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Preparative Biochemistry & Biotechnology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

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To cite this article: Selma Kayacan Çakmakoğlu, Melike Vurmaz, Emine Bezirci, Yasemin Kaya, Hilal Dikmen, Hamza Göktaş, Fatmanur Demirbaş, Betül Encu, Esra Acar Soykut, Filiz Alemdar, İbrahim Çakır, M. Zeki Durak, Muhammet Arıcı, Osman Sağdıç, Mustafa Türker & Enes Dertli (2023) Isolation and characterization of yogurt starter cultures from traditional yogurts and growth kinetics of selected cultures under lab-scale fermentation, Preparative Biochemistry & Biotechnology, 53:4, 454-463, DOI: 10.1080/10826068.2022.2098325

To link to this article: https://doi.org/10.1080/10826068.2022.2098325

Published online: 18 Jul 2022.

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PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
2023, VOL. 53, NO. 4, 454–463
https://doi.org/10.1080/10826068.2022.2098325

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Isolation and characterization of yogurt starter cultures from traditional yogurts and growth kinetics of selected cultures under lab-scale fermentation

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ABSTRACT

The development of new starter cultures is a crucial task for the food industry to meet technological requirements and traditional products are important reservoirs for new starter cultures. In this respect, this study aimed to isolate, identify, and determine the technological characteristics of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains originated from traditional yogurt samples. Genotypic discrimination of 200 isolates revealed the presence of distinct 19 *S. thermophilus* and 11 *Lb. delbrueckii* subsp. *bulgaricus* strains as potential starter cultures. Strain-specific properties determined the acidification capacity of the yogurt starter cultures and a higher acidification capacity was observed for *S. thermophilus* strains compared to *Lb. delbrueckii* subsp. *bulgaricus* strains. Proteolytic activity was found between 0.012–0.172 and 0.078–0.406 for *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains, respectively. 4 of *S. thermophilus* and 3 of *Lb. delbrueckii* subsp. *bulgaricus* strains were found resistant to all tested bacteriophages. The antibiotic susceptibility tests of the isolates revealed that a very low antibiotic resistance was observed for the yogurt starter cultures. Finally, the growth kinetics of selected strains were determined and the maximum specific growth rate of selected *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* was calculated as 0.527 h^{-1} and 0.589 h^{-1}, respectively.

KEYWORDS

Growth kinetics; *Lb. delbrueckii* subsp. *bulgaricus*; *S. thermophilus*; yogurt starter culture

Introduction

The technological, physicochemical, and sensorial properties of fermented products including yogurt rely on the function and properties of starter Lactic Acid Bacteria (LAB).[1–3] Two LAB species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are responsible for yogurt production. The typical yogurt texture, aroma, and other physicochemical properties can be achieved following the symbiotic growth of these cultures in milk environment.[4,5] Different factors such as milk quality and processing conditions can be important for the properties of yogurt, but the selection of starter cultures is the crucial step for the final properties of yogurt such as aroma, acidification level, and viscosity.[5,6] Commercial yogurt starter cultures are available in the market with distinct technological properties such as high acid or low acid producers and ropy or non-ropy cultures.[4] Dairy farms aim to diversify their culture collections with respect to reaching different preferences of consumers as well as in terms of technological requirements. But bacteriophage contamination is one of the main problems for the dairy industry.[7] Rotation of yogurt starter cultures is still one of the main strategies to overcome the bacteriophage contamination risks.[8] These necessities motivate food scientists and researchers to find new *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains with distinct technological properties that can be used for yogurt production at industrial scale. In this respect, traditionally produced yogurt and yogurt-like products are important reservoirs to isolate and identify autochthonous yogurt starter cultures.[4,5,9,10] Finding new *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains from traditional products are not only important for the utilization of these cultures for industrial purposes but also important for the transfer of the traditional organoleptic properties of yogurt originating from these cultures to an industrial scale.[4,5] Turkey is one of the main countries that consume high levels of yogurt which was recently described as a cultural characteristic. It has been stated that 96% of households consume yogurt.[5] Also, the etymological background of the term yogurt as a Turkish word is another factor demonstrating the role of yogurt in Turkish food culture.[11] Together with the high level of industrial yogurt production, the traditional production of yogurt by back-slopping methodologyby individuals under household conditions is still an important part of yogurt production in Turkey.[12] Therefore, distinct *Lb.*

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delbrueckii subsp. *bulgaricus* and *S. thermophilus* strains can be isolated and identified with different technological properties from traditionally produced yogurts. Also, it has been stated that genetic modifications were applied to strains that had the potential to be used as a starter culture to develop their starter characterization. But, consumers generally prefer what is natural both in organisms and/or foods. Therefore, isolation of cultures from natural sources is a popular approach.[5]

In this regard, yogurt samples produced under traditional back-slopping conditions were collected from villages in ten distinct provinces of Turkey to isolate and identify the potential yogurt starter cultures. Isolation of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were conducted under specific medium and incubation conditions and isolates were subjected to genotypic discrimination using specific primer sets previously developed for these strains. Distinct *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were identified genotypically and the technological properties of both strains were determined in terms of acidification ability, acid production, proteolytic acitivity, phage resistance and antibiotic susceptibility. Finally, the growth kinetics of selected *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were observed under laboratory-scale fermentation conditions and growth parameters; μ max (specific growth $rate_{max}$), g (doubling time), n (number of generations), ϕ and ϕmax (productivity) were determined. This study demonstrates the diversity and wide range of technological performances of traditional yogurt starter cultures and reveals the growth characteristics of potential starter cultures for industrial utilization.

Materials and methods

Materials

All chemicals used in PCR were supplied from Thermo Fisher Scientific (US) and the other chemicals used in this study were obtained from Merck (Darmstadt, Germany).

