Isolation and characterization of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* from plants in Bulgaria

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Abstract

One of the traditional ways of preparation of yogurt starter in Bulgaria is placing a branch of a particular plant species into boiled sheep's milk maintained at about 45 °C, which is further incubated until a dense coagulum is obtained. To investigate the possible origin of the yogurt starter bacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*), the traditional way of yogurt-starter preparation was followed. Hundreds of plant samples were collected from four regions in Bulgaria and incubated in sterile skim milk. The two target bacteria at low frequencies from the plant samples collected were successfully isolated. Phenotypic and genotypic characteristics of these bacterial isolates revealed that they were identified as *L. bulgaricus* and *S. thermophilus*. Twenty isolates of *L. bulgaricus* and *S. thermophilus*, respectively, were selected from the isolated strains and further characterized with regard to their performance in yogurt production. Organoleptic and physical properties of yogurt prepared using the isolated strains from plants were not significantly different from those prepared using commercial yogurt-starter strains.

It was therefore suggested that *L. bulgaricus* and *S. thermophilus* strains widely used for commercial yogurt production could have originated from plants in Bulgaria. To our knowledge, this is the first report on the isolation and characterization of *L. bulgaricus* and *S. thermophilus* strains from plants.

Introduction

Yogurt is a fermented milk product obtained by fermentation of milk by the action of symbiotic starter cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*). The first bacteriological study of yogurt originating from Bulgaria was performed by Grigoroff (1905) who isolated and characterized '*Bacille A*' currently known as *L. bulgaricus*. However, the popularity of Bulgarian yogurt, currently known simply as yogurt, and consumption of it throughout the world, could be attributed to the Nobel Prize winner Ilya Metchnikoff. He observed the long life-span of Bulgarian peasants who consumed the traditional fermented milk, and introduced the probiotic concept for the first time. He suggested that lactobacilli might counteract the putrefactive effects of the gastrointestinal metabolism (Metchnikoff, 1907). The microorganism that Metchnikoff referred to in his famous hypothesis was the '*Bulgarian bacillus*'.

There are some opinions concerning the natural habitat or distribution of yogurt-starter bacteria. Yogurt was indicated as the only known habitat of *L. bulgaricus* (Davis, 1975) and this bacterial species is highly adapted to milk (Norbert *et al.*, 1983). However, one of the traditional ways for home-made yogurt starter preparation in Bulgaria is placing a branch of specific plants, such as *Cornus mas*, which was used most frequently into sheep's milk, which is boiled and cooled to about 45 °C. After maintaining at this temperature, a dense milk coagulum is obtained and used to prepare home-made yogurt. This practice, however, is rarely applied at present.

The origin of this traditional custom to prepare yogurt starter is not known precisely. According to Markoff (1925), until more than 200 years ago the shepherds from the Rodopi region of Bulgaria prepared yogurt starter by squashing the roots of *Ononis spinosa* and mixed the juice with sheep's milk. Also *Berberis vulgaris* or *Paliurus aculeatus* were used for the same purpose (Katrandjiev, 1962). Girginoff (1959) pointed *Matricaria chamomilla*, *Prunus spinosa*

and *Alfalfa* as possible sources for isolation of yogurt-starter bacteria. Stefanova (1985) also mentioned that *L. bulgaricus* and *S. thermophilus* could be isolated from plants.

Although all these authors indicated plants as possible sources for isolation of yogurt-starter bacteria by examining the historical aspects of the custom to prepare home-made yogurt in Bulgaria, none of them presented any results of purposive isolation of *L. bulgaricus* and *S. thermophilus* from plants.

One of the major determinants of epiphytic colonization of leaves by bacteria is the availability of carbon and other nutrients (Andrews & Harris, 2000). Glucose, fructose and sucrose are the dominant carbon sources on leaves or stems surface (Tukey, 1970; Mercier & Lindow, 2000). The preliminary investigation by Michaylova *et al.* (2005) suggested the presence of mannose and glucose on the surface of *Cornus mas* and *Prunus spinosa*. Schaffner & Beuchat (1986) and Lee (2001) reported that the two yogurt-starter bacteria can grow on plant materials.

The aims of this study were, first, to investigate the possibility for isolation of *L. bulgaricus* and *S. thermophilus* from plants in Bulgaria, second, to collect initial information concerning geographic locations and plant species from which the yogurt-starter bacteria could be isolated, and third, to compare the taxonomic and fermentation characteristics of the plant-isolates with those of commercial yogurt starter strains. This paper reports, for the first time, the successful isolation of *L. bulgaricus* and *S. thermophilus* strains from plants in Bulgaria, and also shows that the characteristics of the isolated strains were indistinguishable from those of commercial yogurt-starter strains. Based on the results, we speculate that at least one possible origin of the yogurt-starter strains is plants in Bulgaria.

A part of the results in this paper was reported previously (Michaylova *et al.*, 2002).

