Bacteriocin producing lactic acid bacteria isolated from Boza, a traditional fermented beverage from Balkan Peninsula – from isolation to application

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Boza’s origin dates back to the ancient populations that lived in Anatolia and Mesopotamia. The preparation formula was taken by the Ottomans and spread over the countries they conquered. Boza is a low-alcohol beverage produced from the fermentation of barley, oats, millet, maize, wheat or rice. The cooked cereal is strained to remove most of the solids, sugar is added to taste and inoculated with a starter culture, either from yogurt or sourdough. The sludge is fermented at 30°C for 24 hours, cooled and kept refrigerated for 3-5 days. Although a number of Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Oenococcus and Weissella spp. have been isolated from boza, only a few papers addressed the selection of starter cultures. LAB (lactic acid bacteria), and presumably yeast, can produce a number of hydrosoluble vitamins and thus increases the nutritional value of the product. Many of the LAB isolated from boza produce antimicrobial compounds, including bacteriocins, increasing the shelf life of the product and possibly demonstrating health benefits.

In this work the microbiological properties of boza, isolation and characterization of the bacteriocinogenic LAB is discussed and highlighting this with potential probiotic and health promoting properties.

Keywords: bacteriocin; probiotic; fermented cereals; boza

1. History of Boza

Boza’s origin dates back to the ancient populations that lived in Anatolia and Mesopotamia. The preparation formula was taken by the Ottomans and spread over the countries they conquered. The Greek historian Xenophon records that boza was made in eastern Anatolia in 401 BC and stored in clay jars that were buried beneath the ground. The local specialty remained confined to the region until the arrival of the Turks, who took this nourishing drink and spread it far and wide under the name boza, a word derived from the Persian word “buze”, meaning millet. Boza enjoyed its golden age under the Ottomans, and the preparation of boza became one of the principal trade items in towns and cities from the early Ottoman period [1].

Beer is supposed to have originated from boza, a drink considered as old as 8000-9000 years. Although the alcohol and acid content of boza was not known at that time, boza was described in clay tablets as a stimulant and also as a medicine. Its consumption was initially widespread in the Islamic countries, but production was prohibited in the 18th century because of its high alcohol content. It is still produced and consumed widely in Anatolia, South Russia, East European countries, Middle East, and Northern Persia [2].

Boza is a low-alcohol beverage produced from the fermentation of barley, oats, millet, maize, wheat or rice. Cooked cereal is strained to remove most of the solids then sugar is added to taste and inoculated with a starter culture, either consisting of yogurt or sourdough. The sludge is fermented at 30°C for 24 hours, cooled and kept refrigerated for 3-5 days [3, 4]. Boza contains about 0.50 - 1.61% protein, 12.3% carbohydrate, and 75 – 85% moisture [4, 5]. In general the pH of the boza samples ranges from 3.16 to 4.02 [5] and the average alcohol content is 0.13% (w/v) [6].

Besides improving organoleptic quality and digestibility by fermentation, it is a nutritious food because of its lactic acid, fat, protein, carbohydrate, fiber, and vitamin contents, and thus a valuable fermented food that contributes to human nutrition [7]. Boza can be classified as either sweet or sour depending on its acid content. The chemical properties of boza during the fermentation and storage time are significantly affected by the raw material (p < 0.01) [8]. The compositional differences of boza samples may result from the use of different cereals as the raw material and their amounts in the recipe [9, 10]. The raw material affects the amount and quality of carbohydrates available as primary fermentation substrates, nitrogen sources, and growth factors for microbial activity.

Total titratable acidity in terms of lactic acid was found to be lowest in millet with 0.32 ± 0.04% and highest in wheat boza (0.61 ± 0.07%) due to the probable high fermentable carbohydrate content of wheat compared to other raw materials [8]. The pH varied between 3.43 ± 0.08 and 3.86 ± 0.17 [8]. The acidity of the samples increased during storage (being highest at 192 hours with 0.68 ± 0.06%) in accordance with a decrease in the pH. Furthermore, the alcohol content was lower in wheat boza (0.46 ± 0.04%) and showed fluctuations during storage depending on microbial and
enzymatic activities [8]. The acidity and alcohol content depended mainly on the fermentation period; it was demonstrated that with longer fermentation periods, the acidity increases as does the concentration of alcohol [8].