Methods

Isolation of Lb. delbrueckii *subps.* bulgaricus *and* S. thermophilus *from traditional yogurt samples*

For the isolation of autochthonous yogurt starter cultures, yogurt samples were collected from 10 distinct provinces of Turkey selected based on their location, Bayburt, Bursa, Bingol, Malatya, Kayseri, Ordu, Erzurum, Erzincan, Trabzon and Elazıg. In these provinces, specific villages were selected based on their distances to the central districts, and the farthest was selected to increase the potential to isolate the autochthonous yogurt starter cultures. Traditional yogurt samples were collected in sterile, temperature-controlled bags. They were transferred to the laboratory on the same day and stored at 4 °C until the bacterial isolation process.

Serial dilutions were prepared from yogurt samples and from each dilution, aliquots were plated to modified growth agar mediums to specifically growt of yogurt starter cultures. For the isolation of *Lb. delbrueckii* subps. *bulgaricus*, a modified De Man, Rogosa and Sharpe (MRS) agar medium enriched with fructose (peptone from casein 18 g L^{-1}, D(+) glucose 20 g L^{-1}, yeast extract 4 g L^{-1}, di-Potasium hydrogen phosphate 2 g L^{-1}, tween 80 3 g L^{-1}, ammonium sulfate 2 g L^{-1}, sodium acetate 5 g L^{-1}, magnesium sulfate 0.2 g L^{-1}, fructose 10 g L^{-1}, cysteine 0.5 g L^{-1} and agar-agar 15 g L^{-1}) was used whereas for *S. thermophilus* isolation, M17 agar modified with 1% lactose was utilized. The corresponding agar plates were incubated at 45 °C for 24 h aerobically and 45 °C for 72 h anaerobically for *S. thermophilus* and *Lb. delbrueckii* subps. *bulgaricus* isolation, respectively.

Following the incubation period, colonies were selected based on their morphological features and typical colonies were subjected to Gram staining and catalase treatment. Gram + and catalase—colonies were then subjected to genotypic differentiation, discrimination, and identification.[1]

Genotypic detection, characterization, and identification of yogurt starter cultures

At first, *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* strains were PCR detected genotypically with specific primers developed previously for both strains.[13,14] For this, in total 200 colonies for each yogurt starter culture were subjected to genomic DNA extraction and genomic DNAs of LABs were extracted by phenol: chloroform: isoamyl alcohol methodology as described previously.[15] Genomic DNAs from each LAB isolates were then used as the templates for PCR analysis and PCRs were conducted with the primers specific for *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* to detect the corresponding strains.[16] For *Lb. delbrueckii* subps. *bulgaricus* primer set delF188 (forward 5′-CAACATGAGTCGCAT GATTCAAG-3′) and delR1042 (reverse 5′-GGAACCA CCTCTCTCTAGCTGTAG-3′)[14] and for *S. thermophilus* P1 (forward 5′-CACTATGCTCAGAATACA-3′) and P2 (reverse 5′-CGAACAGCATTGATGTTA-3′) were used and PCR conditions were described elsewhere.[16]

For the genotypic discrimination of the *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* strains, RAPD-PCR analysis using GTG5 primer were applied using the previously described methodology.[16] For this, PCR mixtures containing genomic DNA, 0.25 μL Taq polymerase (Thermo Fisher Scientific, US), 1.5 μL, 20 mM of GTG5 primer (5′-GTGGTGGTGGTGGTG-3′), 5 μL PCR buffer (Thermo Fisher Scientific, US), 4 μL, 20 mM dNTPS (Thermo Fisher Scientific, US), were prepared. The amplification was carried out with a model T100 thermal cycler (Bio-rad, US) and the RAPD-PCR was performed with the following program: 95 °C for 10 min, 35 cycles of 94 °C for 1 min, 40 °C for 1 min, 65 °C for 8 min, then final step of 65 °C for 16 min. The PCR products were separated with electrophoresis on 1% (wt/vol) agarose gels at 120 V for 1.5 hr and band patterns were visualized to discriminate potential distinct strains. Finally, distinct yogurt isolates were identified by 16S rRNA gene sequencing and details for 16S rRNA gene PCR and sequencing were given elsewhere.[15] The phylogenetic relationship of the isolates was tested by MEGA7

program, and the evolutionary history was inferred using the Neighbour-Joining methodology.[17]

Characterization of technological properties of yogurt starter cultures

Acidification ability of yogurt cultures. The acidification activity of *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* strains were determined by pH measurements during 10 h of time period (ΔpH). For this, following the activation of yogurt cultures from their glycerol stocks, cultures were inoculated to 100 mL skim milk at 2% ratio and left for incubation at 42 °C for 10 h. The pH of the skim milk broth was determined through the 10 h of incubation period at 2 h intervals and the ΔpH was determined with the following formula:

$$\Delta pH = pH \text{ at the initial measurement} - pH \text{ at the measurement time} \quad (1)$$

Acid production of yogurt cultures. To determine acid production of *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* strains in terms of lactic acid production, cultures were activated in MRS and M17 broths, respectively. From the activated cultures 2% of inoculum was applied to the corresponding broths and cultures were incubated at 42 °C for 24 h. Supernatants of the cultures were obtained following the centrifugation and acid production ability of the cultures were determined by titration which was applied with 0.1 N NaOH in the presence of phenolphthalein. The acid production ability of the cultures was expressed as % lactic acid. Because 1 mL of 0.1 mol/L NaOH neutralizes 0.009 g of lactic acid, TA was calculated by converting the volume of NaOH using equation in 2.

$$Lactic\ acid(\%) = \frac{V_{NaOH} \times 0.009g \times 0.1}{W} \times 100 \quad (2)$$

