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# ISOLATION, IDENTIFICATION AND COMPARISON OF SOME PROPERTIES OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* STRAINS FROM TRADITIONAL BULGARIAN AND ITALIAN YOGURTS

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**ABSTRACT**

An important step in the development of successful technological schemes for production of yogurt and other functional foods is the selection of appropriate *Lactobacillus strains* with useful properties that are resistant to antibiotics and bacteriocins. In the present study eleven *Lactobacillus* strains were isolated from fourteen homemade Bulgarian and Italian yogurts. The isolates were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) by 16S rDNA sequence analysis. The results from comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 92-99% pairwise similarity of the isolates to the reference *L. bulgaricus* strains. The antimicrobial activity, antibiotic susceptibility and nisin resistance of the isolated *L. bulgaricus* strains were examined. Bulgarian *L. bulgaricus* strains 3-BG, 5-BG and 8-BG were characterized by highest antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Listeria ivanovii* ATCC 19119, *Listeria innocua* ATCC 33090, *Enterococcus faecalis* ATCC 19433 and *Enterococcus faecuim* ATCC 19434. *L. bulgaricus* 8-BG was active also against the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 9027, *Proteus vulgaris* ATCC 6380, *Salmonella enteritidis* ATCC 13076, *Salmonella abony* NTCC 6017 and *Escherichia coli* ATCC 25922. In contrast, Italian *L. bulgaricus* strains demonstrated low antimicrobial activity. Bulgarian *L. bulgaricus* strains showed moderate sensitivity or resistance to most of the antibiotics used in the screening, while Italian *L. bulgaricus* strains were sensitive. Bulgarian *L. bulgaricus* strains 1-BG and 6-BG were resistant to 10 and 13 of a total of 24 antibiotics tested, respectively. Nisin resistance test showed that 10 of a total of 11 *L. bulgaricus* strains were highly sensitive to nisin (MIC values varying from 0.078 mg/mL to 0.156 mg/mL), except of Italian strain *L. bulgaricus* 6-IT which was resistant to nisin.

## 1. Introduction

Lactobacilli are lactic acid bacteria (LAB) that have been successfully used for many centuries in fermentation processes in the production of various foods. The life of modern people and associated "diseases of the 21-st century" have focused the scientific and commercial interests on the use of lactobacilli in a number of functional foods and probiotic products that have a positive impact on intestinal microflora, thus exert beneficial effect on the physiological status. Nowadays, they are widely accepted as useful component of the food chain, with a substantial role in

maintaining a healthy balance and preventing diseases in humans and animals.

*Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*), isolated and described for a first time by the Bulgarian physician Dr. Stamen Grigoroff (1905), is the most economically important representative of the heterogeneous group of LAB with global application in the manufacture of fermented and functional foods, in particular yogurt. One of the most popular varieties of yogurt is the traditional Bulgarian yogurt, which has been consumed for millennia as a traditional food on the Balkans, known also as sour milk or "kiselo mlyako" due to its specific taste. Yogurt is recognized as a nutritious, natural and safe component of the healthy diet, which has beneficial effects on the gastrointestinal microflora and underlies the concept of probiotic foods. The benefits for human health from the consumption of yogurt are well known, and in recent years numerous studies have proven its therapeutic effects on various disorders (Adolfsson et al., 2004; McKinley, 2005; Fisberg and Machado, 2015).

The selection of appropriate *Lactobacillus* strains with useful properties is of paramount importance in the development of successful technological schemes in the dairy industry. *Lactobacillus* strains used as starter cultures or in composition of probiotic foods, should be carefully selected not only for their contribution to the organoleptic properties of the product (aroma, taste and texture), but also for their biological activities. In addition to the generally accepted requirement to be "generally recognized as safe" (GRAS), the probiotic strains must meet some other basic criteria related to their survival *in vivo* and probiotic role, such as resistance to the conditions in the gastrointestinal tract (Chen et al., 2017), adhesion to the intestinal epithelial cells, antimicrobial activity (Silva et al., 2020), and resistance to antibiotics (Havenaar et al., 1992; Amara et al., 2019).

The use of *L. bulgaricus* strains and their metabolites as biopreservatives in order to improve the shelf-life of various foods requires selection of strains that possess high antimicrobial activity (Mohammed et al., 2013). Another desired feature in the selection of *L. bulgaricus* strains is their resistance to antibiotics. The susceptibility of *L. bulgaricus* to different antibiotics used in the clinical practice for treatment of intestinal and other infections is considered undesirable, and in these cases antibiotic therapy reduces the effectiveness of probiotics and functional foods. On the other hand, the natural resistance of some *L. bulgaricus* strains may also have a negative side, as they can serve as sources of genes responsible for antibiotic resistance to be genetically transferred to the pathogenic bacteria, thus to threat human and animal health. Therefore, before including the lactobacilli strains in the composition of starter cultures or probiotic products, it is necessary to check whether these strains do not have transferable genes for antibiotic resistance (Danielsen and Wind, 2003; Karapetkov et al., 2011).

The application of some LAB bacteriocins (nisin) as biopreservatives in fermented products to prolong the shelf-life and control the pathogenic and spoilage microorganisms may have a negative impact on the strains in the starter culture and lactic acid fermentation. In this regard, the selection of *L. bulgaricus* strains resistant or weakly sensitive to nisin is also important for the technological process in the manufacture of quality fermented products.