The history of boza and similar beverages dates back to 6000 - 7000 years B.C. - nearly 9000 years of background history. However, the original boza is different from what is produced nowadays, which has high alcohol content (up to 7% w/v). In Egypt, a traditional beverage called bouza is still produced. In the South African region, boza production has become an important section of the beverage industry. Boza and similar beverages are produced with different recipes and methods in various countries. Boza is called boussa or bouza in Nigeria and some other African countries, and it is similar to beer due to its high alcohol content [11]. In Bulgaria, boza is produced either plain or with cocoa, either in winter or summer [12]. In the Balkan region of Europe, this beverage is also called boza. In Turkey, boza is mostly produced and consumed in winter but because of the refreshing cooling effect of lactic acid, it can also be consumed in summer; however high temperatures in the summer season can lead to the growth of yeast and acetic acid bacteria [2]. Thus, organoleptic qualities of the product can change rapidly causing a dramatic decrease in the shelf-life [2].

The steps for boza production can be summarized as (i) preparation of the raw materials, (ii) boiling, (iii) cooling and straining, (iv) sugar addition, and (v) fermentation. Boza (2 - 3%) from a previous batch should is usually used as a starter culture. The mixture is left to ferment in wooden barrels. The ratio of the starter culture depends on the season and temperature at which it was produced. Inoculated mixture is incubated at 15 - 25°C for nearly 24 hr before it is ready for use [2].

Two different types of fermentation occur simultaneously during boza fermentation. The first being the alcoholic fermentation that produces carbon dioxide bubbles and increases the volume, whereas the second, lactic acid fermentation, produces lactic acid and gives the acidic character to boza. Due to the increase in volume during fermentation, the wooden barrels should not be fully filled. After being produced, boza should be consumed within a couple of days to prevent excess sour taste. In practice, fermentation is retarded by cold storage to extend the shelf-life of boza. In the first boza production of the season, sourdough or yoghurt is used as a starter culture since fresh boza is not available. When using sourdough, which is less viscous, a more acidic product is obtained when compared to the product that is inoculated with a previous boza batch. If yoghurt is preferred, a viscous but more acidic product is obtained and the characteristic yoghurt taste is easily detected [2].

2. Starter culture & microbiology

Countries of the Balkan region in Europe are famous for their production of food and beverages fermented with lactic acid bacteria. Boza is one such traditional drink, however, only a few reports have been published on the microbial composition of Boza and most of the lactic acid bacteria that have been isolated belong to the genera Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Enterococcus, Oenococcus and Weissella [1-4, 13-19]. Only one study addressed the selection of boza starter cultures [4]. LAB, and presumably yeast, produce a number of vitamins [20] and the adequate selection of starter cultures can increase the nutritional value of fermented products [21].

Several studies have been published on the isolation and identification of LAB and yeast in boza, but to our knowledge only study from Botes et al. [15], Todorov and Dicks [18] and Todorov [1] uses biomolecular approaches to identify these microorganisms. In this studies, the concentration of lactic acid bacteria isolated from three boza samples ranged from 9 x 10^6 to 5 x 10^7 CFU/ml. Carbohydrate fermentation reactions and PCR with species-specific primers classified the isolates as being Lactobacillus paracasei subsp. paracasei, Lactococcus plantarum, Lactobacillus brevis, Lactobacillus rhamnosus, Lactobacillus fermentum, Leuconostoc lactis and Enterococcus faecium.

Only a few papers reported the isolation of yeasts and molds from boza. Most of the yeasts were identified as strains of Candida glabrata, Candida tropicalis, Geotrichum candidum, Geotrichum penicillatum, Saccharomyces carlsbergensis, Saccharomyces cerevisiae and Saccharomyces uvarum [2, 13]. The latter identifications were based on morphological, physiological and biochemical characteristics.

Boza harbours a diverse population of lactic acid bacteria that include strains of Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus corynformis, Lactobacillus plantarum, Lactococcus lactis subsp. lactis, Leuconostoc mesenteroides, Leuconostoc mesenteroides subsp. dextranicum, Leuconostoc raffinolactis, Leuconostoc lactis, Enterococcus faecium, Pediococcus pentosaceus, Leuconostoc oenos (reclassified to Oenococcus oeni), Weissella confusa and Weissella paramesenteroides [1, 2, 13, 18, 19].

