, X-15, Q-5, and Z-12 isolates have antagonistic activity because of a proteinaceous substance. Therefore, the antibacterial activity is lost after treatment with proteinase K. The substances produced by Y-7, Z-2, X-3, X-4, X-11, Q-6, Q-8, Z-7, Z-10, X-7, Q-2, KB4, and Y-8 isolates were neither hydrogen peroxide nor organic acids. The inhibitory activity was not affected by catalase and was retained in neutralized supernatant fluid. Thus, it was concluded that the inhibitory effects were due to the presence of bacteriocin-like metabolites. Those inhibitory effects of the other lactic acid bacteria isolates were due to the presence of other metabolites. The antimicrobial activity was not lost or was stable after treatment with proteinase K. Many of these agents are bacteriocins with a proteinaceous active moiety, while others are non-protein agents (25,26).

L. plantarum Y-11, Y-12, X-8, X-9, X-15, Q-5 and *L. lactis* Z-12 strains from boza were found to have a broad spectrum of inhibitory activity against different bacterial species. Similar results were obtained by other authors (10,27) working with different LAB genera. Many authors have also reported that bacteriocins or bacteriocin-like substances are produced by strains of lactococcus and lactobacillus (4-6,9). Enterobacteriaceae were also inhibited by our LAB isolates. These results agreed with those obtained by Vignolo et al. (28) and Carrasco et al. (27). Vignolo et al. (28) detected a wide inhibitory spectrum of a *L. plantarum* culture supernatant, which also includes gram-negatives. Low molecular mass compounds produced by LAB are active against gram-positive and gram-negative bacteria. Many of these agents are proteinaceous compounds while others are primary metabolites or other non-identified by-products (26). We observed a marked difference between the inhibitory spectrum of crude extract and its proteinaceous fraction (crude bacteriocin-like compounds). It is then important to distinguish between overall culture inhibitory capacity and bacteriocin inhibitory spectrum.

Isolates showed no activities for the enzymes alkaline phosphatase, esterase, esterase lipase, lipase, trypsin, α-chymotrpsin, acid phosphatase, β-galactosidase, β-glucuronidase, α-fucosidase, and α-mannosidase when cultured on MRS agar. All the isolates of LAB displayed activities of leucine arylamidase, valine arylamidase, β- glucosidase, and β-galactosidase. Isolates showed activities for the enzyme α-glucosidase, except for *L. plantarum* X-8, Z-9, and Q-6. These strains are well known to hydrolyse various β-glucosides of vegetable origin (29). These isolates showed a possibility of digesting polysaccharides including damaged starch, dextrin, etc., and thus would impact on the physical properties of starch.

The production of H_2O_2 by lactic acid bacteria is presented in Table 3. The amount of H_2O_2 produced by lactic acid bacteria ranged from 0.55 mg/mL (KB13) to 2.18 mg/mL (X-13). Hydrogen peroxide is one of the primary metabolites that may be produced by lactic acid bacteria and which may contribute to their antagonistic action (30). Raccah and Baker (31) examined the production of H_2O_2 in vitro by 2 commercial strains of meat starter cultures, *Pediococcus cerevisiae* and *Lactobacillus plantarum*, and found a maximal level of 0.55 mg $H_2O_2/10^9$ cells, which could not be considered high enough for antimicrobial activity.

The amount of lactic acid produced by lactic acid bacteria was also shown in Table 3. The ratio of lactic acid for acetic acid varied from strain to strain. The amount of lactic acid produced by these bacteria ranged from 0.16 mg/mL to 7.79 mg/mL.

Table 2. Antagonistic activity of supernatant with different treatments (neutralized supernatant, treated with catalase and protease K) against tested bacteria by the well diffusion test (mm).

Treatment	*L. monocytogenes*	*B. cereus*	*B. subtilis*	*S. aureus*	*Y. enterocolitica*	*E. faecalis*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. typhimurium*	*K. pneumoniae*	*A. hydrophila*
***Lactobacillus plantarum* Y-11**												
Neutralized	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Protease K	+	+	+	+	+	+	+	+	+	+	+	+
***Lactobacillus plantarum* Y-12**												
Neutralized	+	-	+	-	+	+	+	+	+	+	-	+
Catalase	+	-	+	-	+	+	+	+	+	+	-	+
Protease K	-	-	-	-	-	-	-	-	-	-	-	-
***Lactobacillus plantarum* X-8**												
Neutralized	+	+	+	-	+	+	+	+	+	+	+	+
Catalase	+	+	-	-	+	+	+	+	+	+	+	-
Protease K	-	-	-	-	-	-	-	-	-	-	-	-
***Lactobacillus plantarum* X-9**												
Neutralized	+	+	+	-	+	+	+	+	+	+	+	+
Catalase	+	+	+	-	+	+	+	+	+	+	+	+
Protease K	-	-	-	-	-	-	-	-	-	-	-	-
***Lactobacillus plantarum* X-13**												
Neutralized	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Proteinase K	+	+	+	+	+	+	+	+	+	+	+	+
***Lactobacillus plantarum* X-15**												
Neutralized	+	+	-	-	+	+	+	+	+	+	+	+
Catalase	+	+	-	-	+	+	+	+	+	+	+	+
Proteinase K	-	-	-	-	-	-	-	-	-	-	-	-
***Lactobacillus plantarum* Q-5**												
Neutralized	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Proteinase K	-	-	-	-	-	-	-	-	-	-	-	-
***Lactococcus lactis* Z-12**												
Neutralized	+	+	+	-	+	+	+	+	+	+	+	+
Catalase	+	+	+	-	+	+	+	+	+	+	+	+
Proteinase K	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Amount of lactic acid, proteolytic activity, and hydrogen peroxide produced by lactic acid bacteria showing antimicrobial effect by well diffusion assay.

