SİDAS MEDYA
Akademik Gıda®
ISSN Print: 1304-7582, Online: 2148-015X
http://www.academicfoodjournal.com

Akademik Gıda 14(3) (2016) 230-234

Research Paper / Araştırma Makalesi

Biochemical and Molecular Identification of a Strain *Leuconostoc lactis* BT_17, Isolated from a Spontaneously Fermented Cereal Beverage, Boza

Remzi Cholakov[1], ✉, Zapryana Denkova[1], Velichka Yanakieva[1], Yulian Tumbarski[1], Zoltan Urshev[2], Iliyan Dobrev[1]

[1]University of Food Technologies, Department of Microbiology, Plovdiv, Bulgaria
[2]Laboratory "DNA Analysis", ELBI Bulgaricum, Sofia, Bulgaria

Received (Geliş Tarihi): 27.07.2016, Accepted (Kabul Tarihi): 22.08.2016
✉ *Corresponding author (Yazışmalardan Sorumlu Yazar): inj.cholakov@gmail.com (R. Cholakov)*
✆ +359 889 761 720

ABSTRACT

The strain BT_17 was isolated from a naturally fermented cereal beverage (boza), which is traditional in Balkan countries. The colony characteristics and cell morphology of the strain were determined after its cultivation on a solid culture medium and coloring of microscopic preparations. The strain was identified by biochemical methods - API 50 CHL kit system, followed by apiweb software processing, as well as molecular techniques - amplified rDNA restriction analysis (ARDRA) with the endonucleases *Eco* RI, *Hae* III and *Alu* I, and nucleotide sequencing of the 16S rRNA gene. The results obtained from the biochemical identification showed that the isolated strain of BT_17 is related to the species *Lactobacillus plantarum* 1 (the rate of reliability with 59.1%) and *Lactobacillus pentosus* (the rate of reliability with 41.2%). The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 99% pairwise similarity of the studied strain to the bacterial species *Leuconostoc lactis.* The aim of this study was the identification, biochemical and molecular characterization of a strain *Leuconostoc lactis* BT_17, isolated from a spontaneously fermented cereal beverage (boza).

Keywords: *Leuconostoc,* Cereal, Boza, Sequencing, API 50 CHL

Bozadan İzole Edilen *Leuconostoc lactis* BT_17 Suşunun Biyokimyasal ve Moleküler-Genetik Yöntemlerle Tanımlanması

ÖZ

Balkanlar bölgesinde geleneksel bir doğal fermente tahıl ürünü olan bozadan, BT_17 suşu izole edilmiştir. Katı MRS agar besiyerine çizim ve Gram boyama yöntemiyle koloni ve hücre morfolojisi belirlenmiştir. Gram pozitif olarak tespit edilen izolatın biyokimyasal tanımlaması API 50 CHL ve Apiweb®, moleküler-genetik yöntemlerden ise ARDRA (çoğaltılmış rDNA restriksiyon analizi, uç endonükleaz enzimi *Eco*R I, *Hae* III ve *Alu* I kullanılarak, endonüklezlardan elde edilen restriksiyon profillerinin her bir suş için spesifik olduğu analiz yöntemi) ve 16S rRNA dizi sekansı kullanılarak gerçekleştirilmiştir. Biyokimyasal tanımlamadan elde edilen sonuçlarda, izole edilen BT_17 suşunun %59.1 *Lactobacillus plantarum* 1 ve %41.2 *Lactobacillus pentosus* suşuna ait olduğu tespit edilmiştir. Biyokimyasal tanımlama yöntemleri çabuk sonuç vermekte, fakat bazı farklılıklar göstermektedir. 16S rRNA dizi sekansına göre elde edilen sonuçlar izole edilen BT_17 suşunu %99 oranında *Leuconostoc lactis* olarak tanımlarken, ARDRA yönteminden elde edilen sonuçlar izole edilen suşun restriksiyon profillerinin referans suşlarının (*Lactobacillus acidophilus* DSM 20079, *Lactobacillus helveticus* DSM 20075, *Lactobacillus casei* ssp. *casei* DSM 20011, *Lactobacillus plantarum* DSM 20174, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081) profilleriyle eşleşmediği tespit edilmiştir.