Yeasts were isolated from two batches of the boza samples, with cell numbers ranging from 1.3 x 10^7 to 1.8 x 10^7 CFU/mL. Results obtained from sequencing of the D1/D2 rDNA region identified the yeasts as Candida diversa, Candida inconspicua, Candida pararugosa, Issatchenka orientalis, Pichia fermentans, Pichia guilliermondii, Pichia norvegensis, Rhodotorula mucilaginosa and Torulaspora delbrueckii. Saccharomyces cerevisiae, commonly associated with fermented beverages, has not been detected in any of the boza samples [15]. The absence of Saccharomyces cerevisiae suggests that the species was either not present in the inoculum (at least not in high numbers), or that it was repressed by the lactic acid bacteria and other yeasts towards the end of fermentation. Candida inconspicua has been
isolated from human sputum and tongue and is an opportunistic pathogen. *Rhodotorula mucilaginosa* is also an opportunistic pathogen implicated in fungaemia, endocarditis and meningitis. *Pichia norvegensis* has been associated with septicaemia in humans [22, 23]. The presence of potential pathogens emphasizes the importance of developing starter cultures with GRAS status for the commercial production of boza and the necessity of introducing novel methods to prevent their propagation.

### 3. Bacteriocin produced by Lactic acid bacteria isolated from boza

Lactic acid bacteria are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides [24]. Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms [24]. Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis [24, 25]. In recent studies, specific environmental conditions, including those found in food, have been studied to determine their effect on the production of bacteriocins [20]. Bacteriocin production dramatically changes upon altering of environmental conditions and optimum production may require a specific combination of parameters [26]. Little is known about the interactions these factors have on the production of bacteriocins, especially in a complex food environment.

Kabadjova et al. [14] and Ivanova et al. [27] first reported the isolated of bacteriocin producing strains from boza (produced in Sofia, Bulgaria). Of the 80 isolated strains of lactic acid bacteria, a group of 33 showed antibacterial activity against different test microorganisms (*Listeria innocua*, *Lactobacillus plantarum 73*, *Lactococcus cremoris* 117 and even against Gram negative bacteria such as *Escherichia coli*). The strain defined as *Lactococcus lactis* subsp. *lactis* 14 (based on biochemical identification tests) was chosen for the future tests and its growth curve and its ability to produce bacteriocin under different conditions of cultivation have been studied. An attempt has been made for initial purification of bacteriocin by means of a classical method [14].

Todorov and Dicks [16] reported on bacteriocin producer of *Leuconostoc mesenteroides* subsp. *dextranicum* ST99 isolated from boza originated from Belogradchik (Nort-West Bulgaria). The cell-free supernatant of this strain inhibited the growth of *Bacillus subtilis*, *Enterococcus faecalis*, several *Lactobacillus* spp., *Lactococcus lactis* subsp. *cremoris*, *Listeria innocua*, *Listeria monocytogenes*, *Pediococcus pentosaceus*, *Staphylococcus aureus* and *Streptococcus thermophilus*. However, *Clostridium* spp., *Carnobacterium* spp., *Leuconostoc mesenteroides* and Gram-negative bacteria were not inhibited [16]. Maximum antimicrobial activity, i.e. 6 400 AU/ml, was recorded in MRS broth after approximately 5 x 107 CFU/ml. Incubation in the presence of protease IV and pronase E resulted in the loss of its antimicrobial activity, confirming that growth inhibition was caused by a bacteriocin, designated here as mesentericin ST99. No loss in activity was recorded after treatment with α-amylase, SDS, Tween 20, Tween 80, urea, Triton X-100, N-laurylsarcosin, EDTA and phenylmethylsulfonylfluoride. Mesentericin ST99 remained active after 30 min at 121 °C and after 2 h of incubation at pH 2 to 12. Metabolically active cells of *Listeria innocua* treated with mesentericin ST99 did not undergo lysis, and mesentericin ST99 did not adhere to the cell surface of strain ST99. Precipitation with ammonium sulfate (70% saturation), followed by Sep-Pack C18 chromatography and reverse-phase HPLC on a C18 Nucleosil column yielded one antimicrobial peptide [16].

In 2005, these same authors [17] reported that 13 of the 52 strains isolates from boza produced in Belogradchik, Nort-West of Bulgaria inhibited the growth of *Pediococcus* spp., *Listeria innocua* and *Lactobacillus plantarum*. The population of lactic acid bacteria recorded in boza was ca. 2 x 10^6 CFU/ml. One of the strains, identified as *Pediococcus pentosaceus* ST18 (based on biochemical identification tests), produced pediocin ST18 at 3200 AU/ml in MRS broth at the end of logarithmic growth (i.e. after 24 h). Pediocin ST18 was active against all tested strains of *Pediococcus* spp. included in this study and revealed an important anti-listerial activity, including against *Listeria monocytogenes*. From the 54 bacterial strains tested, 29 were sensitive to pediocin ST18. No activity was recorded against *Listeria innocua*.