Therefore, the aim of the present study was to isolate and identify *L. bulgaricus* strains from traditional Bulgarian and Italian yogurts, and to evaluate and compare some important properties of the strains such as antimicrobial activity, nisin resistance and antibiotic susceptibility.

## 2. Materials and methods

### 2.1. Materials

#### *2.1.1. Yogurts*

Fourteen samples of homemade yogurts (eight Bulgarian and six Italian) were used in the study.

### *2.1.2. Test microorganisms*

Six Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecuim* ATCC 19434) and six Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Proteus vulgaris* ATCC 6380, *Salmonella enteritidis* ATCC 13076, *Salmonella abony* NTCC 6017, *Escherichia coli* ATCC 25922, *Klebsiella* sp. – clinical isolate) from the collection of the Department of Microbiology, University of Food Technologies, Plovdiv, Bulgaria were used in the antimicrobial screening.

### *2.1.3. Nisin*

Nisin produced by *Lactococcus lactis* subsp. *lactis* - 2.5% (Sigma-Aldrich, USA), containing 1000000 IU active substance/g was used.

### *2.1.4. Culture media*

***Milk.*** Milk was prepared by the following prescription: 80 g of skim milk was dissolved in 1 L of deionized water, and then autoclaved at 112 °C for 45 min.

***LAPTg10 broth.*** LAPTg10 broth was prepared by the manufacturer's (Laboratorios Conda S.A., Spain) prescription: 45 g of LAPTg10-solid substance mixture (containing 15 g peptone, 10 g yeast extract, 10 g tryptone and 10 g glucose) was dissolved in 1 L of deionized water. pH was adjusted to 6.6-6.8, and then 1mL Tween 80 (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 20 min.

***LAPTg10 agar.*** This medium was prepared by the following prescription: 45 g of LAPTg10-solid substance mixture (Laboratorios Conda S.A.) was dissolved in 1 L of deionized water. The final pH was adjusted to 6.6-6.8, and then 1mL Tween 80 (Sigma-Aldrich) and 15 g of agar (Sigma-Aldrich) were added. The medium was autoclaved at 121 °C for 20 min.

***Luria-Bertani agar medium supplemented with glucose (LBG agar).*** LBG agar was prepared by the manufacturer's (Laboratorios Conda S.A.) prescription: 50 g of LBG-solid substance mixture (containing 10 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g glucose and 15 g agar) was dissolved in 1 L of deionized water. The final pH was adjusted to 7.5, and then medium was autoclaved at 121 °C for 20 min.

***de Man, Rogosa and Sharpe (MRS) agar.*** This medium was prepared by the manufacturer's (Merck, Germany) prescription: 55.2 g of MRS broth (containing 10 g peptone, 5 g yeast extract, 10 g beef extract, 20 g glucose, 2 g potassium phosphate, 5 g sodium acetate, 0.2 g magnesium sulphate, 0.05 g manganese sulfate, 1 g Tween 80 and 2 g ammonium citrate) was dissolved in 1 L of deionized water. The final pH was adjusted to 6.4, and then 15 g of agar (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 15 min.

***Modified de Man, Rogosa and Sharpe (mMRS) agar.*** The modified MRS agar medium was prepared by the following prescription: 55.2 g of MRS broth (Merck) and 0.05 g L-cysteine (Merck) were dissolved in 1 L of deionized water. The final pH was adjusted to 6.4, and then 15 g of agar (Sigma-Aldrich) was added. The medium was autoclaved at 121 °C for 15 min.

***Modified skim milk (MSM) agar.*** This medium was prepared by the manufacturer's (Himedia®, India) prescription: 24.5 g of solid substance mixture (containing 5 g tryptone, 2.5 g yeast extract, 1 g glucose monohydrate, 1 g skim milk powder and 15 g agar) was dissolved in 1 L of deionized water. The final pH was adjusted to 7.0, and the medium was autoclaved at 121 °C for 15 min.

## 2.2. Methods

### *2.2.1. Isolation and cultivation of the strains*

Samples from yogurts were first propagated in milk (1 mL sample + 9 mL milk) and incubated at 42 °C for 3-4 h (until coagulation). Then samples were streaked on LAPTg10 agar medium, and the Petri plates (d=90 mm; Gosselin™, France) were incubated at 37 °C for 48 h. Single colonies were cut and

transferred into 2 mL of LAPTg10 broth, stirred by vortex V-1 plus (Biosan, Latvia) for 5-10 s, and cultured at 37 °C for 48 h. Next, 1 mL of the biomass was transferred into 5 mL of LAPTg10 broth and incubated under identical conditions, then stored at 4 °C for further analyses. The cellular morphology of the isolated strains was determined by microscopic observation of colored smears. The colony morphology of the isolates was described by microscopic observation of single colonies grown on LAPTg10 agar, MRS agar, mMRS agar and MSM agar media.

***2.2.2. Isolation of total DNA***

The isolation of DNA was performed by the method of Delley et al. (1990).

***2.2.3. 16S rDNA amplification***

16S rDNA of the isolates was amplified using the universal primers 27F (5'AGAG TTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') according to the method of Lane (1991). The amplification program included: denaturation – 95 °C for 3 min; 40 cycles – 93 °C for 30 s, 55 °C for 60 s, 72 °C for 2 min; final elongation – 72 °C for 5 min.