Anahtar Kelimeler: *Leuconostoc,* Tahıl, Boza, Sekans, API 50 CHL

INTRODUCTION

Daily foods, which are essential for establishing a balanced human life, consist of various kinds with different origins and process types, and they reflect the taste of the region in which they were produced. Among them, products produced by fermentation have an important place. In general, fermentation is an inexpensive process involving the use of microorganisms to carry out enzyme-catalyzed transformations of wide range of agricultural materials. It is one of the oldest and economical methods of food processing and safety. Although the primary purpose of fermentation was to achieve food safety, it plays different roles such as (a) enhancing the diet through a diversity of flavors, nutritional value and textures in food substrates, (b) preservation of food through lactic acid, acetic acid and ethyl alcohol, (c) biological enrichment of the substrates, (d) detoxification and destruction of undesirable substances present in raw foods such as cyanide, phytates, tannins and polyphenols during food fermentation processing and (e) lowering cooking times and fuel requirements [1].

Boza is a highly viscous and low-alcoholic fermented cereal beverage. It is consumed widely in some Balkan countries (Bulgaria, Turkey, Albania, Serbia, Macedonia and Romania), Middle East (Iran and Iraq), Asia (Turkestan) and Africa (Egypt and Kenya). Boza is a beverage made by adding drinkable water to cereals such as barley, oats, millet, maize, wheat, and rice. The sugar is then added to allow alcohol and lactic acid fermentation. On this base, boza can be classified as sweet or sour boza depending on its acid content [1, 2].

Boza is a beverage characterized by high energy content, specific aroma and original taste. It contains approximately 83% water and 17% dry substances including carbohydrates (starch, dextrin, sugars, cellulose, hemicellulose), proteins, lipids, organic acids, vitamins (mainly B group vitamins) and salts. Carbohydrates are the main part of dry substances. The acidity of boza is between 0.3 and 0.6%. It contains a small amount of alcohol (0.5%).

The nutritional qualities of the foods fermented by lactic acid bacteria - yeast associations are of interest in connection with the new concept concerning the functional foods and probiotics. In this aspect the study of boza is important because it is considered to be a dietetic food [3].

A large number of microorganisms belonging mainly to the genera *Lactobacillus, Leuconstoc, Weisella, Oenococcus, Candida, Geotricum, Torulaspora, Issatchenkia* and *Pichia* have been isolated from boza [3 - 8].

The aim of the present study was identification, biochemical and molecular characterization of a strain *Leuconostoc lactis* $BT_1$7, isolated from spontaneously fermented cereal beverage (boza).

MATERIALS and METHODS

Materials

Microorganisms

In the present study, *Lactobacillus acidophilus* DSM 20079, *Lactobacillus helveticus* DSM 20075, *Lactobacillus casei* ssp. *casei* DSM 20011, *Lactobacillus plantarum* DSM 20174 and *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081 were used Reference microorganisms. LAB (lactic acid bacteria) $BT_1$7 isolated from naturally fermented cereal beverage (boza) was studied.

Culture Media

MRS-Broth

Composition (g/dm^3) of this broth was peptone from casein–10.0, yeast extract–4.0, meat extract–8.0, glucose–20.0, K_2HPO_4–2.0, sodium acetate–5.0, diammonium citrate–2.0, $MgSO_4$-0.2, $MnSO_4$-0.04 and Tween 80-1 cm^3/dm^3. The final pH was adjusted to 6.5. The medium was sterilized at 118ºC for 15 minutes.

Laptg10 Agar

Composition (g/dm^3) of this agar was peptone–15.0, yeast extract–10.0, tryptone–10.0, glucose–10.0 and Tween 80–1 cm^3/dm^3. The final pH was adjusted to 6.6–6.8, and 20.0 g of agar was added. The medium was sterilized at 121ºC for 20 minutes.

Methods

Morphological and Cultural Methods

Cellular Morphology and Colony Characteristics

The description of the cellular morphology of the isolated strain of lactic acid bacteria was obtained by the microscopic observation of a colored smear on a slide. The colony characteristics were determined by microscopic observation of single colonies developed on LAPTg10-agar.

