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ORIGINAL ARTICLE

Non-traditional sources for isolation of lactic acid bacteria

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Abstract In recent years there has been increasing interest in lactic acid bacteria isolated from non-dairy products, due to their diverse metabolic profile, unique flavor-forming activities, and potential for use as starters or starter adjuncts for the dairy industry. Screening of 400 microbial isolates obtained from the herbs *Geranium sanguineum* L., *Hypericum perforatum* L., and *Panax ginseng* C.A. Meyer was performed, and 64 isolates were selected based on milk coagulation and gas formation ability and non-specific odour. Using tests involving multiple transfer and growth in selective and differential media, 258 single colonies were isolated, of which 98 were affiliated with the lactic acid bacteria group. These bacteria are homofermentative cocci and rods with a wide pH (5.0–9.6) and temperature range (15–45 °C), high salt tolerance (3.0–10.0 % NaCl), and high acid-producing activity (3.50–12.00 g/L). With the use of genotype-based methods, the plant isolates were identified at the species level as *Enterococcus faecium*, *Streptococcus thermophilus* and *Lactobacillus rhamnosus*.

Keywords Lactic acid bacteria · Medicinal plants · Isolation · 16S rDNA

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Introduction

Lactic acid bacteria (LAB) are predominantly represented as a non-taxonomic heterogeneous group of Gram-positive and non-spore-forming facultative anaerobic bacteria. This group includes a wide variety of cell types and physiological and biochemical characteristics. LAB are designated "generally recognized as safe" (GRAS status), and have long played an important role in food technology—they are responsible for lactic fermentation in several products, including dairy, meat, and silage. LAB can be isolated from traditional sources such as raw milk, dairy products and fermented foods (Wouters et al. 2002 ; Kimoto et al. 2004; Tamang et al. 2005; Nomura et al. 2006; Kostinek et al. 2007; Tanasupawat et al. 2007; Kivanc et al. 2011; Venturi et al. 2012; Abegaz 2014), and from alternative sources including fecal samples, soil and plants (Hartnett et al. 2002; Magnusson et al. 2003; Cock and de Stouvenel 2006; Siezen et al. 2008; Trias et al. 2008; Di Cagno et al. 2009; Cakir 2010; Chen et al. 2010; Venugopalan et al. 2010; Baradaran et al. 2012; Emerenini et al. 2013; Fhoula et al. 2013; Nguyen et al. 2013; Alemayehu et al. 2014). In recent years, there has been increasing interest in LAB isolated from non-dairy sources due to their diverse metabolic profile and unique flavor-forming activities. Plant-derived strains of lactobacteria have demonstrated tolerance to high pH values and salt concentrations, an ability to ferment more types of carbohydrates, and a high level of stress resistance compared to those of dairy origin. Furthermore, studies have noted no significant differences in fermentation characteristics and profiles of enzymes (lipases, peptidases and phosphatases) required for obtaining various fermented dairy products with plant-derived and commercial strains of lactobacteria (Nomura et al. 2006; Michaylova et al. 2007; Siezen et al. 2008; Venugopalan et al. 2010).

The search for new solutions to improve starter systems for fermented health foods and for opportunities to realize the biological potential of LAB has given rise to initiatives for exploiting biodiversity in unique natural systems (medicinal plants). These plants are an important ecosystem for isolation of LAB (Siezen et al. 2008; Cakir 2010; Venugopalan et al. 2010; Baradaran et al. 2012), and each individual plant species provides a unique environment in terms of competing microorganisms and natural plant antagonists, as well as accessibility, type and concentration of the substrate in the various physical factors. When the plant material is collected and prepared for fermentation, these conditions allow for growth of typical epiphytic flora from which a population and chain of fermentation processes is derived.

Current available information on the use of medicinal plants as a source for isolation of LAB and their subsequent potential application as components to form starters for milk fermentation is scarce (Venugopalan et al. 2010). There are no data in the scientific literature regarding the microbial presence of different types of bacteria—and LAB in particular—isolated from herbs of the genera *Geranium, Panax* or *Hypericum*. Extracts from various parts of *Geranium sanguineum* L., known in Bulgaria by the popular name "bloody geranium," have significant antiviral, antibacterial, anti-inflammatory and antioxidant activity (Serkedjieva and Manolova 1992; Hammami et al. 2011). Extracts of *Hypericum perforatum* L., commonly known in Bulgaria as "yellow wort" and as "St John's wort" in other countries and on other continents, also possess antibacterial and antiviral properties. St John's wort has traditionally been used in the form of homeopathic medicines as an alternative treatment for various depressive disorders of mild to moderate severity (Barnes et al. 2001). The incorporation of plant extracts in preparations for topical wound treatment has also been successful (Newall et al. 1996). Husain et al. (2011) demonstrated significant hypolipidemic activity for the *Hypericum perforatum* L. extract in a rat model. In addition, the plant is classified by the Council of Europe (2000) as a natural seasoning for various foods (Barnes et al. 2001). There have been reports of high levels of antioxidant activity and pharmacological effects for extracts of *Panax ginseng* C.A. Meyer, known worldwide as "ginseng," including stimulation of brain function, pain relief, activation of antitumor immunity and improvement in total immunity, regulation of blood pressure and blood sugar levels in diabetic patients, tonic effects on physical fatigue and mental stress, a positive influence on liver function, male potency and side effects of menopause in women, and the inhibition of growth of the AIDS virus (Choi 2008). A new direction in scientific research that has recently been explored involves obtaining bioactive or biogenic substances extracted from different plants or synthesized during food fermentation, and the subsequent creation of novel foods (defined as healthy and functional) by further introduction of such exogenous functional components into their technological schemes or through the use of microorganisms as producers of biogenic substances or microorganisms with probiotic characteristics (Gobbetti et al. 2010). In this context, Servili et al. (2011) reported the production of a functional milk beverage through fermentation of cow's milk that had been enriched with phenolic extracts from olive vegetable water. For this purpose, the authors used starter cultures including *Lactobacillus delbrueckii* subsp. *bulgaricus* DPPMALDb5 and *Streptococcus thermophilus* DPPMAST1, and used the probiotic strain *Lactobacillus paracasei* 15 N and γ-amino butyric acid-producing-strain *Lactobacillus plantarum* C48 as adjunct cultures.