Concentration by ammonium sulfate precipitation, followed by separation in a Sep-Pack C18 column and reverse-phase HPLC on a C18 Nucleosil column yielded two active antimicrobial peptides, which suggests that pediocin ST18 may be a two-peptide bacteriocin. The peptide had bacteriostatic action towards *Listeria innocua*, but did not cause cellular lyses. Pediocin ST18 remained active for 30 min at 121 °C and after 2 h of incubation at pH 2 - 12. No loss in activity was recorded after treatment with α-amylase, SDS, Tween 20, Tween 80, urea, Triton X-100, N-laurylsarcosin, EDTA and PMSE. Pediocin ST18 does not adhere to the cell surface of the producer strain [17].

The study of Todorov and Dicks [18] is the first report on the isolation of lactic acid bacteria from boza and their identification based on genetic approaches. Boza samples were obtained from same town in Bulgaria has been previously reported by Todorov and Dicks [16, 17]. The population of lactic acid bacteria recorded in Boza was approximately 5 x 10^7 CFU/ml. *Lactobacillus plantarum* (strains ST194BZ, ST414BZ and ST664BZ), *Lactobacillus pentosus* (strain ST712BZ), *Lactobacillus rhamnosus* (strains ST461BZ and ST462BZ) and *Lactobacillus paracasei* (strains ST242BZ and ST284BZ), isolated from boza, produced bacteriocins active against *Lactobacillus casei*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Thus far, only a few bacteriocins with activity...
against Gram-negative bacteria have been reported, but for the second time, a bacteriocin isolated from LAB originated from boza was reported [18]. All eight bacteriocins inhibited Lactobacillus casei, Escherichia coli, Pseudomonas aeruginosa and Enterococcus faecalis. Bacteriocin ST242BZ inhibited the growth of Acinetobacter baumanii and bacteriocins ST194BZ, ST242BZ, ST284BZ and ST414BZ were active against Enterobacter cloacae [18].

Complete inactivation of antimicrobial activity was observed after treatment of the bacteriocins with proteinase K, pronase, papain, chymotrypsin, trypsin, pepsin and Qiagen protease, confirming their proteinaceous nature. Treatment with catalase and α-amylase did not result in any changes of antimicrobial activity, indicating that the inhibition recorded was not hydrogen peroxide and that carbohydrate moieties were not required for antimicrobial activity. The bacteriocins remained stable after 2 h of incubation at pH values between 2.0 and 10.0, and for 120 min at 100 ºC. The bacteriocins were resistant to treatment with SDS, Tween 20, urea and EDTA, but sensitive to Tween 80, Triton X-100 and Triton X-114. All bacteriocins acted bactericidal. The bacteriocins did not adhere to the surface of the producer cells. Production occurred throughout logarithmic growth, with the highest activity recorded at the end of logarithmic and during stationary growth. Based on tricine-SDS-PAGE, the bacteriocins ranged from 2.8 to 14.0 kDa in size. No plasmids were recorded, suggesting that the genes encoding the bacteriocins are located on the genomes [18].

Von Mollendorff et al. [19] reported bacteriocins produced by boza related lactic acid bacteria. Four isolates (JW3BZ, JW6BZ, JW11BZ, and JW15BZ) produced bacteriocins active against a broad spectrum of Gram-positive bacteria. In addition, bacteriocin JW15BZ was shown to inhibit the growth of Klebsiella pneumoniae. The producer strains were identified as Lactobacillus plantarum (strains JW3BZ and JW6BZ) and Lactobacillus fermentum (strains JW11BZ and JW15BZ) based on bio-chemical and bio-molecular approaches and 16s rDNA sequencing. The spectrum of antimicrobial activity, characteristics, and mode of action of these bacteriocins were compared with bacteriocins previously described for lactic-acid bacteria isolated from boza [19].

The highest level of bacteriocin JW3BZ activity (25 600 AU/ml) was recorded after 18 hours of growth in MRS broth (30 ºC), and it remained at this level for the duration of fermentation. The same level of bacteriocin JW6BZ activity (25 600 AU/ml) was recorded after 15 hours of growth, which was followed by a decrease to 12 800 AU/ml during the next 6 hours. Bacteriocins JW11BZ and JW15BZ were produced at lower levels (12 800 AU/ml) and only after 15 hours and 12 hours, respectively. Bacteriocin JW11BZ production decreased to 6 400 AU/ml after 18 hours of fermentation. Bacteriocin JW15BZ decreased to 3 200 AU/ml after 21 hours of fermentation. The end pH (after 24 hours of fermentation) recorded for all four cultures was approximately 4.0 [19].