Materials and methods

Sampling of plant materials

In the period from September 1997 to September 1999, plant samples were collected at four regions in Bulgaria (Fig. 1.) that are famous for the high-quality home-made yogurt preparation. In each of the four regions, samples were taken from several sites that were away from human habitation to avoid potential contamination from home-made or commercial yogurt. Samples were collected from native plants (height range up to 2 m) that looked healthy. The target plant was *Cornus mas* and it was collected from each site. To get information on the relationship between the plant species and the existence of yogurt-starter bacteria, the other plant materials were also collected in an area about 20 m adjacent to the target plant. Plant materials were carefully handled to avoid contamination, and each sample was immediately put in a glass tube containing 10 mL of sterile SM medium, which was then firmly plugged. The SM medium comprised 10% (w/w) skim milk (Oxoid Limited, Basingstoke, Hampshire, UK).

Isolation of lactic acid bacteria

The test tubes containing a plant sample were incubated at 37 °C for between 24 and 48 h. Then a portion of the medium in a test tube showing apparent bacterial growth was frozen in liquid nitrogen after supplementation with 20% glycerol. Each frozen sample was thawed and diluted with sterile 0.85% (w/w) NaCl solution (saline), and 0.1-mL aliquots were spread on both MRS (Oxoid) and M17 agar plates (Oxoid). The plates were incubated anaerobically using the Anaerogen system (Oxoid) at 37, 45 or 50 °C for between 48 and 72 h to obtain Lactic acid bacteria colonies.

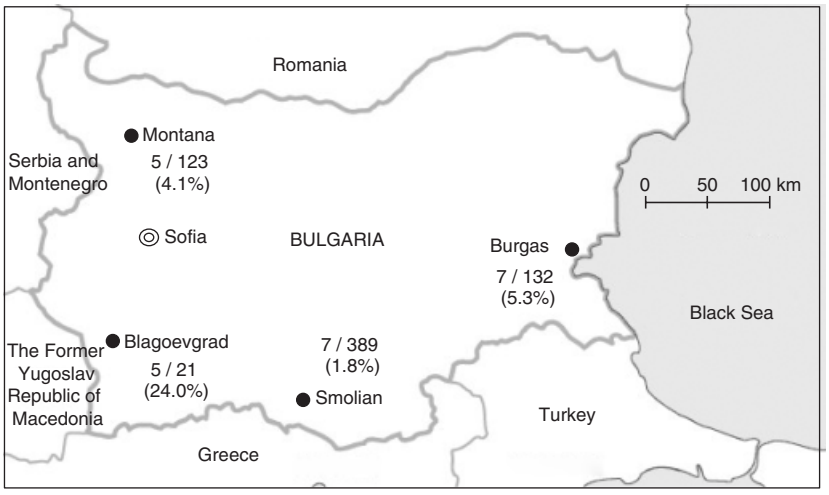

Fig. 1. Sampling areas in Bulgaria and detection of yogurt-starter bacteria. Plant samples were collected in the four regions (●). At each area, the number of plant samples containing *Lactobacillus bulgaricus*, *Streptococcus thermophilus* or both strains and the total sample number are shown, together with the detection rate (%) in the parentheses.

Characterization and identification of the isolated bacteria

The following characteristics of the bacterial isolates were checked using standard protocols: Gram-staining, cell morphology, catalase activity, production of gas from glucose, halotolerance (6.5% NaCl), reduction of litmus milk (Oxoid) and growth at 10, 15, 45 and 50 °C. The isomer of lactate produced was determined with a D-lactic acid/L-lactic acid enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

The bacterial isolates that were judged to be LAB on the basis of the test results were further classified using an API 50 CH system and the manufacturer's databank (bioMerieux SA, Marcy l'Etoile, France). The identification of the isolated LAB strains was confirmed by PCR using the subspecies-specific primer set LB1/LLB1 for *L. bulgaricus* (Torriani *et al.*, 1999) and the species-specific primer set ThI/ThII for *S. thermophilus* (Tilsala-Timisjärvi & Alatossava, 1997).

Selection of bacterial strains used

Twenty strains of *L. bulgaricus* and *S. thermophilus*, respectively, isolated from the plant samples (Table 2) were selected to include diverse strains from different plant samples. Two industrial strains, *L. bulgaricus* 2038 and *S. thermophilus* 1131, were used as controls, which correspond to LBB.B 26 and LBB.T 12, respectively, in the microbial collection of LB-Bulgaricum PLC. They have been used as starter bacteria in the commercial production of 'Meiji Bulgaria Yogurt LB81' (Meiji Dairies Corporation) in Japan since 1993 under Bulgarian license.

Pulsed-field gel electrophoresis (PFGE)

The PFGE method (McCartney *et al.*, 1996) was used, with some minor modifications. Briefly, the cells were embedded in low-melting-point agarose (Sea Plaque GTG; FMC Bio Products, Rockland, ME) before DNA extraction. The restriction endonucleases ApaI, XbaI, EcoRI and BamHI (R-Biopharm AG, Darmstadt, Germany) were used individually to digest *L. bulgaricus* DNA, and ApaI was used to digest *S. thermophilus* DNA. The restriction fragments were separated by PFGE for 17 h using the CHEF-DRII system (Bio-Rad Laboratories, Ontario, Canada). An initial pulse time of 1 s and a final pulse time of 12 s were used, and the gel was run at 5 V cm^{-1} with the buffer maintained at 14 °C. The gels were stained with 200 μg mL^{-1} SYBR Green I and examined by UV transillumination.