The present study was a preliminary evaluation of LAB isolated from the herbs *Geranium sanguineum* L., *Hypericum perforatum* L. and *Panax ginseng* C.A. Meyer as potential starters or adjunct starters for fermented dairy products. The main objective of this work was to evaluate the presence of LAB within the context of microbial diversity in natural biosystems (medicinal plants *Geranium sanguineum* L., *Hypericum perforatum* L. and *Panax ginseng* C.A. Meyer) with desired metabolic activities in order to include them as starter or non-starter components during in situ cultivation in milk, and to identify the isolates at the species level using genotype-based methods including 16S rDNA and phylogenetic tree construction.

Materials and methods

Sample collection

Plant material was collected from following locations: *Geranium sanguineum* L., Eastern Rhodopes (Ivaylovgrad), the Sofia region (experimental field of the Institute of Biodiversity and Ecosystem Research [IBER] at the Bulgarian Academy of Sciences [BAS]) and the Vitosha region (Iskar Dam); *Hypericum perforatum* L., Western Rhodopes (Persenk Chalet); *Panax ginseng* C.A. Meyer, Botanical Garden of the Technical University of Dresden, Germany. All plant samples were collected aseptically in sterile poly-bags, kept under refrigeration, and transported to the laboratory for analyses.

Lactic acid bacteria isolation procedure

Individual parts (flower, leaf and stem) of each plant species were carefully washed in sterile water, then transferred to test tubes with sterile 10 % reconstituted skim milk (RSM; HiMedia Laboratories, Mumbai, India) and incubated at 30 and 37 °C until coagulation of milk occurred. After assessment of milk coagulation, gas formation and non-specific odour, samples were selected for subsequent transfer to M17

broth (pH 6.6; Merck Millipore, Darmstadt, Germany) and MRS broth (pH 5.7; Merck Millipore), with the addition of 100 μg/mL of cycloheximide (Sigma-Aldrich, St. Louis. MO USA) (Hartnett et al. 2002) in order to prevent fungal growth and to select for LAB. Selected samples were cultured in anaerobic jars using the Anaerocult A mini system (Merck Millipore) for a period of 72 h at the above-referenced temperatures. Serial decimal dilutions of broth cultures in 0.85 % (w/v) sterilized NaCl solution were plated by spreading 0.1 mL on MRS and M17 agar plates (Merck Millipore), which were incubated anaerobically at 30 and 37 °C, respectively, for 3–5 days to obtain single bacterial colonies. These colonies were then randomly selected and purified by streaking again and subculturing on fresh MRS and M17 agar plates. The purity of isolated single colonies was evaluated microscopically (MICROS Pink MC50; MICROS Produktions- und HandelsgesmbH St. Veit an der Glan, Austria).

The abbreviations of isolated lactic acid strains from the relevant plant samples include the initials of the relevant plant species, an index indicating the location of the plant species or an initial of a nearby location to the region in question in Bulgaria and, respectively, the abbreviated spelling of Germany (e.g., GsfIV123: Gs [*Geranium sanguineum*], f [flower], IV [Ivaylovgrad], 123 [number of colony]).

Identification of lactic acid bacteria

Phenotypic and biochemical characteristics

The initial study of purified bacterial isolates involved microscopic characterization of the morphology of cells (MICROS Pink MC50) and colonies (shape, color and size) (CETI Digi Steddy II, Medline Scientific, Chalgrove, Oxfordshire, UK) and subsequent differentiating Gram staining. Biochemical tests (catalase test, oxidase test, reaction to indole) were carried out in selected Gram (+) isolates in order to establish their LAB group affiliation.

Gram-positive, catalase-negative, indole-negative and oxidase-negative presumptive LAB were stored in M17 and MRS broth containing 15 % glycerol (Merck Millipore, Darmstadt, Germany) at −80 °C for use in subsequent examinations. For cultivation, bacterial isolates were reactivated in M17 and MRS broth at 37 °C for 24–48 h.

Production of CO_2 from glucose was determined in tubes of MRS broth containing inverted Durham tubes, incubated for 3 days at 37 °C.

The configuration of lactic acid (LA) produced from glucose was determined enzymatically using the D-lactate and L-lactate dehydrogenase test kit K-DLATE 12/12 (Megazyme International Ireland, Bray, Co. Wicklow, Ireland), with incubation for 72 h at 37 °C.

The presumptive LAB that manifested high acid-producing activity were tested for growth at different temperatures (4, 15, 30, 37, 45 and 55 °C) and different pH values (3.0, 5.0, 7.0, 8.0 and 9.6) in M17 broth and MRS broth for 72 h, with initial pH values of 6.6 and 5.7 for the two media, respectively, at a temperature 37 °C. The level of salt tolerance was determined after growth in M17 broth (pH 6.6) and MRS broth (pH 5.7) in the presence of various concentrations of NaCl (3.0, 4.5, 6.5, 8.5 and 10.0 %) at 37 °C for 72 h.

For comparative characterization of the presumptive LAB isolated from *G. sanguineum*, *H. perforatum* and *P. ginseng*, with regard to the above-mentioned properties, these were cultivated in parallel with LAB belonging to the laboratory collection, which were isolated from various dairy products, and were used in our previous studies: *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* LD5 (*Lactococcus diacetylactis* LD5), *Streptococcus thermophilus* ST3, *Lactococcus lactis* ssp. *lactis* LL3, *Lactococcus lactis* ssp. *cremoris* LC1 (*Lactococcus cremoris* LC1), *Enterococcus faecium* EF4, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB1-9 (*Lactobacillus bulgaricus* LB1-9) and *Lactobacillus rhamnosus* LR2.