The molecular size of bacteriocins JW3BZ, JW6BZ, JW11BZ, and JW15BZ ranged from approximately 2.3 to 3.0 kDa. The antimicrobial activity of all four bacteriocins was inhibited after treatment of the cell-free supernatants with proteolytic enzymes. No change in activity levels was recorded when the cell-free supernatants were treated with α-amylase and catalase. All four bacteriocins remained active after incubation at pH 2.0 to 10.0. Bacteriocin JW11BZ resisted incubation at pH 12.0. No decrease in antimicrobial activity was recorded after treatment of the cell-free supernatants at any of the temperatures tested, including 100 ºC for 120 minutes. No change in activity was recorded after treatment of cell-free supernatants containing bacteriocins JW3BZ, JW6BZ, JW11BZ, and JW15BZ with SDS, urea, Tween 20, Tween 80, and EDTA. Treatment of bacteriocins JW3BZ and JW11BZ with Triton X-100 resulted in a loss of activity. Treatment with Triton X-114 destroyed the activity of bacteriocins JW3BZ, JW6BZ, and JW11BZ [19].

Lactococcus lactis subsp. lactis YBD11 was isolated from boza, produced in Turkey [28]. The bacteriocin produced by Lactococcus lactis subsp. lactis YBD11 inhibited the growth of Lactobacillus plantarum, Lactobacillus sake, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Micrococcus luteus, Listeria innocua, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus carnosus, Pediococcus pentosaceus, and Bacillus cereus, however the nisin producing strain Lactococcus lactis SIK83 and Gram-negative bacteria were not inhibited. Different enzyme, pH and heat treatments showed that the bacteriocin produced by Lactococcus lactis subsp. lactis YBD11 exhibited a similar behavior with the nisin control. Maximum antimicrobial activity, 10 240 AU/ml, was recorded in glucose-M17 broth after 18h at 30ºC. Based on the cell lysis treatments, bacteriocin of Lactococcus lactis subsp. lactis YBD11 was determined to have a bactericidal activity against Micrococcus luteus. Bacteriocin production occurred throughout the exponential phase, with the highest activity recorded at the end of this phase. Tricine-SDS-PAGE of partially purified bacteriocin gave the same molecular weight of nisin (3.5 kDa). These results indicate that the antimicrobial compound produced by Lactococcus lactis subsp. lactis YBD11 is a nisin-like bacteriocin [28].

A bacterium isolated from boza produced in Turkey was identified, and the physico-chemical and microbiological properties of its inhibitory compound were characterized [29]. 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 betamercaptoethanol, 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 ºC for 30 min), at acidic and neutral pHs (2.0-7.0), and after storage at -20 to -80 degrees 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 ºC. 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 5.5 kDa, 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 [29].

Five bacteriocin-producing lactic acid bacteria (*Lactobacillus plantarum* ST69BZ, *Enterococcus faeueci* ST62BZ and *Leuconostoc lactis* ST63BZ, ST611BZ and ST612BZ) were isolated from boza originated from Belgratichik, Bulgaria [1]. The bacteriocins of all five isolates inhibited the growth of *Enterococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Lactobacillus* spp., *Lactococcus lactis* subsp. *lactis*, *Listeria* spp., *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Streptococcus caprinus*. The mode of activity of bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ is bactericidal, as determined against *Enterococcus faeueci* HKLHS and *Lactobacillus sakiei* DSM 20017, respectively. Two of the five studied bacteriocinogenic strains (ST69BZ and ST612BZ) exhibited activity against some fungal cultures. In addition, a synergetic effect between bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ and ciprofloxacin were registered [1].

The highest level of bacteriocin ST69BZ activity (12 800 AU/ml) was recorded after 18 h of growth in MRS broth (30 °C) and it remained at this level for the duration of fermentation. The highest level of bacteriocin ST62BZ activity (25 600 AU/ml) was recorded after 21 h of growth, followed by stable production for the next 3 h. Similar results were recorded for bacteriocin ST63BZ. Maximum levels of bacteriocin ST63BZ (1 600 AU/ml) were recorded after 24 h, at the end of stationary growth. Bacteriocin ST611BZ reached 52 600 AU/ml after 15 h, but decreased to 25 600 AU/ml during the following 9 h. Similar results were recorded for strain ST612BZ. Optimum levels of bacteriocin ST612BZ was recorded at 15 h (6 400 AU/ml), followed by a decrease to 3 200 AU/ml during the following 9 h [1]. The size of bacteriocins ST69BZ (3.2 kDa), ST62BZ (10.0 kDa), ST63BZ (10.0 kDa), ST611BZ (3.2 kDa) and ST612BZ (6.5 kDa) [1] was similar to that described for other bacteriocins produced by lactic acid bacteria isolated from boza. Bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ are between 2.3 to 3.3 kDa in size [19]. Similar results were reported for bacteriocins ST194BZ (3.0 kDa and 14.0 kDa), ST242BZ (10.0 kDa), ST284BZ (3.5 kDa), ST414BZ (3.7 kDa), ST461BZ (2.8 kDa), ST462BZ (8.0 kDa), ST64BZ (6.5 kDa), ST712BZ (14.0 kDa) [18]. The sizes recorded for the five bacteriocins (ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ) are within the range reported for most bacteriocins produced by *Lactobacillus* spp. and *Enterococcus* spp. [24].