In this equation, factor, W = weight of sample for titration, V_{NaOH} = volume of NaOH used to neutralize the lactic acid, 0.1 = Normality of NaOH.[18]

Proteolytic activity of yogurt cultures. Proteolytic activity of *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* was determined using *o*-phthaldialdehyde (OPA) method described by Church et al.[19] Briefly, *Lb. delbrueckii* subps. *bulgaricus* and *S. thermophilus* strains were grown in MRS and M17 broths, respectively, at 42 °C for 24 h. After incubation, 5 mL of cells were washed with 0.32 mM sodium phosphate (pH 7.2) and resuspended in the same solvent. Cells were then inoculated 2% into reconstituted skim milk. *Lb. delbrueckii* subps. *bulgaricus* strains were incubated at 42 °C, *S. thermophilus* strains were incubated at 37 °C for 8 h. At the end of the incubation, 2.5 mL of sample was mixed with 10 mL 0.75 M trichloroacetic acid (TCA) and 0.5 mL distilled water. This mixture was incubated at room temperature for 10 min and then filtered Whatman 4 A filter paper. A 50 µL filtered sample was mixed with 1 mL *o*-phthaldialdehyde solution and incubated at room temperature for 2 min. After incubation, the absorbance of the mixture was measured at 340 nm. The determination of amino group was calculated according to the following equation:

$$\Delta OD = \text{Sample } OD_{340\ nm} - \text{Control } OD_{340\ nm} \quad (2)$$

Determination of phage resistance of yogurt cultures. Five *Lb. delbrueckii* subps. *bulgaricus* phages (Φ231LP8, Φ231LP24, ΦLPAA, ΦX19, ΦVILA) and five *S. thermophilus* phages (ΦB3S10, Φ7095-53, Φ46.1, Φ1B3A, Φ231-50) were used in this study which were isolated previously by Acar-Soykut and Tunail.[20] *Lb. delbrueckii* subps. *bulgaricus* strains and their phages were grown in MRS Broth, MRS Agar, and MRS Soft Agar. For better adsorption, 10 mmol/L $CaCl_2$ was added to MRS mediums. *S. thermophilus* and their phages were grown in modified M17 Broth, modified M17 Agar, and M17 Soft Agar. Yogurt starter cultures were grown at 42 °C for 18–24 h and phage resistance of the strains was evaluated according to whether clear plaque (lysis) was formed or not using the previously described methodology.[20]

Antibiotic susceptibility of yogurt cultures. For determination of the antibiotic resistance of yogurt starter cultures, *Lb. delbrueckii* subps. *bulgaricus* strains were cultivated in MRS broth and spread into MRS agar plates whereas *S. thermophilus* strains were cultivated in M17 broth and spread into M17 agar plates. Antibiotic disks, AM10: Ampicillin (10 µg/disk), C30: Chloramphenicol (30 µg/disk), CIP5: Ciprofloxacin (5 µg/disk), DO30: Doxycycline (30 µg/disk), E15: Erythromycin (15 µg/disk), CN10: Gentamicin (10 µg/disk), K30: Kanamycin (30 µg/disk), T30: Oxytetracycline (30 µg/disk), P10: Penicillin (P10 U), S10: Streptomycin (10 µg/disk), TE30: Tetracycline (30 µg/disk) were deposited on the plates which were then incubated at 42 °C for 24–48 h. Following the incubation period, the inhibition zones around the disks if present were calculated to identify the antibiotic susceptibility of tested yogurt cultures.[21]

Growth and fermentation kinetics of selected yogurt starter cultures

Strain and inoculum preparation. *Lb.* subsp. *bulgaricus* and *S. thermophilus*, isolated from yogurt, were stored in 15% glycerol at −80 °C. Frozen cells were precultured in MRS broth for *Lb. delbrueckii* subsp. *bulgaricus* and M17 broth *S. thermophilus* at 42 °C for 16 h before inoculation in the bioreactor.

Fermentation process. MRS broth was used as culture medium for *Lb. delbrueckii* subsp. *bulgaricus* in the bioreactors (Biostat A, Sartorius-Stedim). Batch cultivations were carried out with 1.5 L total volume at 44 °C in two parallels. pH was adjusted to pH 6.1 by the automatic addition of 5 N NaOH. Stirrer speed was 100 rpm and after sterilization, oxygen was removed by sparging N_2 for 30 minutes. The inoculum ratio was 1:20 (v/v) of the initial volume.

M17 broth was used as culture medium for *S. thermophilus* in the bioreactors (Biostat A, Sartorius-Stedim). Fed-batch cultivations were carried out with an initial volume of 1 L and added 500 mL of feeding solutions containing 150 g/L lactose at 40 °C in two parallels. After 3.5 h, the feeding solutions were pumped at a constant speed in the bioreactors during approximately 7 h. pH was adjusted to pH 6.5 by the automatic addition of 5 N NaOH. The stirrer speed was 100 rpm and after sterilization, oxygen was removed by sparging N_2 for 30 minutes. The inoculum ratio was 1:20 (v/v) of the initial volume.