The preliminary identification of selected presumptive LAB was investigated using the API 50 CHL and API 20 STREP (bioMérieux SA, Marcy-l'Étoile, France) galleries. The tests were conducted according to manufacturer instructions, and the results were read after incubation of the strain at 37 °C for 2–3 days.

Genotypic characterization

Each isolate was grown anaerobically on M17 and MRS agar for 48 h at 37 °C. A single colony was suspended in 1 mL Milli-Q water and centrifuged for 1 min at 12,000 *g*. Genomic DNA was isolated from the pellet using the NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), following the manufacturer's instructions. The concentration of the resultant genomic DNA was measured with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) were used to amplify the fragments of the 16S ribosomal gene. Each PCR mixture (50 μL) contained a reaction mix of 25 μL HotStarTaq *Plus* Master Mix Kit, 2× (Qiagen GmbH, Düsseldorf, Germany), 1 μL of each primer (10 μM), 100 ng of DNA template and autoclaved Milli-Q water. The amplification was performed in a Mastercycler® pro (Eppendorf North America, Hauppauge, NY, USA), and the following program was used: initial denaturation at 95 °C for 1 min, followed by 33 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, elongation at 72 °C for 100 s, and a final extension for 3 min at 72 °C. The

amplified product was cooled at 4 °C. The quality of the isolated total DNA and amplification fragments of 16S rDNA (approximately 1500 base pairs [bp]) was analyzed by electrophoresis on a 1 % (*w/v*) agarose gel in 1X TAE buffer (stock 50X TAE: 242 g/L Tris base, 57.1 mL/L acetic acid, 100 mL 0.5 M EDTA, pH 8.5) at 80 V for 45 min. Staining was performed in GelRed (Biotium Inc., Hayward, CA, USA) fluorescent dye (0.05 μg/mL), and the bands were visualized under an ULTima 10si (Hoefer Inc., Holliston, MA, USA). The size of DNA fragments was estimated using a standard 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA). Amplification fragments from 16S rDNA were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's instructions.

Sequencing was performed by Eurofins MWG Operon LLC (Ebersberg, Germany). Sequence assembly was performed using the BioEdit software program. The sequence homologies were examined by comparing the sequences obtained with those in the National Center for Biotechnology Information (NCBI) database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The results obtained were used to identify isolates to genus or species level. Phylogenetic trees were constructed using MEGA 4.1 software (Tamura et al. 2007).

Statistical analyses

Data represent the mean values of three independent experiments. The errors of experimental data from the mean values were expressed as standard deviations using the Microsoft Excel 2010 program. Standard deviations were illustrated as error bars.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in NCBI GenBank under the following accession numbers: KR054662, KR054663, KR054664, KR054665, KR054666, KR054667, KR054668, KR054669, KR054670, KR054671, KR054672, KR054673, KR054674, KR054675, KR054676, KR054677, KR054678, KR054679, KR054680, KR054681, KR054682, KR054683, KR054684, KR054685 and KR054686.

Results

Bacterial isolates obtained from *Geranium sanguineum* L., *Hypericum perforatum* L. and *Panax ginseng* C.A. Meyer, and their preliminary identification

As a result of large-scale screening of 400 microbial isolates (derived from flower, leaf or stem of *G. sanguineum*, *H. perforatum* and *P. ginseng*) based on visual assessment of milk coagulation, gas formation and non-specific odour, 64 microbial isolates (16.0 %) were selected. After subsequent multiple transfer and growth on selective media, 258 single bacterial colonies were isolated, which were further identified by means of classical techniques to determine their phenotypic characteristics and their LAB group affiliation. Based on the required and confirmatory tests performed, 98 isolates (38 %) showed phenotypic identity with the lactobacteria group, which were Gram-positive, catalase-negative, oxidase-negative and indole-negative. Representatives of LAB were not isolated from the stem of *G. sanguineum*, but the flowers proved to be the most preferred site for habitation by lactobacteria (37 single colonies), followed by the leaves (25 single colonies). Presumptive LAB were isolated only from the flowers of *H. perforatum* (11 single colonies) and from the leaves of *P. ginseng* (25 single colonies).

The presumptive LAB were morphologically defined as cocci and rods. Cocci were isolated from *G. sanguineum* and *H. perforatum*, and rods from *P. ginseng*. Cocci were arranged as single cells and cell pairs, in short and long chains, with cell sizes from 0.8 to 2.9 μm. The relevant colonies were shiny, with white and light beige colors, circular and convex in shape, with entire or undulate margins, and ranging in size from 1.0 to 2.5 mm. Rods were arranged in pairs, in short or long chains, with cells 1.4–2.8 × 0.3–0.8 μm in size. The colonies were shiny, with white and grayish-white colors, circular, convex shape with entire margins, and ranging in size from 1.0 to 2.1 mm.

All examined isolates were related to the homofermentative LAB group, as determined from testing of their ability to produce CO_2 from glucose indicating the absence of such metabolic activity.

Determination of isomeric forms of lactic acid, level of halotolerance, and temperature and pH ranges for growth of the bacteria

The data for isomeric forms of lactic acid produced from plant-derived lactobacteria showed that 82.7 % synthesized L(+)-LA, and 17.3 % synthesized D(−)/L(+)-LA (Figs. 1, 2 and 3). Cocci isolated from *H. perforatum* and rods from *P. ginseng* synthesized only L(+)-LA (Figs. 2 and 3). Among the isolates from *G. sanguineum* are both representatives synthesizing only L(+)-LA and those synthesizing D(−)/L(+)-LA (Fig. 1a–c). Moreover, the presence of L(+)-LA is dominant for isolates producing both isomeric forms of lactic acid. The only exception is the isolate GslIV213, which synthesizes 6.17 g/L D(−)-LA and a lower amount of L(+)-LA. Representatives expressing only D(−)-LA are missing. The highest activity level for synthesis of lactic acid was present in isolates obtained from *G. sanguineum*, which reached maximum concentrations of 11.99 and 10.27 g/L (Fig. 1a and b).