***2.2.4. Purification of the product of the PCR-reaction from TAE agarose gel***

The purification of 16S rDNA was conducted using a DNA-purification kit in a Microspin$^{TM}$ column following the standard protocol (Denkova et al., 2012).

***2.2.5. Sequencing of the 16S rRNA gene***

The partial sequencing of the 16S rRNA gene with two universal primers (27F and 1492R) was implemented according to the method of Sanger et al. (1977) at "Macrogen Europe Laboratory", The Netherlands.

The entire sequence of the 16S rRNA gene was obtained using the CLC Sequence Viewer software, and the resulting whole sequence was compared with the on-line database sequences via the BLASTn algorithm. Thus, the studied strains were identified to the species level with the corresponding confidence level.

***2.2.6. Antimicrobial activity***

The antimicrobial activity of *L. bulgaricus* strains was determined by the agar well diffusion method. The test bacteria were cultivated on LBG agar medium at 37 °C for 24 h. To prepare bacterial inocula, a small amount of biomass of each test microorganism was suspended in 5 mL of sterile 0.5% NaCl, and then vortexed (V-1 plus, Biosan) for 5-10 s. The number of viable cells in each bacterial inoculum was determined using the counting chamber Thoma (Poly-Optik GmbH, Germany), and the final concentration was adjusted to $1.0\times10^8$ cfu/mL. Next, bacterial suspensions were inoculated in preliminarily melted and tempered at 45–48 °C LAPTg10 agar media. The inoculated media were transferred in quantity of 18 mL in sterile Petri plates (Gosselin™) and allowed to harden. Then six wells (d=6 mm) per plate were cut.

*L. bulgaricus* strains (cultured in LAPTg10 broth at 37 °C for 48 h) were pippeted into the agar wells in quantity of 60 μL. As a control, the antibiotic Ampicillin (10 mg/mL) was used. The Petri plates (Gosselin™) were incubated at identical conditions. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zones around the wells at the 24-th and 48-th h after incubation. The results were interpreted as follows: high antimicrobial activity - inhibition zones of 18 mm or more; moderate antimicrobial activity - inhibition zones from 12 to 18 mm; low antimicrobial activity - inhibition zones under 12 mm (Tumbarski et al., 2018a).

***2.2.7. Nisin resistance test and minimal inhibitory concentration***

Nisin resistance and minimal inhibitory concentration (MIC) of nisin were determined by the dilution method. Two-fold serial dilutions of nisin in sterile distilled water, ranging from 10 mg/mL to 0.0049 mg/mL were prepared. 60 μL of dilution was pipetted in wells cut in LAPTg10 agar media inoculated with each *L. bulgaricus* strain. The Petri plates (Gosselin™) were incubated at 37 °C for 48 h. The MIC values were determined as the lowest concentration of nisin inhibiting completely the growth of each *L. bulgaricus* strain around the agar well (Tumbarski et al., 2017).

### *2.2.8. Antibiotic susceptibility test*

Antibiotic susceptibility test was performed by *in vitro* disc diffusion method of Bauer et al. (1966) with impregnated paper discs of 24 antibiotics (Bul Bio - NCIPD Ltd., Bulgaria). The strain suspensions (0.1 mL) were spread plated on LAPTg10 agar medium, and then four discs of different antibiotics per Petri plate (Gosselin™) were put on the surface of the agar medium. The plates were incubated at 37 °C for 48 h. Zones of inhibition were measured and recorded at 24-th and 48-th h of incubation. Strains with no inhibition zones were considered resistant; with inhibition zones from 7 to 16 mm – intermediate sensitive; with zones >16 mm – sensitive.

### *2.2.9. Statistical analysis*

Data from triplicate experiments for the antimicrobial activity were processed with MS Office Excel 2010 software, using statistical functions to determine the standard deviation (±SD), and maximum estimation error at significance level ά <0.05.

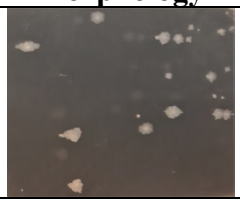
## 3.Results and discussions

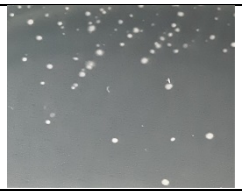
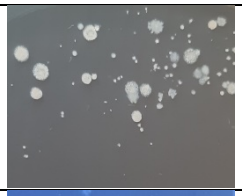
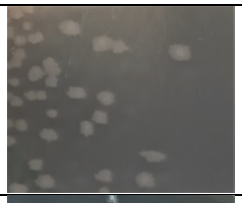
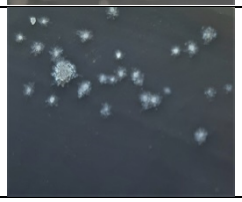
### 3.1. Strain isolation, cellular and colony morphology

