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Original Article

Phenotypic and molecular identification of lactobacilli isolated from vaginal secretions

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Background and Purpose: Twenty lactobacilli isolated from vaginal samples of healthy volunteers were characterized according to polyphasic taxonomy. A broad spectrum of activity and protective properties of these vaginal isolates has been reported.

Methods: Phenotypic and genotypic methods such as random amplified polymorphic DNA (RAPD), species-specific and BOX polymerase chain reaction (PCR), amplified ribosomal DNA restriction analysis (ARDRA), and 16S rRNA sequence analyses were applied for *Lactobacillus* spp. identification.

Results: On the basis of carbohydrate utilization profiles using API 50 CHL kits (fermentation pattern), the strains were divided into 7 groups. RAPD- and BOX-PCR analyses revealed heterogeneity within the established phenotypic groups and discriminated successfully between the vaginal strains. The combination of species-specific PCR, ARDRA, and 16S rRNA sequence analyses allowed the species identification of 7 strains as *Lactobacillus fermentum*, 2 as *Lactobacillus gasseri*, 1 as *Lactobacillus brevis*, and 1 as *Lactobacillus salivarius*. These results were not in agreement with data obtained by classical and molecular methods for some clinical isolates.

Conclusion: This is the first polyphasic taxonomy study on vaginal lactobacilli from Bulgarian women of reproductive age. These data add to the limited information existing in Bulgaria on the true identity of *Lactobacillus* spp. in the vaginal microbiota.

Key words: Databases, genetic; DNA, ribosomal; *Lactobacillus*; Molecular sequence data; Random amplified polymorphic DNA technique; Vagina

Introduction

Lactobacilli are predominant in, and a critically important component of, the vaginal ecosystem in healthy women. Data on species affiliation of vaginal lactobacilli are necessary for understanding of the dynamic equilibrium and the complex mechanisms involved in ecological homeostasis, a key component in the health and well-being of women.

The time-consuming and ambiguous classical methods used for lactobacilli classification based on physiological and biochemical criteria do not always correspond to the actual genetic relativity of the species, and are not discriminative enough to distinguish strains of the same species. Methods of identification of lactobacilli, previously based on culture-dependent methods, have recently been supplemented with molecular techniques. A large number of genotyping analyses (such as DNA-DNA homology, polymerase chain reaction [PCR]-based methods, pulsed-field gel electrophoresis, ribotyping, and sequencing) have enabled taxonomic studies of these bacteria [1-3]. Although new applications of molecular methods, such as PCR denaturing gradient gel electrophoresis [2] and thermal gradient gel electrophoresis [4], have become available for the detection and analysis of vaginal microbiota, improvements are still required, especially in the areas of sensitivity, cost, and quantitative

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power. In addition, the newer cultivation-independent methods [5] and the combination of different genotyping methods represent powerful tools for strain-specific identification, thus facilitating differentiation of exogenous from indigenous strains in the complex ecosystem of the human vagina [6].

Polyphasic taxonomy is a new approach combining morphological, biochemical, and physiological characteristics with molecular-based genomic techniques [7]. This method has been successfully applied for differentiation of vaginal lactobacilli and for elucidation of the complexity and dynamics of vaginal microbiota. As a result, reliable information for species affiliation of vaginal microflora from different geographical regions has been accumulated that complements knowledge of the diversity of vaginal lactobacilli and their taxonomy [4,8-11]. However, limited data exist on the species affiliation and biology of vaginal lactobacilli from Bulgarian woman. During our recent study on the vaginal microflora of 100 healthy Bulgarian premenopausal volunteers, a laboratory collection of vaginal *Lactobacillus* strains was created. A group of randomly selected vaginal isolates were characterized with regard to inhibitory activity and antibiotic susceptibility [12,13]. The present study was undertaken to identify these newly isolated vaginal lactobacilli.

Methods

Microorganisms, media and growth conditions

The 20 vaginal strains used in this study were isolated from vaginal swab samples obtained from healthy Bulgarian women of reproductive age. Selective media de Man, Rogosa, Sharpe (MRS) agar (Difco, Detroit, MI, USA) and Rogosa agar (Difco) were used for initial colony selection and isolation of pure cultures.

Pure cultures were stored at –70°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% v/v glycerol. The strains were subsequently grown on MRS agar or broth media, incubated for 24 to 48 h at 37°C under anaerobic conditions (BBL® GasPak Anaerobic System; Becton Dickinson, Cockeysville, MD, USA). For DNA studies, *Lactobacillus* isolates and reference strains from the American Type Culture Collection (ATCC) were grown in MRS broth overnight and were checked for purity by plating a drop onto plate count agar (Difco).

The following reference ATCC strains were used: *Lactobacillus johnsonii* ATCC 33200^T, *Lactobacillus crispatus* ATCC 33820^T, *Lactobacillus gasseri* ATCC 9857, *Lactobacillus fermentum* ATCC 14931^T, *Lactobacillus acidophilus* ATCC 4356^T, *Lactobacillus brevis* ATCC 14869^T, *Lactobacillus jensenii* ATCC 25258^T, and *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741^T.