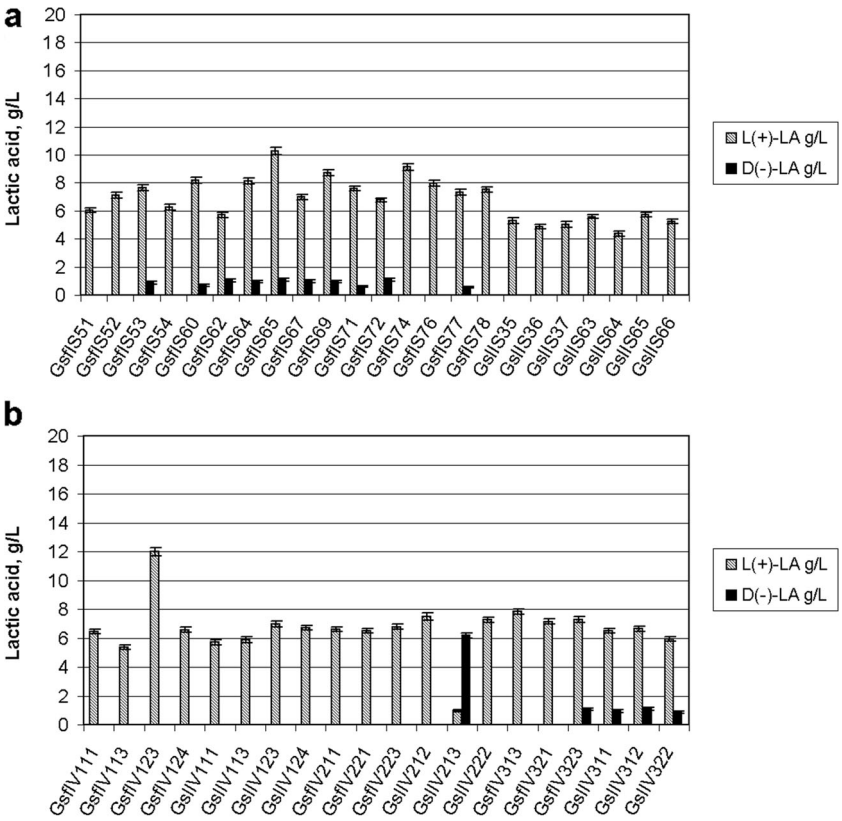

Fig. 1 Isomeric forms of lactic acid produced by lactic acid bacteria isolated from *Geranium sanguineum* L. collected from **a** the Vitosha region (Iskar Dam), **b** Eastern Rhodopes (Ivaylovgrad), and **c** the Sofia region (experimental field of the Institute of Biodiversity and Ecosystem Research [IBER] at the Bulgarian Academy of Sciences [BAS]): ▨ - L(+)-lactic acid; ■ - D(−)-lactic acid. *Bars* represent standard deviation

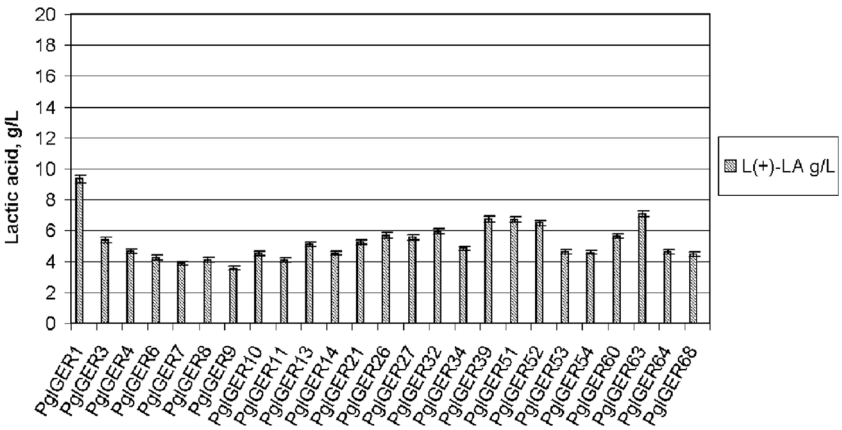

Fig. 2 Isomeric forms of lactic acid produced by lactic acid bacteria isolated from *Panax ginseng* C.A. Meyer: ▨ - L(+)-lactic acid. *Bars* represent standard deviation

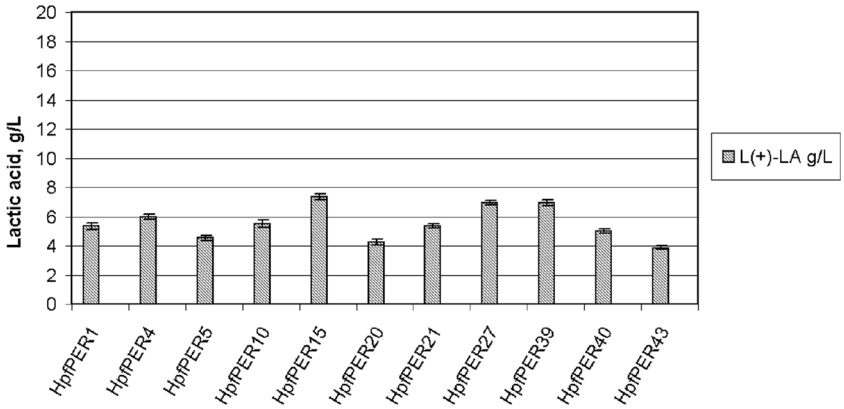

Fig. 3 Isomeric forms of lactic acid produced by lactic acid bacteria isolated from *Hypericum perforatum* L.: ▨ - L(+)-lactic acid. *Bars* represent standard deviation