Determination of cell numbers and lactic acid levels during scale-up fermentation and. Lactic acid was determined with HPLC using Hi-Plex H, 300 × 7.7 mm column (Agilent Technologies) with 0.6 mL/min flow rate at 55 °C column temperature and 5 mM H_2SO_4 as mobile phase. Glucose and lactose were determined with HPLC using Sugar SH1011, 8.0 mm x 300 mm column (Shodex) with 0.8 mL/min flow rate at 50 °C column temperature using 0.01 N H_2SO_4 as mobile phase. Lactic acid, glucose and lactose were obtained from Sigma Aldrich, Germany. Optical density of the cells was measured at 600 nm. The number of colony forming unit (cfu) was determined after 2 days of incubation on MRS agar for *Lb. delbrueckii* subsp. *bulgaricus*, on M17 agar for *S. thermophilus* at 42 °C under the anaerobic conditions. Finally, growth kinetic parametes μ max (specific growth $rate_{max}$), g (doubling time), n (number of generations), ϕ and ϕmax (productivity) were calculated for selected *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* as described previously.[22]

Results and discussion

Identification of yogurt starter cultures from traditional yogurt samples

In total sixty traditionally produced yogurt samples were tested for the isolation of yogurt starter cultures *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains and the isolation process was performed for both strains under specific conditions in terms of culture broth and incubation conditions. It should be noted that the PCR detection of the grown colonies with specific primer sets for both strains revealed that the methodology was working as the selected 200 colonies belonged to *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains. Following the genotypic discrimination of the selected isolated belonging to yogurt starter cultures, 19 and 11 distinct *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains respectively were identified and selected for further studies (Figure 1). The phylogenetic relationship of the *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains were tested by the alignments of the 16S rRNA genes of distinct strains using MEGA7 program[23] and as can be seen in Figure 1 two separate groups were formed. Importantly, the reference *Lb. delbrueckii* subsp. *bulgaricus* strain formed a subgroup in comparison to the traditional isolates suggesting the presence of nucleotide substitution in the 16S rRNA genes among the tested strains. Three strains were separated from the other *S. thermophilus* strains and reference *S. thermophilus* strain was formed together with some of the traditional isolates. Overall, distinct *S. thermophilus*[20] and *Lb. delbrueckii* subsp. *bulgaricus*[11] strains were isolated from traditional yogurt samples. Previously potential yogurt starter cultures were isolated from traditional yogurt and yogurt-like samples[4,5,10] but the numbers of distinct *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were lower in previous studies in comparison to this study.

To determine the technological properties of distinct *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains isolated from traditional yogurt samples, their acidification ability, lactic acid production, proteolytic activity, phage resistance, and antibiotic susceptibility were determined. One of the important parameters for the selection of yogurt starter cultures is their acidification capacity and, in this respect, the acidification ability of yogurt starter cultures was tested in skim milk environment. Figure 2 demonstrates

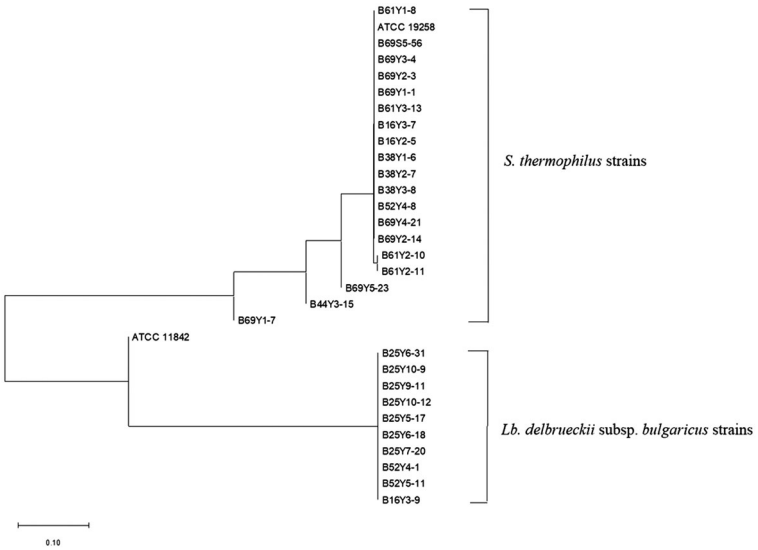


Figure 1. Phylogenetic relationship of the *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains.

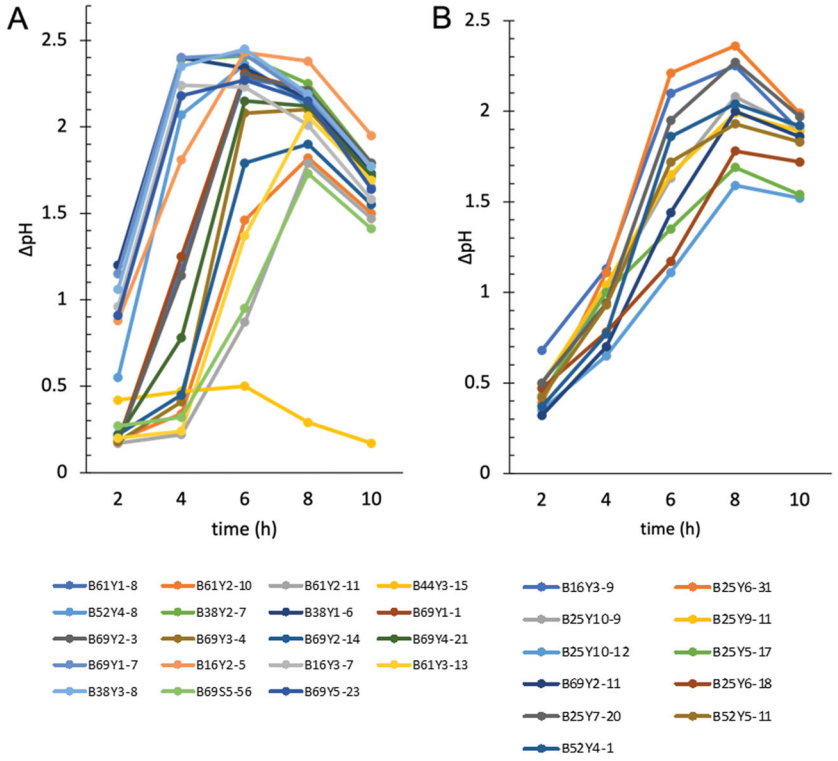


Figure 2. ΔpH values of distinct *S. thermophilus* (A) and *Lb. delbrueckii* subsp. *bulgaricus* (B) strains during 10 h of growth period.