Analysis of PFGE band-pattern similarities

Image analysis of the band patterns, and calculations of the molecular weight of the DNA fragments based on markers, the density of the fragments and the matching among the lanes were carried out using RLFP SCAN software (Scanalytics, Inc., Fairfax, VA). TREECON software (Scanalytics, Inc.) was used to prepare dendrograms. Similarities among the strains were determined based on the genetic distance, which was calculated using the formula described by Nei & Li (1979). The generated matrix was subjected to clustering by the unweighted pair-group method with arithmetic means.

Measurement of acidification properties of the isolated strains

Each *L. bulgaricus* or *S. thermophilus* strain was subcultured twice at 37 °C for 16 h in SMY medium comprising 10% (w/w) SM supplemented with 0.1% (w/w) yeast extract. The preculture was inoculated (1%; w/w) into fresh SMY medium and incubated at 37 °C. The acidity (% lactate) of the culture after 16 and 40 h of incubation was measured by titrating a 9 g sample against 0.1 N NaOH using phenolphthalein as an indicator. The pH of the culture was measured with a pH meter (HM-50 V, DKK-TOA Corp., Tokyo, Japan)

Kinematic viscosity of the whey

The culture used for the measurement of acidity after 40 h of incubation was also used to obtain the whey by filtration through a paper filter (No. 2; Toyo Roshi Kaisha, Ltd, Tokyo, Japan). The kinematic viscosity of the whey was measured at 25 °C using an ubbelohde viscosimeter (Sibata Scientific Technology, Ltd, Tokyo, Japan).

Proteolytic activity of *L. bulgaricus*

The method described by Church *et al.* (1985) was used to measure the proteolytic activity of *L. bulgaricus*. The strains were cultured in SMY medium for 18 h at 37 °C. An equal volume of 12% TCA solution was then added to the culture medium and incubated for 10 min at room temperature. A 100 μL sample of the supernatant obtained after centrifugation (13 684 g; 5 min; 10 °C) was mixed with 2 mL *o*-phthaldialdehyde solution in a square cuvette (1 × 1 cm), and the absorbance at 340 nm was measured after incubation for 2 min at room temperature. The proteolytic activities were expressed as leucine equivalents, according to a standard curve obtained using leucine in a concentration range of 0–10 mM.

Urease activity of *S. thermophilus*

The strains of *S. thermophilus* were cultured twice at 37 °C for 16 h in TPY medium comprising 8 g trypticase peptone (BBL), 3 g phytone peptone (BBL), 5 g yeast extract (Difco), 2 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g L-cysteine · HCl, 10 mg $FeSO_4 \cdot 7H_2O$ and 20 g glucose per liter of distilled water (pH 6.5). After growth in the same

medium at 37 °C for 16 h, the cells were washed twice and suspended in saline to reach an absorbance of 1.5–2.0 at 660 nm. The cell suspension (0.1 mL) was mixed with 0.85 mL of 0.2 M phosphate buffer (pH 6.0), and 0.05 mL of 5 M urea was added to begin the reaction. After incubation at 37 °C for 30 min, the reaction was stopped by adding a deproteinizing reagent (Ammonia Test Wako; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) and the released ammonia was measured using the same kit. One unit of urease activity was defined as the amount of enzyme decomposing 1 μmol urea min^{-1}. The specific activity was defined as the number of units per absorbance of the cell suspension at 660 nm.

Preparation of yogurt

The method for yogurt preparation was based on the laboratory-scale manufacturing process for yogurt at the Food Research and Development Center, Meiji Dairies Corporation. A yogurt mix containing 3.0% (w/w) fat and 9.5% (w/w) solid-not-fat (SNF) was obtained by mixing commercial pasteurized milk [3.6% (w/w) fat], skim-milk powder, whey protein isolate (WPI; ALACEN 898; Fonterra, Ltd, Auckland, New Zealand) and water. The concentration of WPI was fixed at 0.1% (w/w). The yogurt mix was heated to 95 °C and immediately cooled to 45 °C. One percent of the yogurt-starter culture was inoculated to the mix. After mixing, *c.* 90 g of the mixture was placed into fifteen 100 mL polystyrene cups. Fermentation at 43 °C was stopped by cooling when the acidity of each culture reached 0.75% and the cups were stored at 5 °C.

Characterization of yogurt

The acidity (% lactate), pH, curd-tension, viscosity and particle size of the yogurt samples were measured after 24 h storage at 5 °C. Curd-tension, viscosity and particle size were measured using a curd-meter (ME-305; I. Techno Engineering, Tokyo, Japan), a viscometer (RC-100; Toki Sangyo Co., Ltd, Tokyo, Japan), and a laser-diffraction particle-size analyzer (SALD-2100; Shimadzu Corp., Kyoto, Japan), respectively.

Enumeration of viable bacterial cells in yogurt

To count the viable cell numbers of LAB, aliquots of the diluted yogurt sample after 1 day storage at 5 °C were poured onto plates and mixed with 15 mL of bromocresol purple (BCP) plate count agar (Eiken Chemical Co., Ltd, Tokyo, Japan). The plates were incubated at 37 °C for 48 h. *Lactobacillus bulgaricus* and *S. thermophilus* were identified by their rough-shaped and smooth-shaped colonies, respectively, in the agar plates.