One-third of the isolates from *G. sanguineum* also showed high acid-producing activity, with lactic acid concentrations above 7.00 g/L (Fig. 1a–c). Lower concentrations of lactic acid, in a range of 3.87–7.34 g/L, were determined for other cocci-shaped isolates of *H. perforatum* (Fig. 3). The highest acid-producing activity of the rod-shaped isolates of *P. ginseng* was present in PglGER1 (9.36 g/L), while the remainder of the isolates synthesized lactic acid at lower concentrations (3.50–7.00 g/L) (Fig. 2). Data for isomeric forms of LA produced by LAB isolated from dairy products are shown in Fig. 4. With the exception of *Lactobacillus bulgaricus* LB1-9, which produced only D(−)-LA, in a concentration of 19.00 g/L, all LAB produced L(+)-LA: *Lactococcus diacetylactis* LD5, 10.00 g/L; *Lactococcus lactis* LL3, 8.08 g/L; *Lactococcus cremoris* LC1, 7.47 g/L; *S. thermophilus* ST3, 9.39 g/L; *E. faecium* EF4, 6.85 g/L; and *Lactobacillus rhamnosus* LR2, 10.51 g/L.

Other phenotypic characteristics of the presumptive LAB include the capacity for growth over a wide temperature and pH range and a high level of halotolerance (Table 1). The presumptive LAB that demonstrated high acid-producing activity (data shown in Figs. 1, 2 and 3) in combination with the milk coagulation time parameter (data not shown) were tested for the given criteria. Almost all isolates—cocci from *G. sanguineum* and rods from *P. ginseng*—showed very good growth over a wide temperature range (15–45 °C), good at 4 °C and weak at 55 °C. A narrower range (30–45 °C) for very good growth and absence of growth at 55 °C were established for the cocci isolated from *H. perforatum*. The pH range for the growth of most isolates of the three plants was also extended, i.e., good and very good growth in the range of 5.0–9.6, and no growth at pH 3.0. Exceptions were observed for rod-shaped isolates from *P. ginseng*, some of which showed weak growth at pH 3.0. With regard to halotolerance, the majority of plant isolates grew well and very well in medium containing NaCl at concentrations of 3.0–6.5 %. At 8.5 and 10 % concentrations of NaCl, about one-half of the isolates from *G. sanguineum* and *H. perforatum* grew, but to a lesser extent, while the majority of the isolates from *P. ginseng* showed good growth at relevant concentrations of NaCl.

Identification of lactic acid bacteria by PCR and construction of a phylogenetic tree

Phenotyping of LAB using morphological, physiological and biochemical (API 20 STREP; bioMérieux) characteristics identified representatives from several species belonging to the genera *Streptococcus* and *Enterococcus*, with similarity of approximately 90 and 80 %, respectively. Thus the identification system used did not yield 100 % accuracy of identity. With the API 50 CHL system, *Lactobacillus rhamnosus* were identified with high similarity, over 99 %.

Genotyping is known to provide for more accurate identity of microorganisms. As such, genotype-based methods such as

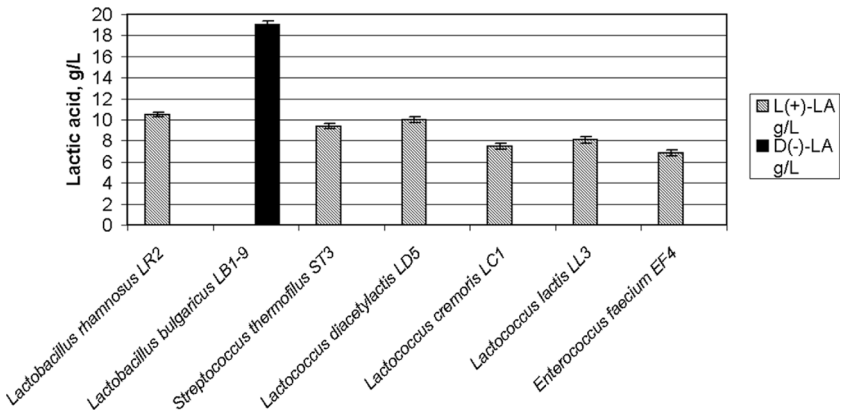

Fig. 4 Isomeric forms of lactic acid produced by lactic acid bacteria isolated from dairy products: ▨ - L(+)-lactic acid; ■ - D(−)-lactic acid. *Bars* represent standard deviation

Table 1 Effect of temperature, pH and NaCl concentration on survival of bacterial isolates and LAB of dairy origin