Biochemical Methods

The system API 50 CHL (BioMerieux SA, France) was used for the identification of the species of the genus *Lactobacillus* based on their ability to utilize 49 carbon sources. Fresh 24-hour culture of the studied strain was centrifuged for 15 min at 5000 rpm. The obtained sludge, containing biomass, was washed twice with PBS-buffer and re-suspended in API 50 CHL medium, an integral part of the used kit. The API strips were placed in incubation boxes, and the microtubules were inoculated with the prepared cell suspension and then sealed with sterile liquid paraffin. The results were reported on the 24th and the 48th hour of incubation at 37±1°C. Reporting of results was based on the color change of each microtubule in comparison to the color

of the control microtubule (microtubule 0). Positive results were recorded in the cases of color change from blue to green or bright yellow. Results were processed with apiweb® identification software.

Molecular - Genetic Methods

Isolation of Total DNA

The isolation of DNA was performed by the method of Delley *et al.* [9].

16S rDNA Amplification and ARDRA Analysis

All PCR reactions were performed using the PCR kit – PCR VWR in a volume of 25 µL in a Progene cycler (Techne, UK) according to the instructions of the manufacturer. In each PCR reaction 50 ng of total DNA of the studied strain and 10 pmol of the primers were used. The partial 16S rDNA of the strain was amplified using universal primers for the 16S rDNA gene - 27f (5'AGAGTTTGATCMTGGCTCAG3') and 1492r (5'ACCTTGTTACGACTT3') [10]. The amplification program included: denaturation at 95°C for 3 minutes; 40 cycles at 93°C for 30 s, 55°C for 60 s and 72°C for 2 minutes and final elongation at 72°C for 5 minutes. The PCR amplification product (the gene for the 16S rRNA) was subjected to overnight restriction with the restriction enzymes *Eco* RI, *Hae* III and *Alu* I (Boehringer Mannheim GmbH, Germany) each with concentration 10 units/µL. The resulting products from the restrictions were visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg/mL), using an UVP Documentation System (UK).

Purification of Product of PCR-Reaction –16S rDNA– from TAE Agarose Gel

The purification of the 16S rDNA was conducted using DNA-purification kit (GFX MicrospinTM) according to the manufacturer's instructions.

Sequencing of 16S rRNA Gene

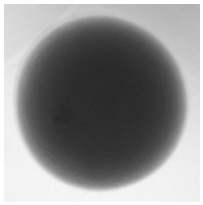
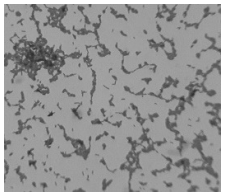
The sequencing of the 16S rRNA gene was conducted by "Macrogen Europe Laboratory" (Netherlands) based on the method of Sanger.

RESULTS and DISCUSSION

The strain LAB $BT_1$7 was isolated from naturally fermented cereal beverage (boza) by spread plating on LAPTg10.

The colony characteristics of the strain were determined by the microscopic observation of single colonies after spread plating on LAPTg10-agar and incubation at 37±1°C for 24 hours. A colored smear was prepared and observed under the microscope in order to determine the cellular characteristics of the strain LAB $BT_1$7 (Table 1).

Table 1. Colony characteristics and cell morphology of the strain LAB $BT_1$7

Strain	Colony Characteristics		Cell Morphology	
	Colony Description	Visualization	Cell Description	Visualization
LAB $BT_1$7	Round shape with smooth edges, convex surface, whitish or cream-colored, sticky consistency and size of 1 - 4 mm		Gram positive, ovoid cocci, arranged singly, in pairs or forming long chains	

The colony characteristics and cell morphology of the studied strain LAB $BT_1$7 were typical for the genus *Leuconostoc* representatives – Gram positive, ovoid cocci, arranged singly, in pairs or forming long chains; round shape colonies with smooth edges, convex surface, whitish or cream-colored and size of 1-4 mm [11, 12]. The comparison of the biochemical profile of the isolate LAB $BT_1$7 with the apiweb database showed similarity with the biochemical profiles of *Lactobacillus plantarum* 1 (59.1%) and *Lactobacillus pentosus* (41.2%).

Lactic acid bacteria are of great economic importance for dairy and other fermented food industries. For both basic research on LAB and their application in industrial food fermentations, reliable and simple methods for identification of such bacteria are required. Because many LAB have similar nutritional and growth requirements, it is very difficult to identify them by classical methods.