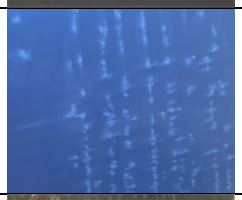
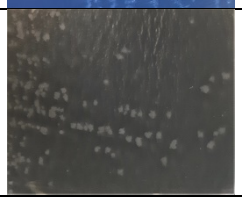
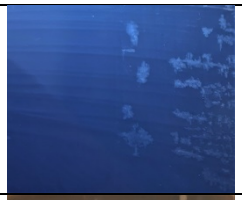
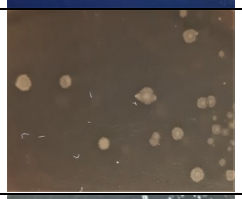
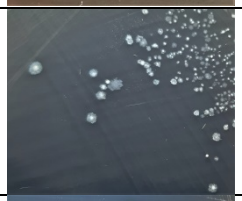
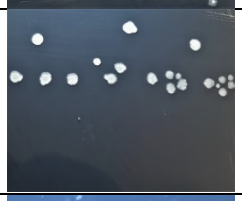
In the present study, six *L. bulgaricus* strains were isolated from eight traditional Bulgarian yogurts (1-BG, 3-BG, 5-BG, 6-BG, 7-BG and 8-BG) and five *L. bulgaricus* strains were isolated from six Italian yogurts (1-IT, 3-IT, 4-IT, 5-IT and 6-IT).

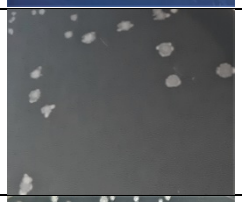
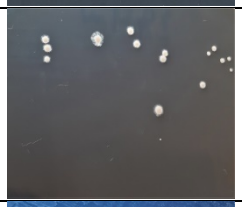
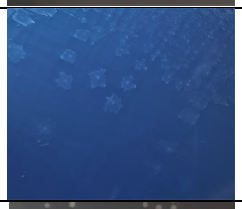
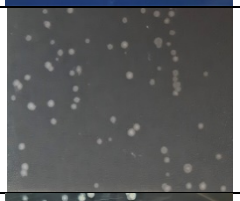
The microscopic observation of colored smears showed that all isolated strains possessed the typical cellular morphology for *L. bulgaricus* – Gram-positive, rod shaped with rounded ends (0.5–0.8 μm × 2–9 μm) or filamentous (0.5–0.8 μm × 20–25 μm) arranged singly, in short or longer chains (figures not provided), which depends on the age of the culture and the composition of the growth medium (Teixeira, 2014). The colony morphology of the isolates was determined by microscopic observation of single colonies. To study the influence of the medium composition on *L. bulgaricus* colony characteristics, the strains were cultured on different agar media used for isolation, enumeration and differentiation of lactobacilli from milk, yogurt, cheese, and other fermented milk products - LAPTg10, MRS, mMRS and MSM. The colonies of all isolated strains exhibited the typical characteristics for *L. bulgaricus colonies* – 1-6 mm in diameter, whitish, round or irregular shaped, flat profile, with or without dot-like center, serrated edges, rough surface, and soft texture, similar to those observed by Tabasco et al. (2007), Nwamaioha and Ibrahim (2018), and Oyeniran et al. (2020). In the present study, *L. bulgaricus* strains were affected by the type and composition of the agar media, and formed colonies with different morphology parameters (mainly size and shape), or did not grow on all agar media. Good growth of all strains was observed only on LAPTg10 agar medium (Table 1).

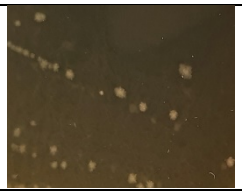
**Table 1.** Colony morphology of *L. bulgaricus* strains on different agar media for lactobacilli

| Strain | Culture medium | Colony morphology | Colony description |
|---|---|---|---|
| **1-BG** | LAPTg10 agar |  | 1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar | | No growth |
| | MSM agar | | No growth |

| | | | |
|---|---|---|---|
| **3-BG** | LAPTg10 agar |  | 1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar |  | 1-3 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |
| | MSM agar |  | 1-3 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture |
| **5-BG** | LAPTg10 agar |  | 1-4 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar |  | 2-3 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture |
| | MSM agar | | No growth |
| **6-BG** | LAPTg10 agar |  | 1-6 mm in diameter, whitish, flat, irregular shaped, serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar | | No growth |
| | MSM agar | | No growth |
| **7-BG** | LAPTg10 agar |  | 2-6 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture |
| | MRS agar |  | 1-3 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |

| | | | |
|---|---|---|---|
| | mMRS agar |  | 1-5 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |
| | MSM agar |  | 1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |
| **8-BG** | LAPTg10 agar |  | 1-2 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar | | No growth |
| | MSM agar |  | 1-5 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |
| **1-IT** | LAPTg10 agar |  | 1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture |
| | MRS agar |  | 1-3 mm in diameter, whitish, flat, round or irregular shaped with dot-like center, serrated edges, rough surface, soft texture |
| | mMRS agar |  | 1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture |
| | MSM agar |  | 1-2 mm in diameter, whitish, flat, round shaped with dot-like center, serrated edges, rough surface, soft texture |
| **3-IT** | LAPTg10 agar |  | 1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |

| | | | |
|---|---|---|---|
| | MRS agar | | No growth |
| | mMRS agar | | No growth |
| | MSM agar |  | 1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture |
| **4-IT** | LAPTg10 agar |  | 1-6 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture |
| | MRS agar |  | 1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |
| | mMRS agar |  | 1-4 mm in diameter, whitish, flat, round or irregular shaped, serrated edges, rough surface, soft texture |
| | MSM agar |  | 2-6 mm in diameter, whitish, flat, round or irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture |
| **5-IT** | LAPTg10 agar |  | 1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |
| | MRS agar |  | 1-2 mm in diameter, whitish, flat, round shaped with dot-like center, serrated edges, rough surface, soft texture |
| | mMRS agar |  | 1-3 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |
| | MSM agar |  | 1-2 mm in diameter, whitish, flat, round shaped, serrated edges, rough surface, soft texture |

| | | | |
|---|---|---|---|
| **6-IT** | LAPTg10 agar |  | 1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like) with dot-like center, serrated edges, rough surface, soft texture |
| | MRS agar | | No growth |
| | mMRS agar | | No growth |
| | MSM agar |  | 1-4 mm in diameter, whitish, flat, irregular shaped (snowflake-like), serrated edges, rough surface, soft texture |

### 3.2. Strain identification

Besides the standard methods used for characterization, the species and strain identification require application of some rapid and reliable molecular genetic techniques such as polymerase chain reaction (PCR), amplified ribosomal DNA restriction analysis (ARDRA) and nucleotide sequencing.

The results obtained from conventional PCR amplification of 16S rDNA, followed by nucleotide sequencing and comparative 16S rRNA gene sequence-based phylogenetic analysis revealed that all of the studied isolates belonged to the bacterial species *Lactobacillus delbrueckii* subsp. *bulgaricus*. The isolates exhibited 92% (strain 5-BG), 97% (strain 6-BG) and 99% (strains 1-BG, 3-BG, 7-BG, 8-BG, 1-IT, 3-IT, 4-IT, 5-IT and 6-IT) pairwise similarity of the sequence of 16S rDNA to the partial sequence of 16S rDNA of the relevant reference *L. bulgaricus* strains (Table 2).

Molecular genetic methods have been successfully applied in a number of previous studies related to the identification, genotyping and grouping of *Lactobacillus* strains isolated from different dairy and non-dairy fermented products (Andrighetto et al., 1998; Markiewicz et al., 2010; Denkova et al., 2012; Yu et al., 2015).

**Table 2.** Pairwise similarity of the sequences of 16S rDNA of the isolated *L. bulgaricus* strains and the partial sequences of 16S rDNA of the reference *L. bulgaricus* strains

| Isolate | Reference strain | Identity/similarity | Gaps | Strand |
|---|---|---|---|---|
| **1-BG** | *L. delbrueckii* subsp. *bulgaricus* IMAU40160 | 1398/1407 (99%) | 3/1407 (0%) | Plus/Plus |
| **3-BG** | *L. delbrueckii* subsp. *bulgaricus* 3641 | 1435/1445 (99%) | 4/1445 (0%) | Plus/Plus |
| **5-BG** | *L.delbrueckii* subsp. *bulgaricus* A320 | 967/1053 (92%) | 15/1053 (1%) | Plus/Minus |
| **6-BG** | *L. delbrueckii* subsp. *bulgaricus* 4640 | 707/731 (97%) | 8/731 (1%) | Plus/Plus |
| **7-BG** | *L. delbrueckii* subsp. *bulgaricus* LJJ | 1424/1434 (99%) | 2/1434 (0%) | Plus/Minus |
| **8-BG** | *L. delbrueckii* subsp. *bulgaricus* 4133 | 1423/1431 (99%) | 1/1431 (0%) | Plus/Plus |

| | | | | |
|---|---|---|---|---|
| **1-IT** | *L. delbrueckii* subsp. *bulgaricus* 4119 | 1441/1449 (99%) | 4/1449 (0%) | Plus/Plus |
| **3-IT** | *L. delbrueckii* subsp. *bulgaricus* 3789 | 1436/1454 (99%) | 8/1454 (0%) | Plus/Plus |
| **4-IT** | *L. delbrueckii* subsp. *bulgaricus* KLDS1.1011 | 1410/1427 (99%) | 6/1427 (0%) | Plus/Minus |
| **5-IT** | *L. delbrueckii* subsp. *bulgaricus* 3913 | 1447/1458 (99%) | 8/1458 (0%) | Plus/Plus |
| **6-IT** | *L. delbrueckii* subsp. *bulgaricus* 4017 | 1419/1420 (99%) | 0/1420 (0%) | Plus/Plus |

### 3.3. Antimicrobial activity

Antimicrobial activity is an important criterion for selection of starter and probiotic cultures as natural antagonists of food spoilage and pathogenic microorganisms (Georgieva et al., 2015).

The results demonstrated that Bulgarian *L. bulgaricus* strains were characterized by higher antimicrobial activity compared to the Italian strains, expressed mainly against Gram-positive bacteria (Table 3). *L. bulgaricus* strains 3-BG, 5-BG and 8-BG exhibited the highest antimicrobial activity inhibiting the growth of *S. aureus* ATCC 25923, *L. monocytogenes* NBIMCC 8632, *L. ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *E. faecalis* ATCC 19433 and *E. faecuim* ATCC 19434. *L. bulgaricus* strains 3-BG and 8-BG were active also against Gram-negative *P. aeruginosa* ATCC 9027, *P. vulgaris* ATCC 6380 (only 8-BG), *S. enteritidis* ATCC 13076, *S. abony* NTCC 6017 and *E. coli* ATCC 25922. Among Italian strains, *L. bulgaricus* 4-IT showed highest antimicrobial activity against all Gram-positive bacteria used in the study, and Gram-negative *S. enteritidis* ATCC 13076 and *S. abony* NTCC 6017. The rest of the *L. bulgaricus* strains (1-BG, 6-BG, 7-BG, 1-IT, 3-IT, 5-IT and 6-IT) demonstrated weak antimicrobial effect. None of *L. bulgaricus* strains inhibited the test microorganism *Klebsiella* sp.

