ANTIMICROBIAL ACTIVITY OF STARCH-DEGRADING LACTOBACILLUS STRAINS ISOLATED FROM BOZA

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ABSTRACT
The proved probiotic effects of boza consummation prompted the increasing interest in the microflora taking part in its fermentation. Boza is a rich source of probiotic lactic acid bacteria that are able to produce bacteriocins active against a number of pathogens. In the present study, three strains – Lactobacillus paracasei B41, Lactobacillus plantarum Bom 816, and Lactobacillus pentosus N3, isolated from boza and possessing significant amylolytic activity, were studied for their ability to repress the growth of common foodborne pathogens. All strains demonstrated antimicrobial activity against Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae and Bacillus subtilis. The results indicated possible bacteriocin production and made the strains desirable starters with application in cereals processing. None of the tested lactobacilli inhibited the growth of Saccharomyces cerevisiae or Pichia strains, suggesting the existence of stable microbial community of yeasts and lactobacilli in boza starters.


Keywords: Lactobacillus, antimicrobial activity, foodborne pathogens, boza

Introduction
Lactic acid fermentation is a centuries-old method of food preservation and processing. It is the main approach to increase the nutritional value and the taste and to save energy for the preparation of foods and beverages of plant origin, cereals, and bread. Million tonnes of fermented foods, based on manioc, taro, sorghum, rice, millet and maize in the form of over 90 different products such as bakery bread, pastas, traditional drinks or snacks are obtained with the participation of amylolytic lactic acid bacteria (ALAB) (1, 8). ALAB were initially found in tropical starch-containing fermented foods, prepared mainly from manioc and cereals (maize and sorghum). Consumer’s expectations that besides the improved aroma and taste the LAB fermented cereal foods possess probiotic properties are not always justified. The majority of the LAB strains, evaluated as probiotic do not possess amylolytic activity, i.e. their survival and growth in starchy environment are questionable. In addition, the amylase-positive strains usually are not probiotic. Driven by the fact, that some of the starch-based beverages, as boza, yosa or ogi, have been claimed for their reference to the functional foods because of LAB content (1), we focused our investigation on the finding of potentially probiotic, and at the same time, amylolytic LAB strain.

Boza is an ancient cereal beverage which originated in Mesopotamia about 9000 years ago. Starting from the 13th century, with the Ottoman invasion of Anatolia, the Turks introduced the drink under the name “boza” to the Balkans. The name derives from the Persian word “buze”, meaning “millet”. Indeed, it is a malt drink, made from maize, bulgur, millet, barley or chick peas in Albania, fermented wheat in Turkey and wheat or millet in Bulgaria and Romania. It has a creamy light to dark yellow colour and is rather thick in consistency.

Boza has been found to have several health benefits: the drink helps to balance blood pressure, to increase milk production in lactating women and to facilitate digestion. It is a valuable nutrient to physically active people, as it contains vitamins A, C, E and four types of vitamin B. Boza is especially suitable for vegetarians, as it is entirely plant-based and a good source of vitamins and thus constitutes a good substitute for dairy-based drinks.

Recently, boza is being increasingly introduced to the EU countries. This requires an implementation of scientific assessment of the potential probiotic microbial strains taking part in boza manufacturing. The species diversity of Lactobacillus strains isolated from boza included L. sanfrancisco, L. coryniformis, L. fermentum, L. confusus, L. plantarum (6), L. paracasei, L. pentosus, L. brevis, and L. rhamnosus (2). Some of them are potential or proved probiotics (5, 10). The isolation of a Lactobacillus strain, holding both features – amylolytic and antibacterial activities would contribute to the development of new kind of valuable starters for cereal foods manufacturing.

Materials and Methods

Bacterial strains
Three Lactobacillus strains were included in this study: L. paracasei B41, L. plantarum Bom 816 and L. pentosus N3. The first strain was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under registration
The test microorganisms' cultures were 24 h old. The wells were triplicated.

Enzyme, 37 °C and 30 min incubation time. All assays were done using 20 μg/ml final concentration of the enzyme. Halo around the agar well in mm.

Antagonistic activity was estimated by measurement of a sterile treated supernatant transferred in each well was 100 μL. The quantity of the cell-free catalase-treated supernatants transferred in each well was 100 μL. The antagonistic activity was estimated by measurement of a sterile halo around the agar well in mm.

The treatment of the supernatants with proteinase K (Promega) was done using 20 μg/ml final concentration of the enzyme, 37 °C and 30 min incubation time. All assays were triplicated.

Results and Discussion

The contemporary requirements to the starch-containing foods demand the addition of functional compounds during their manufacturing. The fermented cereals must be highly nourishing, easily digestible, gratifying in taste and fragrance, and desirably with beneficial effects on the human health due to their probiotic microbial content. Unlike fruit and milk fermentations, cereal fermentation requires a saccharification process, which is accomplished with some difficulty. Before centuries, the primitive method of cereal saccharification was the chewing of raw materials and spitting them into a vessel in order to allow saccharification to occur through the action of salivary amylase followed by alcoholic fermentation by natural yeasts. Today, cereal saccharification takes place during the process of malting. It occurs naturally through wet damage of cereals during storage and is used for beer making in Europe.