The classical phenotypic methods for the identification of *Leuconostoc* species are unreliable. Some advanced molecular taxonomic techniques, developed to overcome the defect of the classical phenotypic methods, are also labor-intensive, time-consuming and sometimes unreliable. These techniques include DNA-DNA hybridization, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins, and randomly amplified polymorphic DNA analysis. To overcome these disadvantages, polymerase chain reaction (PCR)-based methods using species-specific primers have been considered [13].

Lee *et al.* [13] established a multiplex polymerase chain reaction (PCR) assay for rapid and reliable identification

of *Leuconostoc* species, by using species-specific primers targeted to the genes encoding 16S rRNA. This assay has been developed to detect and differentiate *Leuconostoc* species from mixed populations in natural sources as well as from pure cultures, within 3 hours.

For the correct identification of the strain in the present study, amplified rDNA restriction analysis (ARDRA) with the enzymes *Eco* RI, *Hae* III and *Alu* I was performed. The profiles of the strain with each of the three enzymes were specific to the particular strain tested. Results indicated that the restriction profiles of the strain LAB BT_17 did not coincide with the profiles of any of the reference strains, included in the present study: *Lactobacillus acidophilus* DSM 20079, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081, *Lactobacillus casei* ssp. *casei* DSM 20011, *Lactobacillus helveticus* DSM 20075 and *Lactobacillus plantarum* DSM 20174 (Figures 1-3).

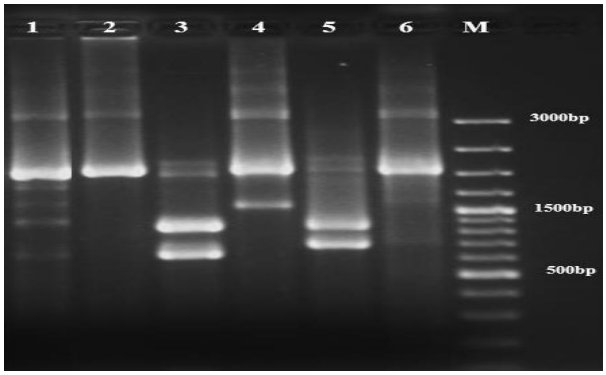


Figure 1. Restriction profile with *Eco* RI (1: LAB BT_17, 2: *Lactobacillus acidophilus* DSM 20079, 3: *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081, 4: *Lactobacillus casei* ssp. *casei* DSM 20011, 5: *Lactobacillus helveticus* DSM 20075, 6: *Lactobacillus plantarum* DSM 20174, M: Marker 100bp Plus DNA Ladder)

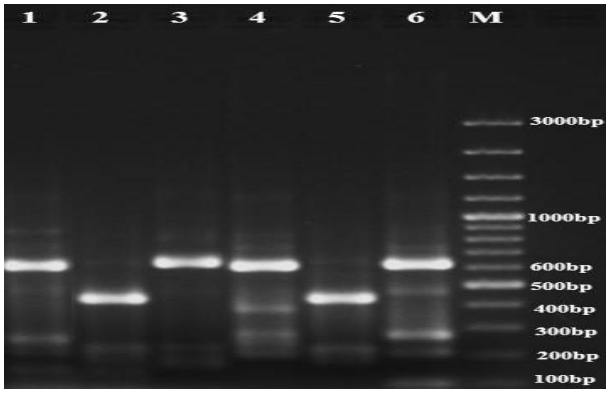


Figure 2. Restriction profile with *Alu* I (See Fig.1 for the description of numbers in profiles)

In order to identify the strain LAB BT_17, another molecular-genetic technique for genotyping (sequencing of the gene for the 16S rDNA) was used. The results of the sequencing analysis of the 16S rDNA of LAB BT_17 identified the strain as a representative of the species *Leuconostoc lactis* with 99% of complementation between the sequence of the 16S rDNA of LAB BT_17 and the partial sequence of the 16S rDNA of *Leuconostoc lactis* NBRC 102477 (Figure 4).