In recent years, several studies have revealed the technological properties and probiotic characteristics of *L. bulgaricus* strains such as antimicrobial activity, nisin resistance, and antibiotic susceptibility. Akpinar et al. (2011) ascertained that 17 of

25 *L. bulgaricus* strains isolated from homemade yogurts possessed antimicrobial activity against *Klebsiella pneumoniae*, 16 of 25 were active against *Bacillus cereus* and *Pseudomonas fluorescens*, 18 of 25 were active against *L. monocytogenes*, 6 of 25 were active against *S. aureus*, 3 of 25 were active against *Bacillus coagulans*, and all of them inhibited the growth of *E. coli*.

In contrast, Erdogrul and Erbilir (2006) reported weak antimicrobial activity (inhibition zones <12 mm) of *L. bulgaricus* strain isolated from the probiotic dairy product against *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 18833, *Salmonella typhimurium* ATCC 13311 and *Enterobacter cloacae* ATCC 13047. The strain was inactive against *Bacillus subtilis*. Georgieva et al. (2015) investigated antimicrobial activity of five *L. bulgaricus* strains isolated from homemade yogurts, and found that all isolates had significant inhibitory effect on all tested bacteria - *S. aureus* NBIMCC 3703, *B. cereus* NBIMCC 1085, and *E. coli* NBIMCC 3702.

**Table 3.** Antimicrobial activity of the isolated *L. bulgaricus* strains

| Test microorganism | *L. bulgaricus strains*, inhibition zones (mm) | | | | | | | | | | | Control * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1-BG | 3-BG | 5-BG | 6-BG | 7-BG | 8-BG | 1-IT | 3-IT | 4-IT | 5-IT | 6-IT | |
| **Gram (+) bacteria** | | | | | | | | | | | | |
| ***S. aureus* ATCC 25923** | 8±0.15** | 13±0.21 | 12±0.23 | 8±0.17 | - | 11±0.26 | 8±0.11 | 10±0.23 | 9±0.12 | 8±0.11 | 8±0.17 | 40±0.29 |
| ***L. monocytogenes* NBIMCC 8632** | - | 12±0.23 | 12±0.25 | 8±0.18 | 8±0.12 | 12±0.29 | - | 8±0.15 | 8±0.18 | - | - | 38±0.27 |
| ***L. ivanovii* ATCC 19119** | 8±0.11 | 10±0.2 | 12±0.29 | - | 10±0.23 | 12±0.24 | 10±0.26 | 10±0.29 | 10±0.24 | 8±0.16 | 10±0.2 | 40±0.24 |
| ***L. innocua* ATCC 33090** | - | 11±0.24 | 12±0.2 | 8±0.16 | 11±0.28 | 13±0.2 | 8±0.17 | 10±0.21 | 10±0.28 | 8±0.13 | 10±0.23 | 40±0.22 |
| ***E. faecalis* ATCC 19433** | - | 10±0.22 | 10±0.19 | 10±0.27 | 9±0.19 | 12±0.22 | 8±0.13 | 9±0.14 | 10±0.22 | 8±0.18 | 8±0.15 | 37±0.2 |
| ***E. faecuim* ATCC 19434** | 8±0.14 | 9±0.16 | 8±0.18 | 8±0.14 | 9±0.2 | 10±0.21 | - | - | 8±0.16 | - | - | 30±0.21 |
| **Gram (-) bacteria** | | | | | | | | | | | | |
| ***P. aeruginosa* ATCC 9027** | 8±0.12 | 8±0.17 | - | - | - | 8±0.18 | - | - | - | - | - | 13±0.19 |
| ***P. vulgaris* ATCC 6380** | - | - | - | - | - | 11±0.25 | - | - | - | - | - | 25±0.2 |
| ***S. enteritidis* ATCC 13076** | - | 9±0.14 | 8±0.13 | - | - | 10±0.22 | 8±0.16 | - | 8±0.19 | 8±0.12 | - | 32±0.25 |
| ***S. abony* NTCC 6017** | - | 11±0.25 | 8±0.11 | 8±0.15 | - | 10±0.28 | - | - | 8±0.14 | - | - | 29±0.28 |
| ***E. coli* ATCC 25922** | - | 8±0.19 | - | - | - | 8±0.15 | - | - | - | - | - | 30±0.23 |
| ***Klebsiella* sp. (clinical isolate)** | - | - | - | - | - | - | - | - | - | - | - | - |

*Ampicillin (10 mg/ml); **standard deviation (±SD); $d_{well}$=6mm

### 3.4. Nisin resistance and minimal inhibitory concentration (MIC)

In our study, nisin resistance test showed that 10 of a total of 11 *L. bulgaricus* strains were highly sensitive to the action of nisin (MIC values varying from 0.078 mg/ml for *L. bulgaricus* 3-BG, 6-BG, 1-IT, 3-IT, and 4-IT to 0.156 mg/ml for *L. bulgaricus* 1-BG, 5-BG, 7-BG, 8-BG, and 5-IT) with the exception of Italian strain *L. bulgaricus* 6-IT which was resistant to nisin (Table 4).