Results

Isolation of lactic acid bacteria from plants in Bulgaria

To isolate yogurt-starter LAB from plants in Bulgaria, we analyzed 665 plant samples collected from four regions (Fig. 1). The target plant *Cornus mas* was included in 22% of the total samples tested. About half (319 among 665) of the test tubes showed bacterial growth (Table 1) judged by the apparent change or coagulation of SM medium after incubation at 37 °C. Nine hundred and eighty six single colonies from the 319 test tubes on MRS or M17 agar plates were isolated after incubation for 48 h. All the bacterial cultures were preliminarily examined by simple taxonomic tests of Gram-staining, cell morphology, acid production and catalase test. About 80% (784) of the 986 single colonies were rejected because they were not regarded as LAB based on the results obtained.

The remaining 202 bacterial isolates containing 70 rod-shaped bacteria (hereafter referred to as rods) and 132 coccal bacteria (cocci) were judged as LAB. Rods were isolated from *Calendula officinalis, Cornus mas*, *Galanthus nivalis*, *Prunus spinosa* and other unidentified plant species. Cocci were isolated from *Calendula officinalis, Capsella bursa-pastoris, Chrysanthemum, Cichorium intybus, Colchicum, Cornus mas, Dianthus, Galanthus nivalis, Hedera, Nerium*

Table 1. Isolation of yogurt starter bacteria from plant samples

Region	Collected samples	Samples with bacterial growth	Samples with strains of			
			Lb.	St.	Both	Sum
Smolian (24)*	389	173	0	6	1	7
Montana (6)*	123	80	2	2	1	5
Burgas (8)*	132	49	1	4	2	7
Blagoevgrad (1)*	21	17	0	4	1	5
Total (39)*	665	319	3	16	5	24

*Number of sampling sites.

Lb., *Lactobacillus delbrueckii* sp. *bulgaricus*; St., *Streptococcus thermophilus*; Both, Both *L. bulgaricus* and *S. thermophilus*.

oleander, Plantago lanceolata, Prunus spinosa, Rosa, Tropaeolum and other unidentified plant species. Both rods and cocci were simultaneously isolated from *Calendula officinalis, Cornus mas, Galanthus nivalis* and *Prunus spinosa*.

Identification of isolated bacteria as *L. bulgaricus* and *S. thermophilus*

The phenotypes of the selected 70 rods and 132 cocci were characterized using the standard methods. All of them had no catalase activity and produced no gas from glucose. Most of the rods (63 out of 70) produced D-lactate, grew at 45 °C but not at 15 °C and utilized glucose, fructose, mannose and lactose. These characteristics, as well as the results obtained using the API system (data not shown), identified them as *L. bulgaricus*. The remaining seven rods utilized glucose, galactose, mannose and lactose and produced DL-lactate and were classified as *Lactobacillus helveticus*. Most of the cocci (124 out of 132) grew at 50 °C but not at 10 °C and utilized glucose, lactose and sucrose. These characteristics, as well as the results obtained using the API system (data not shown), identified them as *S. thermophilus*. The remaining eight cocci were tentatively identified as *Lactococcus lactis* based on their characteristics (data not shown). These results show that *L. bulgaricus* and/or *S. thermophilus* were isolated from about 4% of all the plant samples tested. The results of detection of yogurt-starter bacteria from plants are summarized in Table 1.

Confirmation of identification results by PCR

Twenty representative strains of *L. bulgaricus* and *S. thermophilus*, respectively, were selected for further experiments (Table 2). The selection process was designed so as to include the most diverse strains isolated from the plant samples.

The PCR results using subspecies-specific primers LB1/LLB1 (Torriani *et al*., 1999) revealed that all 20 strains identified as *L. bulgaricus* produced a single DNA fragment of the expected size (1065 bp). Moreover, all 20 strains identified as *S. thermophilus* produced a single DNA fragment of the expected size (250 bp) after PCR using species-specific primers ThI/ThII (Tilsala-Timisjärvi & Alatossava, 1997).

These findings verified the identification of the 20 selected strains from plants in Bulgaria as *L. bulgaricus* and *S. thermophilus*, respectively.

PFGE pattern analysis of the selected strains

To gain further insight into the relationships among the 20 selected strains of *L. bulgaricus* and *S. thermophilus*, respectively, PFGE was used to compare their genomic DNA (Fig. 2). The industrial yogurt-starter strains, *L. bulgaricus* 2038 and *S. thermophilus* 1131, were analyzed as controls that have been used in Japan to produce commercial yogurt since 1993.