Isolate	Temperature, °C						pH					NaCl, %				
	4	15	30	37	45	55	3.0	5.0	7.0	8.0	9.6	3.0	4.5	6.5	8.5	10
PglGER1	+	++	++	++	+	+^{w}	+	++	++	++	++	++	++	++	+	+
PglGER3	+	++	++	++	+	+^{w}	+	++	++	++	++	++	++	+	+	+
PglGER26	+	++	++	++	+	+^{w}	+	++	++	++	+	++	++	+	+	+
PglGER32	+^{w}	+^{w}	++	++	+	−	−	++	++	++	++	++	++	+	+	−
PglGER39	+	++	++	++	+	−	−	++	++	+	+	++	++	+	+	+
PglGER51	+^{w}	++	++	++	+	−	−	+	+	+	+	++	++	+	+	−
PglGER63	+^{w}	+	++	++	+	−	−	++	++	++	++	++	++	+	+	−
GsfIS60	+	+	++	++	++	+^{w}	−	+	++	++	+	+^{w}	+^{w}	+^{w}	−	−
GsfIS65	+^{w}	+	++	++	++	+	−	+	++	++	+	+^{w}	+^{w}	−	−	−
GsfIS69	+^{w}	+	++	++	++	+	−	+	++	++	+	+^{w}	+^{w}	−	−	−
GsfIS76	+	+	++	++	++	+^{w}	−	+^{w}	++	++	+^{w}	+^{w}	+^{w}	+^{w}	−	−
GsfIV123	+	++	++	++	++	+^{w}	−	+	++	++	++	++	+	+	+^{w}	+^{w}
GsfIV124	+	++	++	++	++	+	−	+	++	++	+	++	++	++	+	+^{w}
GslIV123	+	++	++	++	++	+^{w}	−	+	++	++	+	++	++	++	+	+^{w}
GslIV124	+	++	++	++	++	+	−	+	++	++	+	++	++	+	+	+^{w}
GslIV213	+	++	++	++	++	−	−	+^{w}	++	++	++	+	+	+^{w}	−	−
GsfIV313	+	++	++	++	++	+^{w}	−	+	++	++	++	++	+	+	+^{w}	+^{w}
GslIV312	+	++	++	++	++	+^{w}	−	+	++	++	++	++	+	+	+^{w}	+^{w}
GsfIB2101	+	++	++	++	++	+^{w}	−	+	++	++	++	++	++	++	+	+^{w}
GsfIB2115	+	++	++	++	++	+^{w}	−	+	++	++	+	++	++	++	+	+^{w}
GslIB2212	+	++	++	++	++	+^{w}	−	+	++	++	++	++	++	++	+	+^{w}
GslIB2227	+	++	++	++	++	+^{w}	−	+	++	++	+	++	++	+	+^{w}	+^{w}
HpfPER15	+	+	++	++	+	−	−	+	++	++	+	++	++	+	+	+^{w}
HpfPER27	+	+	++	++	+	−	−	+	++	++	+	++	++	+	+	+^{w}
HpfPER39	+	+	++	++	+	−	−	+	++	++	+	++	++	+	+	+^{w}
Lactobacillus bulgaricus LB1-9	+^{w}	+	++	++	+	−	−	++	++	++	+^{w}	+	+	+^{w}	+^{w}	−
Lactobacillus rhamnosus LR2	+^{w}	+	++	++	+	−	−	++	++	++	+	++	+	+^{w}	+^{w}	−
Lactococcus lactis LL3	+^{w}	+^{w}	++	++	−	−	−	+	++	+	+^{w}	++	+	+^{w}	−	−
Lactococcus cremoris LC1	−	+^{w}	++	++	−	−	−	+	++	+	−	+	−	−	−	−
Lactococcus diacetylactis LD5	+^{w}	+	++	++	−	−	−	+	++	+	+^{w}	+	+	+^{w}	−	−
Streptococcus thermophilus ST3	+^{w}	+^{w}	++	++	++	+^{w}	−	+	++	+	+^{w}	+^{w}	+^{w}	+^{w}	−	−
Enterococcus faecium EF4	+	++	++	++	++	+^{w}	−	+	++	++	++	++	++	+	+	+^{w}

Growth: ++ very good, + good, − none, ^{w} weak

16S rDNA represent a valuable addition to phenotypic methods for precise identification of lactobacteria isolated from certain microbial communities (Kostinek et al. 2007; Tanasupawat et al. 2007; Chen et al. 2010; Kpikpi et al. 2010; Venturi et al. 2012; Baradaran et al. 2012; Alemayehu et al. 2014). Despite the widespread use of LAB, each plant habitat is characterized by a specific microbocenosis with unique biological activity. The isolated DNA from phenotypically characterized bacterial isolates was used as a matrix for PCR amplification of 16S rDNA. The combination of PCR amplification of variable regions from the 16S rDNA, sequencing of the resulting PCR products, and the subsequent comparison of their sequences with those existing in the NCBI database allowed precise identification of the presumptive LAB to species level, as well as their phylogenetic grouping. All isolates of *H. perforatum*, as well as the majority (73.3 %) of isolates from *G. sanguineum*, were shown to belong to the genus *Enterococcus*. Strain HpfPER39 isolated from

H. perforatum showed 99 % similarity to the species *E. faecium*, while the other two isolates (HpfPER15 and HpfPER27) showed 97 % similarity with this species. The strains GsfIB2115, GslIB2227, GsfIV123, GslIV123 and GslIV213 were related to the species *E. faecium*, based on full conformity of phenotypic characteristics and the results of PCR analysis, reaching 100 % homology of nucleotide sequences. The strains GsfIB2101, GslIB2212, GsfIV124, GsfIV313, GslIV124 and GslIV312 were also found to be related to the same species, indicating 99 % homology. A smaller portion (26.7 %) of isolates from *G. sanguineum* were identified as representatives of the genus *Streptococcus*. High similarity (99.0 %) with *S. thermophilus* was registered for the strains GsfIS60, GsfIS65, GsfIS69 and GsfIS76. The isolates from *P. ginseng* were identified as *Lactobacillus rhamnosus*. The strains PglGER3, PglGER26, PglGER32, PglGER39 and PglGER63 showed 99.0 % homology, and 100 % similarity was reached with strains PglGER1 and PglGER51.

Based on the data from 16S rDNA sequence analysis, a phylogenetic tree was constructed, using the computer program MEGA 4.1, in order to determine the phylogenetic position of the isolated sequences of the strains of plant origin (Fig. 5a–c).

Discussion

Each plant species provides unique conditions for different species of lactobacteria. Among the medicinal plants that we studied, only cocci were isolated from *G. sanguineum* and *H. perforatum*, while only rods were isolated from *P. ginseng*. Cocci have been isolated from the leaves of 14 different plant species, and rods have been identified from four plant species (Michaylova et al. 2007). In naturally fermented herbs used in traditional herb cheese in Turkey, lactobacilli (76.2 % vs. 23.8 % cocci) have predominantly been isolated (Cakir 2010), and only rods have been isolated from the herbal surface of *Phyllanthus niruri* (Venugopalan et al. 2010). Cocci and rods have been isolated from the surface of *Polygonum minus* leaves, a local Malaysian herb (in a ratio of 2:1) and from various plants (clover, grass, dandelion, lilac flowers, chestnut flowers, Hepatica flowers, coltsfoot flowers and rowan leaves) (Magnusson et al. 2003; Baradaran et al. 2012). Cocci have been isolated from grass varieties and vegetables (Alemayehu et al. 2014).