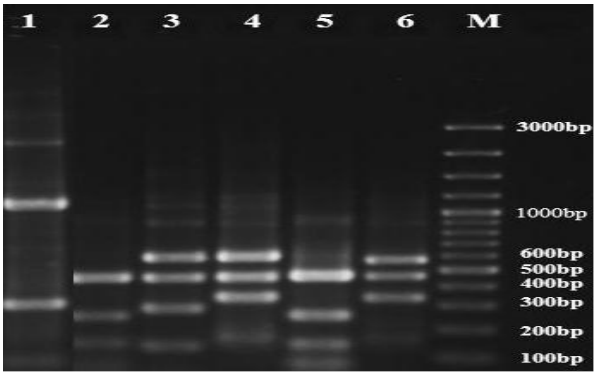


Figure 3. Restriction profile with *Hae* III (See Fig.1 for the description of numbers in profiles)

CONCLUSION

The strain *Leuconostoc lactis* BT_17 isolated from naturally fermented cereal beverage (boza) was identified by morphological, biochemical and molecular-genetic methods.

The comparison of the biochemical profile of the isolate LAB BT_17 with the apiweb database, showed similarity with the biochemical profiles of *Lactobacillus plantarum* 1 (59.1%) and *Lactobacillus pentosus* (41.2%). However, the apiweb database sometimes is insufficient for the identification of newly-isolated strains of microorganisms and does not provide reliable identification results. Therefore, for the exact species identification molecular genetic methods (ARDRA and sequencing of the gene for 16S rDNA) are required.

Figure 4. Comparison of the nucleotide sequence of the 16S rDNA of LAB BT_17 and the partial sequence of the 16S rDNA of *Leuconostoc lactis* NBRC 102477.

REFERENCES

[1] Tanguler, H., 2014. Traditional Turkish fermented cereal based products: Tarhana, boza and chickpea bread. *Turkish Journal of Agriculture - Food Science and Technology* 2(3): 144-149.

[2] Zorba, M., Hancioglu, O., Genç, M., Karapinar, M., Ova, G., 2003. The use of starter cultures in the fermentation of Boza, a traditional Turkish beverage. *Process Biochemistry* 38: 140–141.

[3] Moncheva, P., Chipeva, V., Kujumdzieva, A., Ivanova, I., Dousset, X., Gocheva, B., 2003. The composition of the microflora of boza, an original Bulgarian beverage. *Biotechnology and Biotechnology* 17(1): 164-168.

[4] Arici, M., Daglioglu, O., 2002. Boza: a lactic acid fermented cereal beverage as a traditional Turkish food. *Food Research International* 18: 39-48.

[5] Botes, A., Todorov, S.D., Von Mollendorff, J.W., Botha, A., Dicks, L.M.T., 2007. Identification of lactic acid bacteria and yeast from boza. *Process Biochemistry* 42: 267-270.

[6] Caputo, L., Quintieri, L., Baruzzi, F., Borcakli, M., Morea, M., 2012. Molecular and phenotypic characterization of *Pichia fermentans* strains found among Boza yeasts. *Food Research International* 48: 755-762.

[7] Hancioğlu, O., Karapinar, M., 1997. Microflora of Boza, a traditional fermented Turkish beverage. *International Journal of Food Microbiology* 35: 271-274.

[8] Todorov, S.D., Dicks, L.M.T., 2006. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. Comparison of the bacteriocins. *Process Biochemistry* 41: 11-19.

[9] Delley, M., Mollet, B., Hottinger, H., 1990. DNA probe for *Lactobacillus delbrueckii*. *Applied and Environmental Microbiology* 56: 1967-1970.

[10] Lane, D.J., 1991. 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, Edited by E. Stackebrandt and M. Goodfellow, John Wiley and Sons, New York, NY, 115- 75p.

[11] Dicks, L.M.T., Dellaglio, F., Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov. comb. nov. *International Journal of Systematic Bacteriology* 45(2): 395-397.

[12] Kim, D.S., Fogler, H.S., 1999. The effects of exopolymers on cell morphology and culturability of *Leuconostoc mesenteroides* during starvation. *Applied Microbiology and Biotechnology* 52: 839-844.

[13] Lee, H.J., Park, S.Y., Kim, J., 2000. Multiplex PCR-based detection and identification of *Leuconostoc* species. *FEMS Microbiology Letters* 193: 243-247.