The 20 selected strains of *L. bulgaricus* were divided into seven clusters (Fig. 2a) based on the results of PFGE with the restriction enzyme ApaI. One of the clusters, which contained five strains (Lb12, Lb13, Lb14, Lb18 and Lb19; Fig. 2a), showed a PFGE pattern almost identical to that of the industrial starter strain *L. bulgaricus* 2038 (data not shown). Moreover, the restriction-fragment patterns obtained with the restriction enzymes XbaI, EcoRI and BamHI

Table 2. Selected strains isolated from plants in Bulgaria

Strains	Location	Isolation source	Sampling period
Lactobacillus delbrueckii ssp. *bulgaricus*			
Lb1	Smolian	Unidentified	a
Lb2	Smolian	Unidentified	a
Lb3	Smolian	Unidentified	a
Lb4	Blagoevgrad	Unidentified	a
Lb5	Burgas	*Prunus spinosa*	b
Lb6	Burgas	*Prunus spinosa*	b
Lb7	Burgas	*Prunus spinosa*	b
Lb8	Burgas	*Cornus mas*	b
Lb9	Burgas	*Cornus mas*	b
Lb10	Burgas	*Cornus mas*	b
Lb11	Burgas	*Cornus mas*	b
Lb12	Montana	*Galanthus nivalis*	c
Lb13	Montana	*Galanthus nivalis*	c
Lb14	Montana	*Galanthus nivalis*	c
Lb15	Montana	*Calendula oficinalis*	d
Lb16	Montana	*Calendula oficinalis*	d
Lb17	Montana	*Calendula oficinalis*	d
Lb18	Montana	*Prunus spinosa*	d
Lb19	Montana	*Prunus spinosa*	d
Lb20	Montana	*Prunus spinosa*	d
Streptococcus thermophilus			
St1	Montana	*Plantago laceolata*	a
St2	Montana	*Colchicum*	a
St3	Blagoevgrad	Unidentified Moss	a
St4	Blagoevgrad	*Rosa*	a
St5	Blagoevgrad	*Cichorium intybus*	a
St6	Blagoevgrad	*Capsella bursa-pastoris*	a
St7	Blagoevgrad	Unidentified	a
St8	Smolian	*Chrysanthemum*	a
St9	Smolian	*Nerium oleander*	a
St10	Smolian	*Hedera*	a
St11	Smolian	*Tropaeolum*	a
St12	Smolian	Unidentified	a
St13	Burgas	*Cornus mas*	b
St14	Burgas	*Cornus mas*	b
St15	Burgas	*Dianthus*	b
St16	Burgas	*Capsella bursa-pastoris*	b
St17	Burgas	*Cornus mas*	b
St18	Burgas	Unidentified	b
St19	Montana	*Calendula oficinalis*	d
St20	Smolian	*Cornus mas*	d

Sampling periods: a, September 1997; b, May 1998; c, March 1999; d, September 1999.

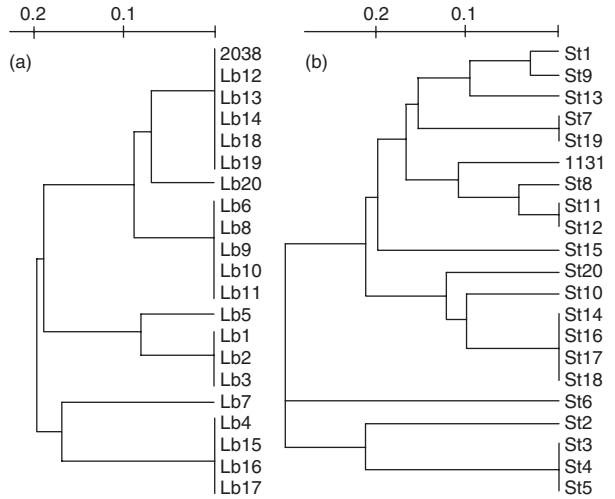

Fig. 2. Cluster analysis of the PFGE patterns of the bacterial isolates. A dendrogram of the cluster analysis of the PFGE DNA fingerprints is shown for 20 strains of *Lactobacillus bulgaricus* (a: Lb1–Lb20) and 20 strains of *Streptococcus thermophilus* (b: St1–St20) isolated from plants in Bulgaria. The results from the industrial strains, *L. bulgaricus* 2038 and *S. thermophilus* 1131, are also shown.

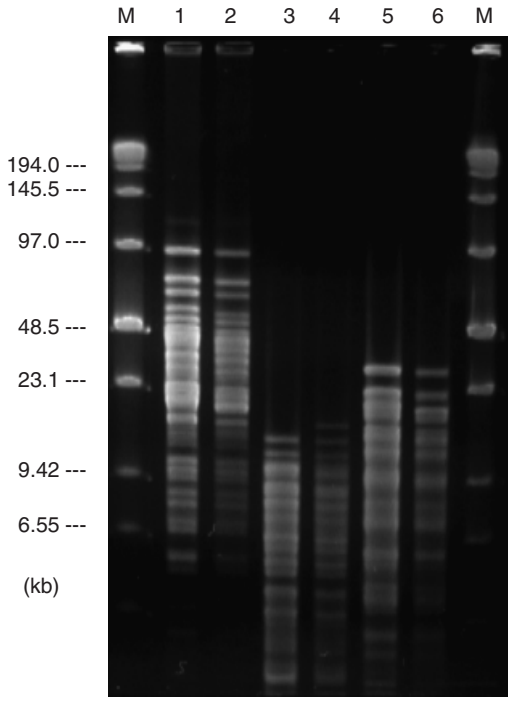

Fig. 3. Comparison of PFGE patterns between 2038 and Lb12 strains. The chromosomal DNA from 2038 or Lb12 strain was digested with a single restriction enzyme: XbaI (lanes 1 and 2), EcoRI (lanes 3 and 4) or BamHI (lanes 5 and 6). The PFGE patterns of the digested DNA from 2038 (lanes 1, 3, and 5) and Lb12 (lanes 2, 4, and 6) strains are shown.

for Lb12, one of the five strains, and *L. bulgaricus* 2038 were indistinguishable (Fig. 3).