**Table 4.** Nisin resistance of the isolated *L. bulgaricus* strains and minimal inhibitory concentration (MIC)

| **Nisin** | ***L. bulgaricus* strains** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1-BG** | **3-BG** | **5-BG** | **6-BG** | **7-BG** | **8-BG** | **1-IT** | **3-IT** | **4-IT** | **5-IT** | **6-IT** |
| **MIC, mg/ml** | 0.156 | 0.078 | 0.156 | 0.078 | 0.156 | 0.156 | 0.078 | 0.078 | 0.078 | 0.156 | Resistant |

Lactobacilli, in particular *L. bulgaricus* strains are essential for food fermentation processes and food preservation, whether they are part of the natural microflora of the products or added as starter cultures.

One of the advanced approaches in food biopreservation is the application of bacteriocins – peptides with antimicrobial activity produced by some LAB and members of genus *Bacillus*. Nisin (E-234) is a bacteriocin synthesized by *Lactococcus lactis* subsp. *lactis*, which is officially approved as a "Generally Recognized as Safe" (GRAS), and widely used as a food additive and biopreservative in the food industry. Nisin is known to possesses strong inhibitory activity against Gram-positive bacteria, including LAB (Tumbarski et al., 2018b). However, the application of bacteriocins can disrupt the fermentation by inhibiting the LAB. In this regard, the selection of *L. bulgaricus* strains resistant to nisin is of great importance for the technological process in the production of stable and high-quality fermented products.

Inhibitory effect of nisin and sensitivity of *L. bulgaricus* strains have been demonstrated in some previous studies. Durlu-Özkaya et al. (2007) examined the exopolysaccharide (EPS) production of 20 *L. bulgaricus* isolates from homemade yogurt and raw milk, and the correlation between EPS production and sensitivity of the strains to bacteriophages and nisin. The authors found that *L. bulgaricus* strains with high EPS-producing capacity were resistant to phages and nisin, and concluded that they are perspective as starter cultures in commercial yogurt production. On the other hand, the antimicrobial activity of nisin against pathogenic microorganisms and LAB depends on the dosage and storage conditions. Benkerroum et al. (2002) stated that in yogurt with addition of nisin at a dose of 10 RU/mL, no *L. monocytogenes* survived at 24-th hour of storage at refrigeration conditions (7 °C). However, the pathogen survived 13 days of storage at the same temperature in the controls (without addition of nisin). The authors concluded that nisin inhibited the yogurt fermentation at concentration higher than 50 RU/mL, and recommended its application in lower doses in order to control the growth of *L. monocytogenes*, and to prevent the excessive acidification normally observed in the end of the storage life of yogurt.

### 3.5. Antibiotic susceptibility

In addition to antimicrobial activity and nisin resistance, the antibiotic resistance is another substantial criterion for selection of appropriate functional strains. The results showed that Bulgarian *L. bulgaricus* strains were moderately sensitive (inhibition zones 7-16 mm) or resistant to most of the 24 antibiotics used in the screening (Table 5). The most resistant among the Bulgarian strains was *L. bulgaricus* 6-BG, which was insensitive to 13 of 24 antibiotics tested (bacitracin, penicillin, oxacillin, amoxicillin, tetracycline, doxycycline, gentamicin, amikacin, rifampin, clarithromycin, chloramphenicol, ciprofloxacin and nalidixic acid), followed by *L. bulgaricus* 1-BG, which was resistant to 10 antibiotics (penicillin, ampicillin, amoxicillin, tetracycline, gentamicin, amikacin, clarithromycin, chloramphenicol, novobiocin and norfloxacin). *L. bulgaricus* 3-BG was resistant to penicillin, ampicillin, tetracycline, lincomycin, novobiocin, ciprofloxacin, nalidixic acid and sulfamethoxazole/trimethoprim. *L. bulgaricus* 5-BG was resistant to bacitracin, penicillin, amoxicillin, vancomycin, tetracycline, gentamicin, rifampin and nalidixic acid. *L. bulgaricus* 8-BG exhibited resistance to piperacillin, vancomycin, cefuroxime, doxycycline, rifampin, erythromycin, clarithromycin and sulfamethoxazole/ trimethoprim, while *L. bulgaricus* 7-BG was resistant to 5 antibiotics – rifampin, lincomycin, tobramycin, novobiocin and norfloxacin. In contrast, Italian *L. bulgaricus* strains were sensitive to almost all of total of 24 antibiotics tested. *L. bulgaricus* 5-IT was resistant to ampicillin, levofloxacin and sulfamethoxazole/trimethoprim; *L. bulgaricus* 1-IT was resistant to levofloxacin and sulfamethoxazole/trimethoprim; *L. bulgaricus* 3-IT and *L. bulgaricus* 4-IT – to sulfamethoxazole/ trimethoprim; *L. bulgaricus* 6-IT – to levofloxacin.