Complete inactivation of the bacteriocins was observed after treatment of the cell-free supernatants with proteolytic enzymes, confirming the proteaceous nature of the antimicrobial compounds [1]. Treatment of cell-free supernatants of the four strains with catalase and α-amylase did not result in activity changes, except for bacteriocin ST63BZ, suggesting that the inhibition recorded was not hydrogen peroxide and that carbohydrate moieties were not required for antimicrobial activity [1]. Inhibition of bacteriocin ST63BZ activity by α-amylase suggests that the bacteriocin is glycosylated and belongs to group IV according to the classification of Klaenhammer [30]. Stability of the other 4 bacteriocins in the presence of α-amylase is not unusual and similar results have also been reported for other bacteriocins isolated from boza [18, 19]. Leuconocin S [31] and carnocin 54 [32] are sensitive to α-amylase, suggesting that their activity is associated with glycosylation of the active peptide.

Termosstability at 100 °C has also been reported for most other bacteriocins [16-19] isolated from boza. The sensitivity of bacteriocins ST63BZ and ST612BZ to 100 °C after 120 min and 121 °C after 20 min, may be a result of their molecular mass (10.0 kDa and 6.5 kDa, respectively), although the stability of bacteriocin ST62BZ, a 10.0 kDa peptide was not affected by treatment for 120 min at 100 °C. A difference in the structures of bacteriocin ST62BZ and ST63BZ may be a reason for these results. Bozacin B14 was inactivated after 10 min at 90 – 121 °C [27].

The mode of activity of bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ is bactericidal, as determined against *Enterococcus faeueci* HKLHS and *Lactobacillus sakiei* DSM 20017, respectively [1]. The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirm that bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ destabilize the permeability of the cell membrane [1].

Cell-free supernatants from 24-h-old cultures of *Lactobacillus plantarum* ST69BZ, *Leuconostoc lactis* ST63BZ, ST611BZ and ST612BZ and *Enterococcus faeueci* ST62BZ (pH neutralized) inhibited the growth of several bacterial strains such as *Listeria monocitogenes*, *Enterococcus faeueci*, *Escherichia coli* [33]. Early log phase cells of test microorganisms treated with bacteriocins resulted in immediate and complete growth inhibition for at least 10 h [33], suggesting that the mode of action is bactericidal. Cells of test microorganisms treated with bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ were clearly deformed or vesiculated as visualised by AFM [33]. Sensitive strains treated with bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ resulted in leakage of DNA, RNA, proteins and β-galactosidase. The results obtained by AFM shown by Meinken and Todorov [33] and leakage of DNA, RNA, proteins and β-galactosidase confirm that bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ are destabilizing the permeability of the cell membrane. Vesiculation was clearly visible on cells of *Lactobacillus sakiei* DSM20017 after treatment with bacteriocin ST62BZ produced by *Enterococcus faeueci* ST62BZ, bacteriocins ST63BZ, ST611BZ and ST612BZ produced by *Leuconostoc lactis* ST63BZ, ST611BZ and ST612BZ, respectively. Changes in morphology, such as collapse and formation of pores of *Enterococcus faeueci* HKLHS were observed after treatment with bacteriocin ST69BZ produced by *Lactobacillus plantarum* ST69BZ. The resulting images clearly showed changes in cell morphology, such as collapse of the apical ends or the cell centre, signs of cytoplasm leakage or vesiculation. Differences observed between the bacteriocins suggests different modes of action, such as the
barrel stave model and the toroidal model, which describe the formation of pores in the cell membrane or the carpet model, which leads to a vesiculation of the outer cell membrane [33]. It was interesting to find that 2 of these 5 strains exhibit antifungal activity. *Lactobacillus plantarum* ST69BZ culture was showing activity against *Absidia* spp., *Aspergillus niger*, *Epicoccum nigrum* and *Penicillium* spp. and *Leuconostoc lactis* ST612BZ produced an antifungal substance active against *Botrytis* spp. [1]. Regarding the fact that these bacterial species are present in boza, we can presume that they could be an important part of the starter cultures and could contribute to the antifungal stability of the product. Todorov [1] reported that in the combined application of the sub-lethal levels of clinical antibiotic (ciprofloxacin) and 5 bacteriocins (bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ), antibacterial activity was strongly increased. These results indicate that the mechanism by which the cationic peptide increases the effectiveness of these antibiotics would be through the dissipation of the proton gradient responsible for the extrusion of these compounds. The synergism between antibiotics, particularly ciprofloxacin and bacteriocins is important in order to reduce the level of the MIC of the antibiotics. Similar synergetic effects may be important for the development of treatments against multidrug resistant strains.