On the other hand, the 20 selected strains of *S. thermophilus* were divided into 13 clusters based on the results of PFGE with ApaI (Fig. 2b). None of the strains showed a PFGE fragment pattern indistinguishable from that of *S. thermophilus* 1131, and the PFGE patterns were more diverse than those observed for the *L. bulgaricus* strains tested.

Judged from the numbers of the PFGE cluster shown in Fig. 2, at least seven different strains of *L. bulgaricus* and 13 different strains of *S. thermophilus* were isolated from plants from the four regions in Bulgaria. Strains with the same PFGE pattern were frequently isolated from different plant species within one region, but were rarely isolated from two or more different regions for both *L. bulgaricus* and *S. thermophilus* (Table 2 and Fig. 2).

Properties of the selected strains in relation to yogurt production

It was examined whether each selected strain of *L. bulgaricus* and *S. thermophilus* has similar yogurt fermentation properties to those of industrial yogurt-starter strains. The acidity and pH of SMY medium, and the kinematic viscosity of the whey, were similar for all the strains of *L. bulgaricus* tested including the industrial strain (Table 3). Moreover, all the selected strains of *L. bulgaricus* showed proteolytic activity ranging from 1.8 to 6.6 mmol L^{-1} (Table 3). These values were consistent with our data so far obtained for *L. bulgaricus* strains used for commercial yogurt production (data not shown).

Similarly, the acidity and pH of SMY medium and the kinematic viscosity of the whey did not significantly differ among all the strains of *S. thermophilus* tested including the industrial strain (Table 4). Furthermore, urease activity was detected in all strains of *S. thermophilus* tested, and the activity (6.5–50 U per OD at 660 nm of culture) was within the normal range for industrial *S. thermophilus* strains used in commercial yogurt production (Table 4).

Based on these results, it was concluded that the 20 strains of *L. bulgaricus* and *S. thermophilus* isolated from plants in Bulgaria have almost the same properties as those of the industrial strains currently used for commercial yogurt production.

Evaluation of yogurt prepared with the selected strains

To evaluate the quality of yogurt prepared with the selected strains, we prepared set-type yogurt using one of the 26 combinations of starter strains as shown in Table 5. Six strains of *L. bulgaricus* together with the industrial strain (2038) and 13 strains of *S. thermophilus* together with the industrial strain (1131) were selected from each cluster of PFGE profiles (Fig. 2). Yogurt mix was fermented at 43 °C with one of the 26 combinations of starters, which included

Table 3. Properties of *Lactobacillus delbrueckii* ssp. *bulgaricus* isolated from plants in Bulgaria

	Acidity (%)		pH			
Strains	16 h	40 h	16 h	40 h	Kinematic viscosity ($mm^2 s^{-1}$)	Proteolytic activity ($mmol L^{-1}$)
Lb1	1.45	1.58	3.78	3.68	1.131	2.37
Lb2	1.40	1.53	3.81	3.79	1.109	2.77
Lb3	1.27	1.52	3.94	3.78	1.177	2.52
Lb4	1.37	1.43	3.83	3.84	1.057	2.65
Lb5	1.39	1.55	3.84	3.77	1.150	3.73
Lb6	1.33	1.44	3.90	3.78	1.061	4.60
Lb7	1.35	1.49	3.85	3.75	1.136	1.81
Lb8	1.36	1.43	3.84	3.78	1.051	3.08
Lb9	1.17	1.53	4.00	3.70	1.085	6.59
Lb10	1.27	1.52	3.90	3.77	1.068	3.96
Lb11	1.08	1.46	4.18	3.79	1.060	4.22
Lb12	1.58	2.03	3.72	3.52	1.028	3.88
Lb13	1.57	2.02	3.73	3.53	1.041	3.85
Lb14	1.58	2.01	3.73	3.53	1.029	3.93
Lb15	1.30	1.45	3.93	3.77	1.065	3.64
Lb16	1.29	1.51	3.92	3.72	1.043	3.46
Lb17	1.32	1.58	3.85	3.69	1.060	3.47
Lb18	1.58	1.95	3.67	3.50	1.026	3.80
Lb19	1.58	1.99	3.67	3.51	1.038	3.40
Lb20	1.59	1.96	3.67	3.50	1.023	3.86
2038*	1.44	1.97	3.79	3.59	1.128	4.20

*Industrial strain for commercial yogurt production.
Strains were incubated at 37 °C in 10% skim milk supplemented with 0.1% yeast extract.
Proteolytic activity was expressed as leucine equivalents.

one control combination composed of the two industrial strains (Table 5). It took between 2.9 and 4.6 h to reach at 0.75% of acidity of yogurt mix, which was within the normal range of fermentation time for commercial yogurt. The viable cell count, acidity, pH, curd tension, viscosity and particle size of the yogurt fluctuated among the preparations. However, all values were within the normal range measured by the authors for yogurt prepared using commercial strains (data not shown).