The defined isomeric forms of lactic acid synthesized from selected LAB from plant species *G. sanguineum*, *H. perforatum* and *P. ginseng* (Figs. 1, 2 and 3), with a dominant presence of L(+) form and concentrations of 3.50–11.99 g/L, are comparable to those produced by lactobacteria from the research team's collection that were isolated from dairy products: *Lactococcus diacetylactis* LD5, *Lactococcus lactis* LL3, *Lactococcus cremoris* LC1, *S. thermophilus* ST3, *E. faecium* EF4 and *Lactobacillus rhamnosus* LR2 (Fig. 4). The exception is *Lactobacillus bulgaricus* LB1-9, which synthesizes only D(−)-LA (Fig. 4). Our results are similar to those reported by Kimoto et al. (2004), who obtained 20 bacterial isolates of raw grass (Napier grass) in Japan, which were subsequently morphologically defined and tested as homofermentative cocci producing only L(+)-LA. Kostinek et al. (2007) isolated homofermentative cocci and rods from fermented cassava that produced L(+)-LA. Homofermentative rods producing the same isomer have been isolated from fermented tea leaves (miang) in Thailand (Tanasupawat et al. 2007), and the same authors isolated homofermentative cocci producing D(−)/L(+)-LA, just as was seen in our study, and which was also proven by Tamang et al. (2005) for pediococci from traditionally fermented vegetable products of the Eastern Himalayas. In contrast to our data, Tanasupawat et al. (2007) and Tamang et al. (2005) isolated homofermentative rods producing D(−)/L(+)-LA, and Kostinek et al. (2007) reported homofermentative rods producing L(+)-, D(−)- and D(−)/L(+)-LA. From these plant sources, heterofermentative cocci producing only D(−)-LA (Tamang et al. 2005; Kostinek et al. 2007) and heterofermentative rods producing D(−)/L(+)-LA (Kostinek et al. 2007; Tanasupawat et al. 2007) have also been isolated. In addition to the determination of isomeric forms of lactic acid synthesized from presumptive lactobacteria present in certain bacterial isolates, as an element of preliminary identification, some authors also report data for concentrations of lactic acid. The results reported by Cock and de Stouvenel (2006) are interesting in this regard. Based on screening of 20 bacterial isolates obtained from the leaves of sugar molasses, the authors found only one strain (with index CC 85–92) with a high potential for synthesis of L(+)-LA, in concentrations of 12.4 and 13.7 g/L and at cultivation temperatures of 36 and 32 °C, respectively—concentrations comparable to those for our isolates (Fig. 1a and b). With this strain, grown within parameters suitable for targeted synthesis of lactic acid, the authors achieved a maximum yield of 35.0 g/L.

Data indicating a wide temperature range for bacterial growth have been reported by Baradaran et al. (2012) and Tanasupawat et al. (2007) for lactococci and lactobacilli isolated from the herb *Polygonum minus* (10–45 °C) and for lactococci from fermented tea leaves (15–45 °C), respectively, which is consistent with our results revealing a temperature range of 15–45 °C for growth of isolated lactobacteria from the three plant species studied (Table 1). Tanasupawat et al. (2007) reported the absence of growth of lactobacilli at 45 °C. The same authors established that these isolates grew well in a wide pH range, 3.0–7.0 and 4.0–8.5, which is consistent with results reported by Tamang et al. (2005) for lactococci of other plant origins. Alemayehu et al. (2014) demonstrated good growth at pH 9.5 for lactococci isolated from grass varieties and vegetables. All isolates obtained from *G. sanguineum*, *H. perforatum* and *P. ginseng* showed good growth at a high