the ΔpH values of distinct S. *thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains during 10 h of the growth period, which revealed that distinct strains had a different acidification ability. Importantly, *S. thermophilus* strains demonstrated a higher level of acidification capacity in comparison to *Lb. delbrueckii* subsp. *bulgaricus* strains and reached the highest ΔpH values in 6 h time whereas this period for *Lb. delbrueckii* subsp. *bulgaricus* strains were 8 h. The higher acidification capacity of *S. thermophilus* strains is important to produce yogurt as they form the relevant environment for the growth of *Lb. delbrueckii* subsp. *bulgaricus* strains during their co-culture in yogurt as previously discussed.[5] Two *S. thermophilus* strains dropped the pH to nearly ΔpH of 2.4 at 4 h of incubation and can be classified for rapid acidification capacity, whereas other 10 and 6 strains required 6 and 8 h of incubation for this level of acidification capacity, respectively. Additionally, one strain did not demonstrate the desired acidification capacity (Figure 2A). A distinct profile for the acidification capacity of traditional *S. thermophilus* strains was also reported previously.[9] *Lb. delbrueckii* subsp. *bulgaricus* strains tested in this study demonstrated similar acidification capacity and reached the highest ΔpH levels at 8 h of incubation period and the highest ΔpH values altered between 1.59 and 2.36 ΔpH during their growth in skim milk (Figure 2B). These findings demonstrated that all isolates except one *S. thermophilus* strain can be classified as yogurt starter culture in terms of their acidification capacity.[5] In addition to the acidification capacity, the total acidity (%) of yogurt cultures in terms of lactic acid (%) production was determined in their corresponding growth mediums (Figure 3). In general, a similar acid production ability was observed for *S. thermophilus* strains (Figure 3A) whereas strain-specific properties determined the acid production ability of *Lb. delbrueckii* subsp. *bulgaricus* strains (Figure 3B) and the acid production ability of *Lb. delbrueckii* subsp. *bulgaricus* strains were lower in comparison to a recent study.[5] This can be a desired factor for *Lb. delbrueckii* subsp. *bulgaricus* strains isolated in this study as with respect to consumer preferences recently *Lb. delbrueckii* subsp. *bulgaricus* cultures with low levels of acid production capacity are selected for the post-acidification process.[5,24]

Another important technological characteristic of yogurt starter cultures is their proteolytic activity and during their symbiotic growth in milk for yogurt production, *Lb. delbrueckii* subsp. *bulgaricus* strains are supposed to provide the free amino acids and peptides to *S. thermophilus* strains with respect to their higher proteolytic activity level in comparison to the *S. thermophilus* strains.[5,25] Our findings were also in accordance with this fact and *Lb. delbrueckii* subsp. *bulgaricus* strains demonstrated a significantly higher level of proteolytic activity compared to the *S. thermophilus* strains (Table 1). For *S. thermophilus* strains, 12 out of 19 isolates did not demonstrate proteolytic activity and for the rest of the isolates, the proteolytic activity altered between 0.012 ± 0.001 and 0.172 ± 0.002 A_{340} level. The proteolytic activity of four *Lb. delbrueckii* subsp. *bulgaricus* strains were changed between 0.078 ± 0.002 and 0.282 ± 0.002 A_{340} level whereas the rest of the isolated showed a proteolytic activity level between 0.312 ± 0.003 and 0.406 ± 0.003 A_{340} level (Table 1). The proteolytic activity of the latter *Lb. delbrueckii* subsp. *bulgaricus* strains were comparable with previous studies[5,26] suggesting the potential of the isolates tested in this study as yogurt starter cultures.

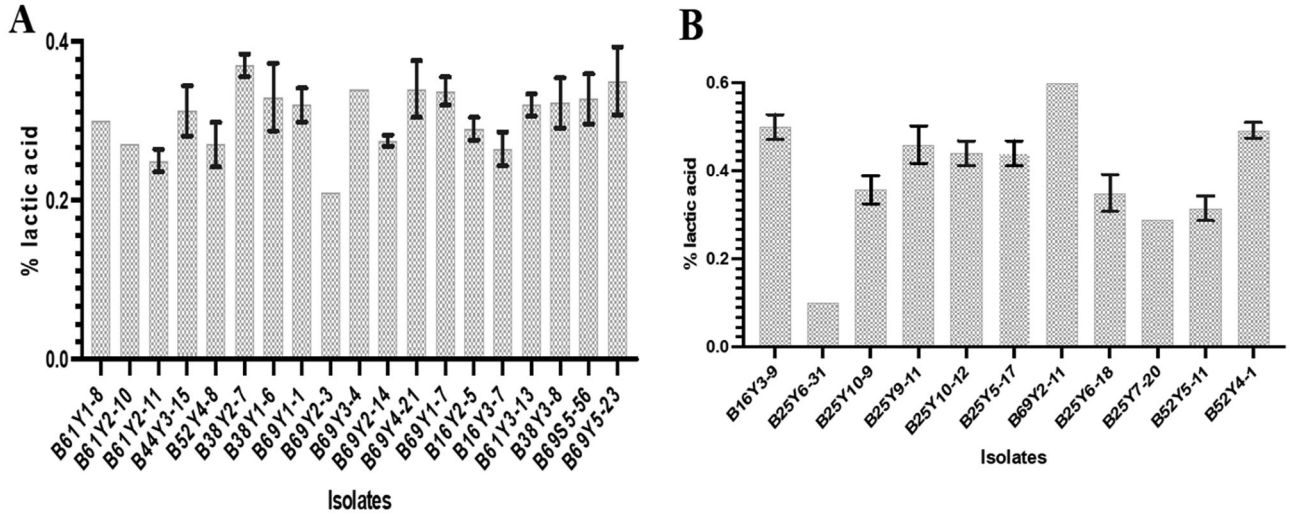


Figure 3. % Lactic acid production of *S. thermophilus* (A) and *Lb. delbrueckii* subsp. *bulgaricus* (B) strains.