Finally, sensory properties of each yogurt was evaluated for its appearance, mouthfeel, flavor and overall quality, using a five-point quality scale ranging from 1 (dislike extremely) to 5 (like extremely). The average scores for each property of the yogurt, assessed by a three-member expert panel and a three-member nonexpert panel, ranged from 3.0 to 4.7 points by the former, and from 2.7 to 5.0 points by the latter. Although a more precise evaluation of yogurt quality may be needed to confirm these findings, results indicate that the quality of all yogurt prepared in this study was at least more than acceptable.

Discussion

The origin of the yogurt-starter strains of *L. bulgaricus* and *S. thermophilus* has been unknown so far. Based on the traditional practice in Bulgaria and the results presented in this paper, it can be assumed that starter strains for home-made yogurt have been primarily derived from plants, and plants in Bulgaria are potential source for *L. bulgaricus* and *S. thermophilus* strains. The results obtained in this study show that *L. bulgaricus* and/or *S. thermophilus* were isolated from 24 plant samples out of 665 samples (Table 1), which means the yogurt starter LAB were detected at a rate of 3.6% on average. The detection rates from plant samples differed apparently from the areas (Fig. 1). The highest rate (24%) was observed in Blagoevgrad, but the number of samples, 21, was much smaller than those in the other areas. Future studies will prove if yogurt-starter bacteria are abundant in some specific areas in Bulgaria or not. On the other hand, the isolation rate (14%) from the samples of *Cornus mas* was much higher than 3.6%, which is in good agreement with the traditional custom in Bulgaria to prepare yogurt starter from this plant. The growth conditions applied in this study (37 °C in SM medium) might have appeared insufficient to efficiently eliminate non-LAB microorganisms, and their growth might have interfered with the isolation of LAB. It was therefore assumed that more yogurt-starter bacteria might have been present in the plant samples than were isolated in this study.

To get more precise insights into the isolated strains, PFGE was applied and the results obtained show the following. First, one of the selected strains of *L. bulgaricus*,

Table 4. Properties of *Streptococcus thermophilus* isolated from plants in Bulgaria

	Acidity (%)		pH			
Strains	16 h	40 h	16 h	40 h	Kinematic viscosity ($mm^2 s^{-1}$)	Urease activity (unit/OD at 660 nm)
St1	0.90	0.90	4.47	4.47	1.054	14.57
St2	1.05	1.15	4.17	4.02	1.039	21.65
St3	0.85	0.86	4.55	4.46	1.066	12.94
St4	0.85	0.85	4.58	4.53	1.053	13.37
St5	0.89	0.89	4.51	4.46	1.063	11.72
St6	1.08	1.13	4.19	4.13	1.095	6.51
St7	1.10	1.10	4.18	4.08	1.075	28.70
St8	1.00	1.01	4.22	4.13	1.047	28.89
St9	1.05	1.11	4.17	4.09	1.071	10.40
St10	1.01	1.04	4.19	4.15	1.048	29.59
St11	1.05	1.10	4.17	4.10	1.067	31.13
St12	1.05	1.06	4.17	4.09	1.046	28.85
St13	1.10	1.21	4.15	4.07	1.177	30.90
St14	1.03	1.08	4.19	4.09	1.063	32.58
St15	0.99	1.06	4.28	4.11	1.061	18.63
St16	1.04	1.09	4.17	4.09	1.055	31.55
St17	1.07	1.08	4.18	4.09	1.075	35.23
St18	1.04	1.09	4.14	4.07	1.059	37.68
St19	1.06	1.10	4.16	4.09	1.071	13.29
St20	0.95	1.06	4.30	4.14	1.040	49.91
1131*	0.95	1.28	4.28	4.01	1.213	10.52

*Industrial strain for commercial yogurt production.

Strains were incubated at 37 °C in 10% skim milk supplemented with 0.1% yeast extract.

One unit of urease activity was defined as the amount of enzyme decomposing 1 μmol of urea per minute.

Lb12, was highly similar to the industrial *L. bulgaricus* strain of 2038 as shown in Fig. 3. It was therefore postulated that the 2038 strain and the five selected strains belonging to the same cluster (Fig. 2a) might have originated from the same ancestral strain, which had lived on wild plants in Bulgaria. Second, at least seven different strains of *L. bulgaricus* and 13 different strains of *S. thermophilus* were isolated from plants in Bulgaria. It was assumed that the distribution of *L. bulgaricus* and *S. thermophilus* strains depends upon the regions rather than upon the plants, because strains with the same PFGE pattern were frequently isolated from different plants within only one region (Table 2 and Fig. 2). As more than seven strains of *L. bulgaricus* and 13 strains of *S. thermophilus* were isolated in this study, it was proposed that many starter strains for yogurt production of *L. bulgaricus* and *S. thermophilus* with distinctive characteristics might have originated mostly from plants in Bulgaria.