### 4. Probiotics properties of Lactic acid bacteria isolated from boza

According to the definition of the World Health Organization, probiotics are live micro-organisms which, when administrated in adequate amounts, confer a health benefit on the host [34]. This includes reduction of gastrointestinal infections and inflammatory bowel disease, and modulation of the immune system [34]. Probiotic strains have to survive harsh conditions in the gastrointestinal tract and adhere to intestinal epithelial cells. They form a defence against the colonization of pathogenic micro-organisms by competing for adsorption to mucus and epithelial cells and, in certain cases, production of hydrogen peroxide and bacteriocins [35-38]. Changes in diet, stress, the use of contraceptives, medicaments and the presence of some fungicides may disturb the microbial balance, which often leads to a decrease in the number of viable lactic acid bacteria [34]. The subsequent uncontrolled proliferation of pathogenic bacteria may lead to diarrhoea and other clinical disorders such as cancer, inflammatory disease and ulcerative colitis [39]. A variety of *Lactobacillus plantarum* strains are presently marketed as probiotics [40]. The best studied probiotic strains are from the species *Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus jensenii, Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus vaginalis* and *Lactobacillus salivarius*. A number of surface-anchored proteins have been described for *Lactobacillus plantarum* [41], suggesting that the species has the potential to associate with many different surfaces and substrates, and adapt to changing environmental conditions.

Criteria for the selection of probiotic strains have only recently been formulated by the Food and Agriculture Organization of the United Nations and the World Health Organization in 2001 [42]. Some of the most important criteria are gastric and bile acid resistance, adhesion to mucus and human epithelial cells, competition with pathogens for adhesion sites, growth inhibition of potentially pathogenic bacteria, bile salt hydrolase activity and, in the case of vaginal applications, resistance to contraceptives [42].

The concept of probiotic foods have been developed extensively since its introduction to clinical nutrition and food science during the 1980’s [43, 44]. Most probiotic foods available today are milk-based while a few attempts have been made using cereals. Cereal grains have a high nutritive value and are distributed worldwide, making it a very suitable raw material for the development of various fermented functional foods [45]. Togwa, a lactic acid-fermented maize and sorghum gruel, inhibits the growth of some enterotoxin-producing bacteria in children under 5 years old. This suggests that togwa may possess probiotic properties [46]. Vogel et al. [47] found that the lactic acid bacteria present in various fermented foods, such as sourdough, is similar or in some cases identical to species found in the gastrointestinal tract of humans and animals. *Lactobacillus plantarum* indigenous to a variety of cereal-based fermented foods, is also associated with the gastrointestinal tract of humans [48, 49]. Colonization of the intestinal mucosa with strains of *L. plantarum* isolated from sourdough has also been reported [50].

A number of lactic acid bacteria with probiotic properties have been isolated from boza, a traditional beverage produced from the fermentation of cereals. *Lactobacillus plantarum* (strains ST194BZ, ST1414BZ and ST664BZ), *Lactobacillus pentosus* (strain ST712BZ), *Lactobacillus rhamnosus* (strains ST461BZ and ST462BZ) and *Lactobacillus paracasei* (strains ST242BZ and ST284BZ) has been described previously as bacteriocin producers with activities against several Gram-positive and Gram-negative test organisms [18]. Strains ST194BZ, ST242BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ, ST664BZ and ST712BZ was showing to have a potential probiotic properties [51], as all of them survived low pH conditions (pH 3.0), grew well at pH 9.0 and were not affected by the presence of 0.3% (w/v) ox bile. Cytotoxicity levels of the bacteriocins, expressed as CC50, ranged from 38 µg/ml for bacteriocin ST194BZ to 3776 µg/ml for bacteriocin ST284BZ. Bacteriocin ST194BZ revealed high activity (EC50 = 735 µg/ml) against the HSV-1 virus that causes encephalitis and oro-facial and genital lesions. Growth of *Mycobacterium tuberculosis* was repressed 69% after 5 days of incubation in the presence of bacteriocin ST194BZ. Various levels of auto (self) aggregation between the probiotic bacteria and co-aggregation with *Listeria innocua* LMG 13568 were observed.
Biogenic amine occurrence in boza