Isolates	pH	Lactic Acid (mg/mL)	Proteolytic Activity (mg/mL)	H_2O_2 (mg/mL)
Leuconostoc citreum				
KB2	4.0	1.01	1.02	0.89
Lactobacillus brevis				
KB12 (1)	4.5	2.69	1.76	0.94
Lactobacillus plantarum				
Y-3	4.6	0.80	0.70	1.63
Y-7	4.8	0.91	0.66	1.18
Y-11	5.1	0.61	0.51	2.03
Y-12	5.9	0.16	0.04	1.35
Z-2	5.6	0.48	0.09	1.49
Z-4	4.6	0.88	0.71	1.59
Z-7	5.2	0.89	0.76	0.75
Z-9	4.2	0.94	0.76	1.04
X-1	4.9	0.73	0.63	1.92
X-3	4.8	0.96	0.77	1.29
X-4	5.2	0.78	0.69	1.78
X-8	5.3	0.57	0.11	1.58
X-9	5.4	0.39	0.06	1.46
X-10	5.2	0.43	0.26	1.02
X-11	5.1	1.15	0.54	1.21
X-13	5.3	0.58	0.46	2.18
X-14	4.6	0.84	0.69	1.71
X-15	6.0	0.82	0.66	0.73
KB13	4.0	7.79	2.50	0.55
Q-1	4.4	0.76	0.64	1.83
Q-5	4.9	1.21	0.63	1.18
Q-6	4.9	0.63	0.49	1.99
Q-7	4.6	0.89	0.72	1.86
Q-8	4.7	0.90	0.75	1.32
Lactobacillus graminis				
X-7	5.9	1.07	0.47	0.97
KB9	5.4	0.72	0.58	0.51
Q-2	5.6	1.06	0.52	0.91
Enterococcus faecium				
KB4	5.1	0.47	0.67	0.90
Lactococcus lactis				
Z-10	5.8	0.95	0.83	0.88
Z-12	5.3	0.86	0.69	0.69
Lactobacillus paraplantarum				
Y-8	4.3	0.82	0.87	1.76

In this study, pH ranges of 4.0 to 6.0 for *L. plantarum*, 5.6 and 5.9 for *L. graminis,* and 5.3 and 5.8 for *L. lactis* were observed. pH decrease during the initial steps of boza preparation has crucial importance in boza manufacture. Lactic acid bacteria strains examined in this study were found to be rather moderate acid producers. Lactic acid bacteria showed inhibitory activity related to organic acids or hydrogen peroxide and bacterial mode of action lyses of sensitive cell. There is evidence of the ability of some antimicrobial substances produced by lactic acid bacteria to limit the growth of many pathogenic organisms (32). The increase in the production of lactic acid with time has been attributed to lower pH, which permits the growth of LAB to the detriment of the competing organism. Lactic acid is known to inhibit the growth of unrelated organisms in a mixed culture. The effect is due to the undissociated form of the acids, which can penetrate the membrane and liberate hydrogen ions in the neutral cytoplasm, thus leading to inhibition of vital cell functions. The undissociated acid can cause the collapse of the electrochemical proton gradient of susceptible bacteria, leading to bacteriostasis and eventual death. The antimicrobial effect was explained by the combined effect of acids, compound sensitive to proteolytic enzymes and other compounds with antimicrobial activity with the acid production being the most important factor (33).

The proteolytic activities of 33 isolates of LAB are presented in Table 3. The amount of tyrosine released by these bacteria ranged from 0.04 mg/mL for *L. plantarum* Y12 to 2.57 mg/mL for *L. plantarum* KB13. According to the results obtained in the present study, lactic acid bacteria strains exhibited low proteolytic activity. The proteolytic ability of lactic acid bacteria depends on species and strain. The highest proteolytic activity was observed in *L. helveticus*, *L. bulgaricus*, and *L. acidophilus* of the thermos-bacteria group, followed by *L. casei* of the streptobacterial group and then the lactic streptococci of the genus *Lactococcus*. The proteolytic enzymes of *L. bulgaricus* degrade casein with the liberation of low molecular weight peptides and amino acids. The amino acids arising from this proteolytic activity have been identified as specific growth stimulants for *S. thermophilus* (12). Rajogopal and Sandine (12) reported 61.0 mg/mL in *L. bulgaricus*. Although the lactic acid bacteria are weakly proteolytic compared with other group of bacteria such as *Bacillus, Proteus*, *Pseudomonas* and *Coliforms* (34) they do cause a significant degree of proteolysis in many fermented dairy products (35). Lactic acid bacteria showed antimicrobial activity towards many of the strains tested. The 6 strains (*Lactobacillus plantarum* Y-12, X-8, X-9, X-15, Q-5, and *Lactococcus lactis* Z-12) that produced antibacterial substances other than acid and H_2O_2 were also evaluated for their potential use as biopreservative agent for boza. The range of applications of antimicrobial metabolites of lactic acid bacteria will certainly grow in future because of their wide spectrum of activity. By using these lactic acid bacteria as starter cultures, controlled fermentation studies can be carried out. These lactic acid bacteria in the production of boza enhance the taste and aroma, the desired texture and shelf life. In addition, it preserves the hygienic quality, due to pH reduction, lactic acid, H_2O_2, and antagonistic activity against food-borne pathogens and microorganisms that can contaminate the boza.

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