The antibiotic susceptibility of *L. bulgaricus* strains varies widely. Georgieva et al. (2015) examined antibiotic susceptibility of five *L. bulgaricus* isolates to nine antibiotics, and found that all tested strains were susceptible toward ampicillin, gentamicin, erythromycin and tetracycline. The results reported by Erdogrul and Erbilir (2006) demonstrated that *L. bulgaricus* strain isolated from probiotic product was moderately sensitive to ampicillin, vancomycin, oxacillin, cephalothin, and cefodizime, but highly sensitive to tobramycin. Kyriacou et al. (2008) investigated the antibiotic resistance of 91 *L. bulgaricus* strains isolated from different commercial Greek yogurts, and found that 97.8% from them were resistant to ciprofloxacin, 65.9% resistant to kanamycin, 62.6% resistant to amikacin, 1.1% resistant to vancomycin, and 1.1% resistant to bacitracin.

**Table 5.** Antibiotic susceptibility of the isolated *L. bulgaricus* strains

| **Antibiotic** | | | ***L. bulgaricus* strains** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **µg/disc** | **MA***** | **1-BG** | **3-BG** | **5-BG** | **6-BG** | **7-BG** | **8-BG** | **1-IT** | **3-IT** | **4-IT** | **5-IT** | **6-IT** |
| **Bacitracin** | 0.07* | Inhibitors of the cell wall synthesis | S | S | R | R | S | S | S | S | S | S | S |
| **Piperacillin** | 100 | | SR | S | S | S | S | R | S | S | S | S | S |
| **Penicillin** | 10* | | R | R | R | R | SR | S | S | S | S | S | S |
| **Ampicillin** | 10 | | R | R | SR | S | SR | SR | SR | S | SR | R | SR |
| **Oxacillin** | 1 | | S | SR | S | R | S | SR | S | S | S | SR | S |
| **Amoxicillin** | 25 | | R | S | R | R | SR | S | S | S | S | S | S |
| **Vancomycin** | 30 | | S | S | R | S | S | R | S | S | S | S | S |
| **Cefuroxime** | 30 | | S | S | S | S | SR | R | SR | SR | S | S | S |
| **Tetracycline** | 30 | Inhibitors of the protein synthesis | R | R | R | R | S | S | S | S | S | S | S |
| **Doxycycline** | 30 | | S | SR | S | R | S | R | S | S | S | S | S |
| **Gentamicin** | 10 | | R | SR | R | R | S | S | S | S | S | S | S |
| **Amikacin** | 30 | | R | S | S | R | S | S | S | S | SR | S | S |
| **Rifampin** | 5 | | SR | SR | R | R | R | R | S | S | S | S | S |
| **Lincomycin** | 15 | | S | R | SR | S | R | S | SR | S | S | S | SR |
| **Tobramycin** | 10 | | SR | S | S | S | R | SR | SR | S | SR | S | SR |
| **Erythromycin** | 15 | | S | SR | SR | S | SR | R | S | S | S | S | S |
| **Clarithromycin** | 15 | | R | S | SR | R | SR | R | S | S | S | S | S |
| **Chloramphenicol** | 30 | | R | S | SR | R | S | S | S | S | S | S | S |
| **Novobiocin** | 5 | Inhibitors of the DNA synthesis or cell division | R | R | SR | SR | R | S | S | S | S | S | S |
| **Ciprofloxacin** | 5 | | S | R | SR | R | SR | S | SR | SR | S | S | SR |
| **Norfloxacin** | 10 | | R | SR | SR | S | R | SR | SR | SR | S | S | SR |
| **Levofloxacin** | 5 | | SR | SR | SR | S | SR | SR | R | S | S | R | R |
| **Nalidixic acid** | 30 | | S | R | R | R | SR | SR | SR | SR | SR | SR | SR |
| **Sulfamethoxazole/ Trimethoprim**** | 23.75/ 1.25 | | SR | R | S | S | SR | R | R | R | R | R | SR |

*E/disc; ** - inhibits also the protein synthesis; ***MA – mechanism of action; R – resistant; SR – intermediate (zone 7-16 mm); S – sensitive (zone >16 mm); $d_{disc}$=6mm

## 4. Conclusions

In the present study, 11 *L. bulgaricus* strains from traditional Bulgarian and Italian homemade yogurts were isolated and identified, and their antimicrobial activity, nisin resistance and antibiotic susceptibility were determined and compared. The antimicrobial screening demonstrated that Bulgarian *L. bulgaricus* strains were characterized by higher antimicrobial activity compared with the Italian strains, which was most pronounced in *L. bulgaricus* 3-BG, 5-BG and 8-BG. The results from nisin resistance test showed that only one of the isolates was resistant to nisin (*L. bulgaricus* 6-IT). The antibiotic susceptibility test revealed that Bulgarian *L. bulgaricus* isolates were resistant or moderately sensitive to most of the antibiotics used, in comparison with the Italian strains, which were sensitive. The obtained results are important from a practical point of view, in the selection of suitable *L. bulgaricus* strains for use as starter cultures, probiotics, or in order to improve the food quality and safety. The future perspectives of the present research will be directed to further investigation of other probiotic and technological properties of the newly isolated *L. bulgaricus* strains, and development of technological schemes for their successful application in the composition of probiotic products.

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