Boza is a fermented beverage produced by a microbial community, containing yeasts and lactobacilli. The majority of the authors assigned the probiotic properties of the drink to the participation of lactobacilli. Some of these Lactobacillus strains were identified as potential probiotic, considering their physiology. None of them, however, was amylolytic, which may impede the strains' growth in starchy media. To assure the viability of LAB probiotic starter for cereals fermentation, the starter has to be capable to utilize starch, i.e. it has to contain LAB with amylolytic abilities. The Lactobacillus strains investigated here were previously described as amylolytic, as their amylases productions were proved and quantitatively estimated (9). The evaluation of their probiotic potential included the selection of test microbes – common food pathogens (E. coli, K. pneumoniae, V. cholerae); food-spoilage bacteria – B. subtilis; and yeast species, naturally participating in boza. The antagonistic activity against the target bacteria was assayed using cell-free neutralized supernatants after 24 h growth of the lactobacilli. All strains possessed antagonistic activity against the common foodborne pathogens. The strongest inhibitory activity was obtained against E. coli and B. subtilis, followed by that against K. pneumoniae and V. cholerae. The most active strain was L. paracasei B41 (Fig. 1), slightly less active were L. plantarum Bom 816 (Fig. 2) and L. pentosus N3 (Fig. 3). The antibacterial activity was completely lost after treatment of the supernatant with proteinase K, suggesting possible bacteriocin production.

![Inhibition zone (mm)](image1)

**Fig. 1.** Antagonistic activity of *L. paracasei* B41 against foodborne pathogens and food-spoilage bacteria. The well-diffusion assay method was used. The clear zone of inhibition around the well, showing no growth of the sensitive strain is presented in mm.

![Inhibition zone (mm)](image2)

**Fig. 2.** Antagonistic activity of *L. plantarum* Bom 816 against foodborne pathogens and food-spoilage bacteria.
Antimicrobial activity of L. pentosus N3

Three different media – MRS-starch, MRS and M1, were used for antagonistic activity tests. The level of pathogens’ growth inhibition was almost equal in MRS and MRS-starch medium, indicating that the antimicrobial activity is not affected by the amylase enzyme produced. Therefore, the putative bacteriocins, produced by the strains, most probably do not possess carbohydrate components. Grown in the poor medium M1, the strains slightly decreased their antagonistic action because of the less accumulated biomass (0.7-1.4 × 10⁷ CFU/ml, compared to 1.8 × 10⁹ - 2.2 × 10⁹ CFU/ml in MRS-starch and MRS, respectively).

Having in mind that lactic acid has an inhibitory effect on bacterial growth, the action of non-neutralized supernatants was also tested. As an example, the activity of two strains (Bom 816 and N3) against V. cholerae is shown (Fig. 4). The results revealed that in all cases the lactic acid enhanced the antimicrobial activity of the strains, increasing the zone of inhibition with approximately 2 mm, but it was not the main antibacterial agent produced by them.

Fig. 4. Antimicrobial activity assay using Vibrio cholerae as a test-microorganism. (A) Growth inhibition by supernatants of L. plantarum Bom 816: 1 – MRS (pH6), 4 – MRS (pH4), 5 – MRS-starch (pH 6), 6 – MRS-starch (pH 4); Controls: 2 – MRS (pH4), 3, 7 – MRS (pH 6). (B) Growth inhibition by supernatants of L. pentosus N3: 3 – MRS (pH 6), 4 – MRS (pH 4), 5 – MRS-starch (pH 6), 6 – MRS-starch (pH 4); Controls: 1 – MRS (pH 4), 2, 7 – MRS (pH 6).

Importantly, no activity was detected against the yeast strains, neither by the neutralized, nor by the acidic supernatants. None of the tested lactobacilli inhibited the growth of Saccharomyces cerevisiae or Pichia strains, suggesting that a stable microbial community of yeasts and lactobacilli exists in boza starters.

The human eating habits increase the risk of new foodborne illnesses. In association with the awareness of not only food safety, but also of the risk derived from the chemical preservatives, there has been an increasing demand for more “natural” and “health-promoting” food (7). Much attention is being directed to food preservation by the use of lactic acid bacteria. The preservative effect is a result of the antimicrobial action of bacteriocins and metabolites, such as lactic acid, produced by LAB (3, 4). The production of antimicrobial substances is one of the principles for probiotic strain selection. Hence, the three Lactobacillus strains described here may have excellent probiotic potential, as they possess remarkable antimicrobial activity both against gram-negative and gram-positive bacteria. They are able to protect starch-containing foods from pathogens’ propagation because of the quite rare combination of antimicrobial and amylolytic characteristics.

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