Table 1. Proteolytic activity of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains.

S. thermophilus strains	Proteolytic activity (A_{340})
B61Y1-8	ND
B61Y2-10	0.084 ± 0.000
B61Y2-11	0.072 ± 0.002
B44Y3-15	ND
B52Y4-8	ND
B38Y2-7	0.064 ± 0.001
B38Y1-6	ND
B69Y1-1	ND
B69Y2-3	0.172 ± 0.002
B69Y3-4	ND
B69Y2-14	ND
B69Y4-21	0.012 ± 0.001
B69Y1-7	ND
B16Y2-5	ND
B16Y3-7	0.042 ± 0.00
B61Y3-13	ND
B38Y3-8	ND
B69S5-56	ND
B69Y5-23	0.032 ± 0.000
Lb. delbrueckii subsp. *bulgaricus*	
B16Y3-9	0.332 ± 0.001
B25Y6-31	0.078 ± 0.002
B25Y10-9	0.175 ± 0.000
B25Y9-11	0.243 ± 0.002
B25Y10-12	0.317 ± 0.001
B25Y5-17	0.282 ± 0.002
B69Y2-11	0.389 ± 0.002
B25Y6-18	0.312 ± 0.003
B25Y7-20	0.332 ± 0.002
B52Y5-11	0.406 ± 0.003
B52Y4-1	0.323 ± 0.002

ND: not detected.

One of the main problems of the dairy industry is the phage attacks on the starter cultures, which results in slow or incomplete milk fermentation and yogurt starter cultures are among mostly affected starter cultures by bacteriophages.[27] In this respect, the phage resistance of yogurt starter cultures isolated in this study was tested against five *S. thermophilus* phages (ΦB3S10, Φ7095-53, Φ46.1, Φ1B3A, Φ231-50) and five *Lb. delbrueckii* subps. *bulgaricus* phages (Φ231LP8, Φ231LP24, ΦLPAA, ΦX19, ΦVILA) were isolated previously.[20] Table 2 shows the susceptibility of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains for the corresponding bacteriophages. For *S. thermophilus* strains, 4 out of 19 strains were found to be resistant whereas 10 strains displayed sensitivity to the tested bacteriophages. Other *S. thermophilus* strains were mainly prone to bacteriophage infection (Table 2). In terms of *Lb. delbrueckii* subsp. *bulgaricus* strains, 3 strains demonstrated resistance whereas one strain was completely sensitive to the tested bacteriophages. Rest of the *Lb. delbrueckii* subsp. *bulgaricus* strains revealed a mixed profile for phage resistance to the corresponding bacteriophages. Previous studies also reported that phage sensitivity of the yogurt starter cultures can be at high levels and several methods including selection of distinct resistance strains and genetic manipulations might be applied to solve this problem.[28,29] It should be also noted that isolation and identification of autochthonous *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains with phage resistance characteristics can be important for the evaluation of these cultures at industrial scale as one of the main phage control strategies is still the natural selection of bacteriophage-insensitive starter cultures as well as the rotation methodology.[8]

Yogurt and other fermented food products have functional roles due to the presence of certain LAB species with some of them are also starter cultures, that can also become an important part of the human microbiome where they are supposed to trigger some metabolic characteristics positively affecting the human health. Antibiotic resistance is a global threat and resistant bacterial species would transfer the antibiotic resistance via horizontal gene transfer of the antibiotic resistance genes to distinct bacterial species especially pathogenic bacteria within the human microbiome.[4,30] As starter cultures can be taken at higher levels, the presence of antibiotic resistance among the starter cultures can increase the risk for the resistance transfer. In this respect, the antibiotic resistance of the yogurt starter cultures was determined against 11 different antibiotics (ampicillin, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, gentamicin, kanamycin, oxytetracycline, penicillin, streptomycin, and tetracycline) (Table 3). We should note that the antibiotic resistance among the yogurt starter cultures was at significantly low levels as only three *Lb. delbrueckii* subsp.

Table 2. Bacteriophage susceptibility of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*.

	Bacteriophage				
S. thermophilus	B3S10	7095-53	46.1	1B3A	231-50
B61Y1-8	+	+	+	+	+
B61Y2-10	⊢	⊢	–	–	+
B61Y2-11	–	–	–	–	–
B44Y3-15	+	+	+	+	+
B52Y4-8	+	+	+	+	+
B38Y2-7	T	–	T	T	–
B38Y1-6	+	+	+	+	+
B69Y1-1	+	+	+	+	⊢
B69Y2-3	+	+	+	+	+
B69Y3-4	+	+	+	+	+
B69Y2-14	+	+	+	+	+
B69Y4-21	–	–	–	–	–
B69Y1-7	+	+	+	+	+
B16Y2-5	⊢	⊢	⊢	⊢	⊢
B16Y3-7	⊢	⊢	+	–	⊢
B61Y3-13	–	–	–	–	–
B38Y3-8	+	+	+	+	+
B69S5-56	–	–	–	–	–
B69Y5-23	+	⊢	⊢	+	⊢