The characteristics of the selected strains isolated from plants was compared in relation to yogurt production with those of the current commercial starter strains. As shown in Tables 3 and 4, no significant differences were detected among them. It was concluded that the selected LAB strains are almost equivalent to the industrial strains in respect to their ability to produce yogurt (Table 5). And from the preliminary sensory examinations, the quality of the yogurt prepared by all 25 starter combinations, which contained either one or two plant-originated strains, was shown to be within the normal range compared with those of commercial yogurt. These results suggest that, at a minimum, yogurt with an acceptable quality could be prepared using starter combinations of *L. bulgaricus* and *S. thermophilus* strains isolated from plants in Bulgaria.

It remains unclear at this moment how *L. bulgaricus* and *S. thermophilus* grow and survive on plants, and if these bacteria are transferred from other materials by insects such as ants (Markoff, 1925). It is also unclear how they have acquired the ability to live on milk and how the symbiosis has been established between the two bacterial species. However, the results in this study clearly show that yogurt bacteria exist, at least at low frequencies, on plants in Bulgaria. Further ecological and biological studies as well as genetic studies using the complete genome sequences of *L. bulgaricus* (van de Guchte *et al.*, 2006) and *S. thermophilus* (Bolotin *et al.*, 2004) will shed light on these questions in the near future.

This is possibly the first report on the isolation of *L. bulgaricus* and *S. thermophilus* strains from nondairy materials, as well as on the characterization of the yogurt prepared using the isolated strains from plants. Findings of this study imply, first, at least one possible origin of *L. bulgaricus* and *S. thermophilus* is plants in Bulgaria and, second, the microbiological and fermentation characteristics of the isolated strains from plants are indistinguishable from

Table 5. Characteristics of yogurts prepared with lactic-acid bacteria isolated from plants in Bulgaria

	Strains			Viable count ($\times 10^7$ CFU g^{-1})		Acidity				
No.	Lb.	St.	Fermentation time (h)	Lb.	St.	(%)	pH	Curd tension (g)	Viscosity (mPa.s)	Particle size (μm)
Control	2038	1131	3.3	18.0	127.0	0.90	4.47	39	2550	18.9
1	Lb1	1131	3.3	21.0	112.0	0.89	4.58	37	2140	22.7
2	Lb4	1131	3.2	25.0	116.0	0.90	4.41	36	2000	19.0
3	Lb5	1131	3.1	15.0	133.0	0.85	4.43	33	1910	18.6
4	Lb6	1131	3.2	13.0	130.0	0.87	4.47	39	2000	16.9
5	Lb7	1131	3.2	13.0	104.0	0.85	4.57	62	1960	20.7
6	Lb20	1131	3.4	8.1	139.0	0.85	4.58	64	2000	16.5
7	2038	St1	3.8	17.0	128.0	0.81	4.47	72	1340	39.3
8	2038	St2	3.5	13.0	151.0	0.92	4.37	80	1250	42.2
9	2038	St3	3.5	15.0	107.0	0.85	4.46	60	1380	31.4
10	2038	St6	4.6	6.0	88.0	0.86	4.43	66	1350	49.1
11	2038	St7	3.5	14.0	137.0	0.84	4.47	62	1310	36.7
12	2038	St8	3.2	12.0	126.0	0.92	4.37	75	1320	39.3
13	2038	St9	3.1	7.0	128.0	0.87	4.46	62	1530	35.0
14	2038	St10	4.5	6.0	136.0	0.80	4.54	90	1250	53.0
15	2038	St11	4.0	28.0	26.5	0.86	4.40	46	1290	52.0
16	2038	St13	3.3	15.0	125.0	0.94	4.39	49	2110	18.4
17	2038	St14	3.4	14.0	126.0	0.82	4.48	67	1290	39.8
18	2038	St15	2.9	8.0	112.0	0.90	4.47	57	1310	38.7
19	2038	St20	3.3	6.0	26.0	0.99	4.24	38	1290	52.0
20	Lb6	St3	3.3	33.0	90.0	0.99	4.12	54	1410	35.9
21	Lb6	St9	3.4	19.0	120.0	0.96	4.13	62	1450	36.7
22	Lb6	St13	3.5	27.0	97.0	1.00	4.12	52	1730	25.3
23	Lb20	St3	3.3	11.0	102.0	0.90	4.17	69	1340	46.9
24	Lb20	St9	3.4	15.0	121.0	0.90	4.24	87	1950	44.1
25	Lb20	St13	3.5	6.0	78.0	0.89	4.31	80	1720	31.4

Lb., *Lactobacillus delbrueckii* ssp. *bulgaricus*; St., *Streptococcus thermophilus*.
The fermentation time indicates the period required for the acidity of the yogurt mix to reached 0.75%.
Strains 2038 and 1131 are industrial starter bacteria for commercial yogurt production.

those of the industrial strains currently used for yogurt production. Therefore, it is assumed that Bulgarian home-made yogurts, originally prepared using LAB from plants, were subsequently transferred to other countries and may have been used as starter bacteria for commercial yogurt production in many countries.

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