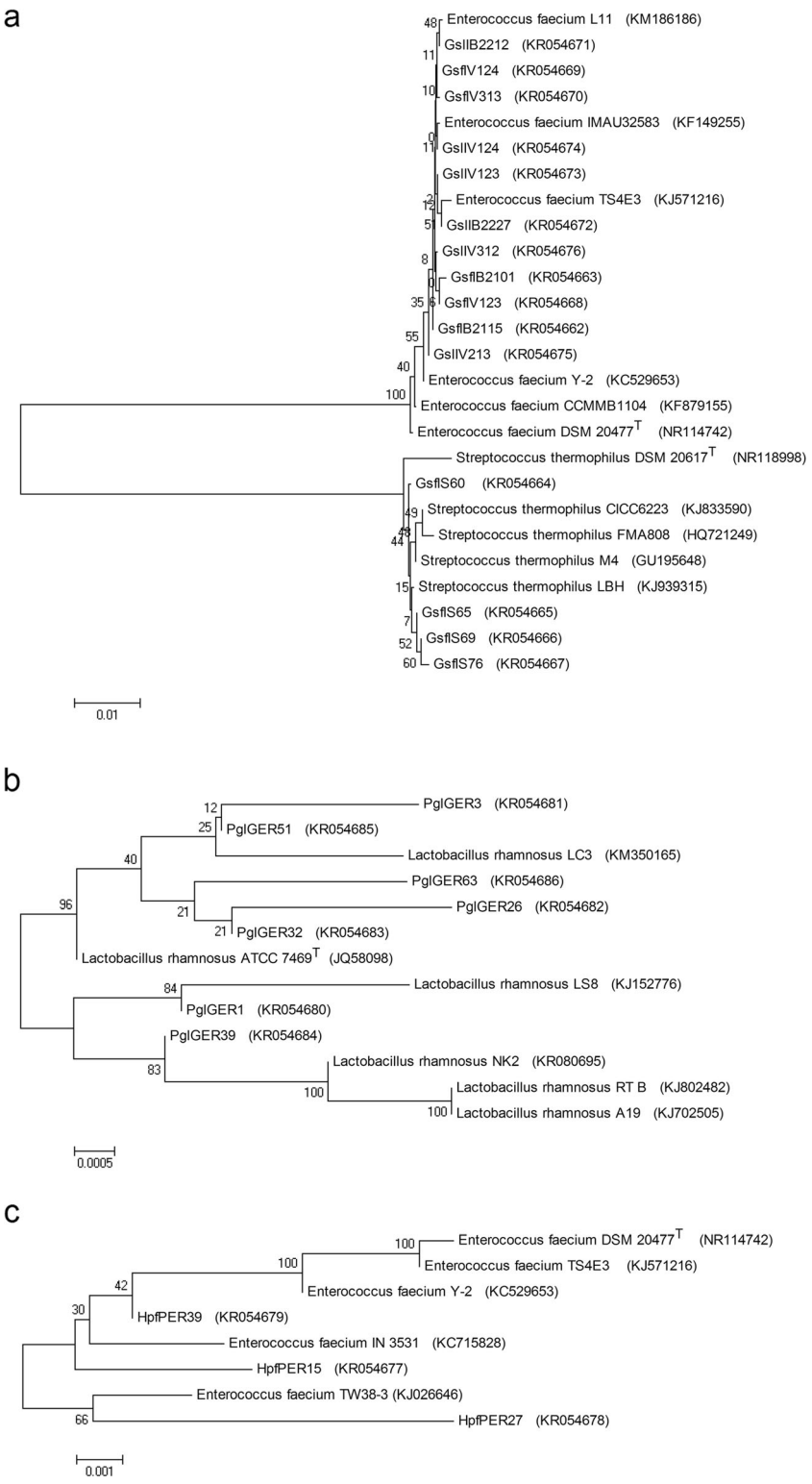

Fig. 5 Phylogenetic trees based on 16S rDNA sequences of identified isolates from medicinal plants (**a** *Geranium sanguineum* L.; **b** *Panax ginseng* C.A. Meyer; **c** *Hypericum perforatum* L.) determined using the neighbor-joining method

pH value (9.6), and some (*P. ginseng*) at a low pH value (3.0) (Table 1). According to the two indicators studied, plant-derived isolates in our study showed better growth characteristics than lactobacteria of a dairy origin (Table 1). Plant isolates also showed substantially higher levels of halotolerance (up to 10 % NaCl) compared to milk (Table 1). Tamang et al. (2005) and Baradaran et al. (2012) established a high level of halotolerance (growth in the presence of 10 % and 6.5 % NaCl, respectively), while Kimoto et al. (2004) and Tanasupawat et al. (2007) found a lower level (growth in the

presence of 4.0 % NaCl) for lactococci of different plant origins. Baradaran et al. (2012) demonstrated good growth of lactobacilli isolated from *Polygonum minus*, and Alemayehu et al. (2014) of lactococci from grass and vegetables, in the presence of 6.5 % NaCl.

All isolates of *P. ginseng* and *H. perforatum* were genetically identified as *Lactobacillus rhamnosus* and *E. faecium*, respectively, with the majority of *G. sanguineum* identified as *E. faecium* and a small minority as *S. thermophilus*. The presence of genotypically identified representatives of species of the genera *Enterococcus*, *Streptococcus* and *Lactobacillus* in isolates from plants of different origins have been reported by other authors (Hartnett et al. 2002; Magnusson et al. 2003; Michaylova et al. 2007; Chen et al. 2010; Baradaran et al. 2012). Hartnett et al. (2002) identified *E. faecium* in isolates from raw barley; in isolates from sorghum, defined species *Enterococcus mundtii* and *E. faecalis* were found—which were undetected in plant isolates that we examined—as well as *Lactococcus lactis* in isolates from raw barley. *Streptococcus thermophilus* has been isolated from leaves from the plant species *Capsella bursa-pastoris*, *Chrysanthemum*, *Cichorium intybus*, *Colchicum*, *Dianthus*, *Hedera*, *Nerium oleander*, *Plantago lanceolata*, *Rosa*, *Tropaeolum*, *Calendula officinalis*, *Cornus mas*, *Galanthus nivalis* and *Prunus spinosa*, and *Lactobacillus bulgaricus* was also identified in some of the plant isolates (Michaylova et al. 2007). Several groups have isolated species of LAB that were not detected in plants that we tested. For instance, Baradaran et al. (2012) identified *Lactococcus lactis*, *Pediococcus pentosaceus* and *Lactobacillus curvatus* in isolates from *Polygonum minus* (a Malaysian herb). Magnusson et al. (2003) isolated representatives of the genera *Lactobacillus* (*L. plantarum*, *L. coryniformis*, *L. acidophilus* and *L. sakei*) and *Pediococcus* and of *Enterococcus hirae* from different parts of plants (grass, dandelion, lilac, chestnut, Hepatica, coltsfoot and rowan). The species *Lactobacillus plantarum*, *Weissella cibaria*, *Leuconostoc pseudomesenteroides* and *Lactococcus lactis* subsp. *lactis* were isolated from ripe mulberries from five countries in Taiwan (Chen et al. 2010). In the present study, it should be noted that each of the plant habitats represents a single ecological niche for the growth of specific lactic acid microflora.

Conclusions

The results of 16S rDNA sequence analysis and phylogenetic position determination of the sequences of strains isolated from the herbs *Geranium sanguineum* L., *Hypericum perforatum* L. and *Panax ginseng* C.A. Meyer indicate that they are appropriate natural ecological niches for the isolation of the lactobacteria *Enterococcus faecium, Streptococcus thermophilus* and *Lactobacillus rhamnosus*. The better growth characteristics of isolated and identified lactococci and lactobacilli in a wide pH and temperature range, as well as their high acid-producing activity, compared to LAB isolated from dairy sources indicates the excellent potential for in situ cultivation of these newly isolated strains in milk, with subsequent creation of starters for new fermented dairy products.

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