	Bacteriophage				
Lb. delbrueckii subsp. *bulgaricus*	231LP8	231LP24	LPAA	X19	VILA
B16Y3-9	+	+	+	+	+
B25Y6-31	⊢	⊢	⊢	⊢	⊢
B25Y10-9	–	–	–	–	–
B25Y9-11	–	–	–	–	–
B25Y10-12	–	–	–	–	–
B25Y5-17	⊢	⊢	⊢	⊢	⊢
B69Y2-11	⊢	+	–	+	⊢
B25Y6-18	⊢	⊢	+	+	+
B25Y7-20	+	–	+	–	+
B52Y5-11	⊢	⊢	⊢	⊢	⊢
B52Y4-1	–	–	+	+	⊢

+: sensitive strain to the corresponding phage; ⊢ : can be sensitive strain with respect to infection with the corresponding phage; - : resistant strain to the corresponding phage.

bulgaricus strains were found to be resistant to ciprofloxacin and three and four *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains, respectively appeared to be resistant to kanamycin (Table 3). No resistance of the yogurt starter cultures was observed to the other nine antibiotics and most of the strains were highly susceptible to the tested antibiotics demonstrating a high level of susceptibility of the *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains isolated in this study. In a previous study all *Lactobacillus* strains including *Lb. delbrueckii* subsp. *bulgaricus* were found to be resistant to ciprofloxacin[30] and this resistance was suggested to be related to the fact that strains of *Lactobacillus* were reported to have a natural resistance to antibiotics as inhibitors of the nucleic acid synthesis including ciprofloxacin.[31] This fact suggests that the resistance of the three *Lb. delbrueckii* subsp. *bulgaricus* isolated to ciprofloxacin in this study was not surprising. Additionally, kanamycin is also one of the antibiotics that LAB can show natural resistance,[31] and the resistance of LAB includes *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains were demonstrated to be at high levels in comparison to our findings.[14,30,32] Overall, very low level of antibiotic resistance among *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains were observed in this study that strengthening their potential to be used as starter cultures.

Following the characterization of yogurt starter cultures for their technological properties, *S. thermophilus* B61y3-13 and *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31 were further selected for determination of their growth kinetics properties. Both strains were tested for their growth kinetics under laboratory-scale fermentation conditions and μ max (specific growth $rate_{max}$), g (doubling time), n (number of generations), ϕ and ϕmax (productivity) were determined. During the 12 h of the exponential growth period, *S. thermophilus* B61y3-13 reached to OD600 nm of 17.0 and 10 log10 cfu ml^{-1} and lactic acid production climbed to the 42 g L^{-1} level at the end of this period (Figure 4A,B). This level of lactic acid production was also reported in another study tested the growth parameters of a *S. thermophilus* strain in bioreactor conditions.[33] Importantly, despite the steady trend of *S. thermophilus* B61y3-13 numbers, an increase in the lactic acid production levels was observed through the final stages of fermentation. Similar observations were reported previously for other LABs including *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains[33,34] suggesting that this level of lactic acid concentration was not at the critical stage for the inhibition of *S. thermophilus* B61y3-13 strain. For *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31, an OD600 of 4.4, and cell numbers of 8.5 log10 cfu ml^{-1} were the highest numbers recorded during the exponential growth period of this strain (Figure 4C,D). Additionally, lactic acid production reached to 12.98 g L^{-1} lactic acid for *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31 during this period. Previous studies also demonstrated the growth kinetics of

Table 3. Antibiotic resistance of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains.

S. thermophilus	AMP10	C30	CIP5	D030	E15	CN10	K30	T30	P10	S10	TE30
B61Y1-8	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B61Y2-10	- - -	- - -	- -	- - -	- - -	- - -	-	- - -	- - -	- -	- - -
B61Y2-11	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B44Y3-15	- - -	- - -	- -	- - -	- - -	- -	–	- - -	- - -	- -	- - -
B52Y4-8	- - -	- - -	- - -	- - -	- - -	- -	- -	- - -	- - -	- - -	- - -
B38Y2-7	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B38Y1-6	- - -	- - -	- - -	- - -	- - -	- -	-	- - -	- - -	- - -	- - -
B69Y1-1	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B69Y2-3	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B69Y3-4	- - -	- - -	- -	- - -	- - -	- -	–	- - -	- - -	- -	- - -
B69Y2-14	- - -	- - -	- -		- - -	- -	-	- - -	- - -	- -	- - -
B69Y4-21	- - -	- - -	- - -	- - -	- - -	- -	+	- - -	- - -	- -	- - -
B69Y1-7	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B16Y2-5	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B16Y3-7	- - -	- - -	- - -	- - -	- - -	- -	+	- - -	- - -	- -	- - -
B61Y3-13	- - -	- - -	- -	- - -	- - -	-	+	- - -	- - -	- -	- - -
B38Y3-8	- - -	- - -	- -	- - -	- - -	- - -	-	- - -	- - -	- - -	- - -
B69S5-56	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- - -	- - -
B69Y5-23	- - -	- - -	- - -	- - -	- - -	- -	-	- - -	- - -	-	- - -
Lb. delbrueckii subsp. *bulgaricus*											
B16Y3-9	- - -	- - -	- -	- - -	- - -	- -	+	- - -	- - -	- -	- - -
B25Y6-31	- - -	- - -	+	- - -	- - -	-	+	- - -	- - -	- -	- - -
B25Y10-9	- - -	- - -	- - -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B25Y9-11	- - -	- - -	+	- - -	- - -	-	+	- - -	- - -	- -	- - -
B25Y10-12	- - -	- - -	-	- - -	- - -	-	-	- - -	- - -	- -	- - -
B25Y5-17	- - -	- - -	+	- - -	- - -	-	+	- - -	- - -	- -	- - -
B69Y2-11	- - -	- - -	- -	- - -	- - -	- -	-	- - -	- - -	- -	- - -
B25Y6-18	- - -	- - -	-	- - -	- - -	-	-	- - -	- - -	- - -	- - -
B25Y7-20	- - -	- - -	–	- - -	–	-	- -	- - -	- - -	- -	- - -
B52Y5-11	- - -	- - -	- - -	- - -	- - -	- -	-	- - -	- - -	- - -	- - -
B52Y4-1	- - -	- - -	-	- - -	- - -	- -	-	- - -	- - -	- - -	- - -