Biogenic amines are basic nitrogenous organic compounds having a recognized activity and which may occur naturally in foods and beverages [53]. They are generated either as a result of endogenous amino acid decarboxylase activity in raw food material or by the growth of decarboxylase-positive microorganisms under conditions favorable to enzyme activity [54, 55]. Biogenic amines are commonly found in many foodstuffs, particularly in products that involve a ripening or fermentation period, such as cheese, meat products, beer, wine and fish [56, 57]. Consumption of foods containing high amounts of biogenic amines may cause problems such as headaches, nausea, hypotension, hypertension, cardiac palpitation, etc. [58, 59]. Fermented foods and beverages are more susceptible to the formation of biogenic amines, since several microorganisms are involved in the fermentation process and the raw materials used contain considerable amounts of proteins [60].

Biogenic amine content of boza has not yet been studied [5]. Since it is a fermented beverage containing protein, which might be used by decarboxylase-positive microorganisms, the formation of various biogenic amines might be expected. Bacterial genera that are known to have decarboxylating ability include Achromobacter, Aerobacter, Betabacterium, Clostridium, Escherichia, Lactobacillus, Proteus, Pseudomonas, Salmonella, Shigella, Streptococcus and Pediococcus [61]. Hancioglu and Karapinar [3], Todorov and Dicks [16-18], Von Mollendorff et al. [19], Todorov [1] isolated several lactic acid bacteria, namely Leuconostoc para-mesenteroides, Lactobacillus sanfrancisco, Leuconostoc mesenteroides subsp. mesenteroides, Lactobacillus corniformis, Lactobacillus confusus, Leuconostoc mesenteroides subsp. dextranicum, Lactobacillus fermentum and Leuconostoc oenos, and the yeasts isolated comprised Saccharomyces uvarum and Saccharomyces cerevisiae, during fermentation of boza. As is evident, the microflora of boza includes decarboxylase-positive microorganisms. Lactobacillus sanfrancisco, Leuconostoc oenos (Oenococcus oenos) [62] and Leuconostoc mesenteroides [60] were mentioned before as biogenic amine-producing bacteria.

Biogenic amine contents of 10 boza samples from different manufacturers in Turkey were analysed using HPLC after derivatisation with benzoyl chloride [5]. Of the 11 biogenic amines under study, putrescine, spermidine and tyramine were detected in all boza samples. Tyramine was the prevailing biogenic amine. Tyramine concentrations of boza samples were between 25 and 69 mg/kg. Consequently, consumption of boza might represent a health risk for patients being treated with drugs containing monoamine oxidase inhibitor (MAOIs). The pH values of boza samples were in the range from 3.16 to 4.02; total dry matters were from 15.3 % to 31.1 % (w/w); protein contents were from 0.50 % to 0.99 % (w/w). No significant
correlations were detected between biogenic amine concentrations and pH, protein content and total dry matter content [5].

In another study [63], 21 samples of boza were analyzed for their tryptamine, beta-phenyl ethyl amine, putrescine, cadaverine, histamine and tyramine contents by HPLC after derivatization with dansyl chloride. The detection limits of HIS, TRY, TYR, PUT, CAD and PHA were 0.03; 0.04; 0.04; 0.09; 0.11 and 0.05 mg/kg, respectively; while the limits of quantification were 0.1; 0.12; 0.12; 0.18; 0.30 and 0.15 mg/kg, respectively. A wide variation was determined in the biogenic amine contents of boza samples. Of the 21 boza samples, 18 (90 %) contained at least one of six screened biogenic amines. Total biogenic amine contents ranged from 1.67 to 101.14 mg/kg. The highest tyramine, cadaverine, tryptamine, putrescine, beta-phenylethylamine and histamine contents were 82.79; 17.69; 13.78; 9.80; 4.53 and 4.07 mg/kg, respectively. Although the biogenic amine contents of boza samples were lower than the recommended toxic limits, this product could be considered as a potentially risky food since the wide variation for biogenic amine contents [63].

6. Conclusions

It can be concluded from all previous findings on boza, that this cereal based fermented beverage is a rich source of bacteriocin-producing lactic acid bacteria with antimicrobial activity against a number of food spoilage and pathogenic bacteria. LAB naturally present in boza may contribute to the increase of the microbiological safety of this product. Bacteriocins produced by these LAB have potential in the reduction of the food spoilage bacteria. The combined application of bacteriocins and antibiotics with synergetic activity may be an answer to increase the control of human and animal pathogens.

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