AM10: Ampicillin (10 μg/disk), C30: Chloramphenicol (30 μg/disk), CIP5: Ciprofloxacin (5 μg/disk), DO30: Doxycycline (30 μg/disk), E15: Erythromycin (15 μg/disk), CN10: Gentamicin (10 μg/disk), K30: Kanamycin (30 μg/disk), T30: Oxytetracycline (30 μg/disk), P10: Penicillin (P10 U), S10: Streptomycin (10 μg/disk), TE30: Tetracycline (30 μg/disk).

- : intermediate susceptible (zone diameter <10 mm), - - : susceptible (zone diameter 10-15 mm), - - - : highly susceptible (zone diameter >15 mm), + : resistant (no zone formation)

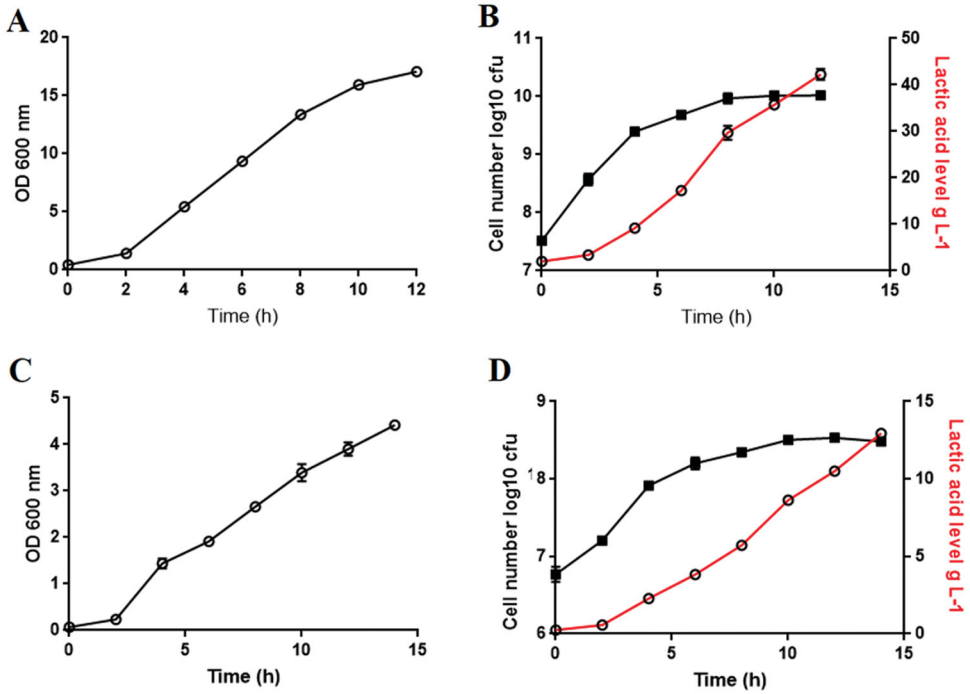


Figure 4. OD values and lactic acid level of S. thermophilus (A,B) and *Lb. delbrueckii* subsp. *bulgaricus* (C,D).

Lb. delbrueckii subsp. *bulgaricus* strains and although the growth kinetics of distinct *Lb. delbrueckii* subsp. *bulgaricus* strains could alter depending on the fermentation medium and growth conditions similar numbers for the bacterial numbers and lactic acid production levels were obtained.[35–37] The μmax value was calculated to be 0.589 h^{-1} for *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31 with a doubling time (g) of 1.18 h^{-1}. Additionally, number of

generations (n) was calculated to be 5.1 for strain B25y6-31 and productivity (ϕ) and maximum specific productivity (ϕmax) values were 0.6 g L^{-1} and 0.787 g L^{-1}, respectively. In terms of growth kinetics of *S. thermophilus* B61y3-13, μmax was calculated to be 0.527 h^{-1} whereas the doubling time (g) was determined to be 1.31 h^{-1}. During the exponential growth phase, the number of generations was calculated to be 4.6, and productivity (ϕ) and maximum specific productivity (ϕmax) values for *S. thermophilus* B61y3-13 were 0.392 g L^{-1} and 0.55 g L^{-1}, respectively. These findings revealed that maximum specific growth rate of both strains was higher in comparison to the growth kinetics of other LAB strains including *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains[22,36,38,39] suggesting the promising characteristics of *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31 and *S. thermophilus* B61y3-13 for industrial growth applications.

Conclusion

Distinct autochthonous *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains were isolated and identified from traditional yogurts in this study. Important numbers of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains were characterized for their high level of acidification capacity and proteolytic activity, respectively. In terms of phage resistance, four *S. thermophilus,* and three *Lb. delbrueckii* subsp. *bulgaricus* strains were resistant to the tested corresponding bacteriophages suggesting their technological potential. Additionally, antibiotic resistance among the yogurt starter cultures was at extremely low levels. Finally, determination of the growth kinetics of *S. thermophilus* B61y3-13 and *Lb. delbrueckii* subsp. *bulgaricus* B25y6-31 demonstrated their suitability for industrial-scale growth applications. More studies are underway to test the functions of these starter cultures during the yogurt production process.

Funding

This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) KAMAG 1007 Program with Project Number 116G012.

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