Phenotypic characterization

The initial identification of obtained pure cultures was based on colony and cell morphology determined by light microscopy, Gram staining, and catalase reaction. Gas production from glucose was observed in MRS broth (with 10% glucose and meat extract omitted) containing inverted vials (Durham). The growth effects of temperature were studied in MRS broth at 15°C to 45°C.

Carbohydrate fermentation profiles were obtained with API Rapid CH fermentation strips (bioMérieux, Marcy l'Etoile, France) in duplicate at 37°C, in *Lactobacillus* identification medium (CHL broth, API 50 CHL; bioMérieux) as specified by the manufacturer. The identification software API LAB 50 CHL version 5.0 (bioMérieux) was used to facilitate the interpretation of fermentation patterns.

Total DNA from reference *Lactobacillus* strains and vaginal isolates was isolated as described by Delley et al [14].

Polymerase chain reaction analyses

All PCR reactions were done in a Progene cycler (Techne, Cambridge, UK) in 25 µL volume, using Ready-To-Go™ PCR beads (Amersham Biosciences, Little Chalfont, England). Random amplified polymorphic DNA (RAPD)-PCR analysis with primer M13V (5'-GTTTTCCCAGTCACGAC-3') [MWG-Biotech, Ebersberg, Germany] was performed as described by Ehrmann et al [15]. BOX-PCR amplification (genomic fingerprinting) was performed with primer BOX A1R (5'-CTACGGCAAGGCGACGCTGAC-3'), according to Louws et al [16]. The species-specific PCR analyses with primers for *L. fermentum*, *L. acidophilus* and *L. crispatus* were performed as described by Walter et al [17] and for *L. salivarius* as described by Chagnaud et al [18].

Amplified rDNA restriction analysis

DNA from the vaginal and ATCC reference strains was used as a template for PCR amplification using universal primers corresponding to the 5' end-fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 3' end-rD1 (5'-TAAGGAGGTGATCCAGGC-3') of the 16S rRNA gene [19]. The PCR products were digested

with endonucleases *Sau*3A and *Hae*III, following the manufacturer's instructions (Boehringer Mannhem GmbH, Mannheim, Germany). The patterns were analyzed in 2% agarose gel (Sigma type II), 0.5 × 4, 5, 6, 7-tetrabromobenzotriazole. A 100-bp molecular mass marker was used (New England BioLabs, Beverly, MA, USA).

Cluster analysis

Computer cluster analysis was performed based on the calculation of Sorensen's coefficient and average linkage analysis (unweighted pair-group method with arithmetic averaging; UPGMA) using the MVSP Plus 3.0 Package (supplier details, UK).

16S rDNA amplification and sequencing

DNA from strains 303, 712, 713, 831, and 832 was amplified using the primer set fD1 and rD1 [16]. Obtained PCR products were purified by the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences) and the DNA was used as a template for the standard sequencing procedure (Macrogen Inc, Seoul, Republic of Korea). The sequencing reactions using universal (27F or 1492R) primer were carried in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) [Applied Biosystems, Foster City, CA, USA]. Sequences were edited to exclude the PCR primer-binding site and manually corrected with Sequence Scanner 1.0 (Applied Biosystems).

The 16S rDNA sequences determined in this study have been deposited in the GenBank database. The accession numbers assigned to vaginal strains were as follows: strain 713, DQ456489; strain 712, EU402394; strain 303, EU402395; strain 831, EU 401990; and strain 832, EU391635.

Results

Phenotyping characterization of vaginal lactobacilli

Twenty strains isolated from vaginal secretions of healthy Bulgarian women were subjected to polyphasic taxonomy study. The interest for their identification was provoked by recently established broad spectrum of antagonistic activity and their protective properties [12]. In a recent investigation, the strains were also evaluated for antibiotic susceptibility [13]. All isolates were Gram-positive, non-motile, non-spore forming, catalase-negative rods, with the classical characteristics of *Lactobacillus* [20]. They grew well at 37ºC and were mesophilic. Twelve of the 20 strains grew at 15ºC and 16 at 45ºC. Eleven vaginal lactobacilli did not produce gas from glucose (Table 1).

As a first step, isolates were classified into 7 groups, according to the results of carbohydrate utilization testing using API 50 CHL systems (API LAB

Table 1. Biochemical characterization and API 50 CH grouping of vaginal *Lactobacillus* strains

Sugar[a]	Vaginal strain group						
	A (301, 302, 303, 304)	B (362, 364, 361)	C (832)	D (711, 712, 713, 732, 821)	E (831, 622)	F (611, 612, 613, 811)	G (822)
Glycerol	–	–	–	–	–	–	+
Erythritol	–	–	–	–	–	–	–
D-Arabinose	–	–	–	–	–	–	–
L-Arabinose	+	–	–	–	+	–	–
Ribose	+	+	–	–	+	+	+
D-Xylose	+	–	–	–	+	–	–
L-Xylose	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–
β-Metil-D-xiloside	–	–	–	–	–	–	–
Galactose	+	+	+	–	–	+	+
D-Glucose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	–	+	+
D-Mannose	–	–	+	+	–	+	+
L-Sorbose	–	–	–	–	–	–	–
Rhamnose	–	–	+	–	–	–	–
Dulcitol	–	–	–	–	–	–	–

(Table continued on page 472)

(Table continued from page 471)

Inositol	–	–	–	–	–	–	–
Mannitol	–	–	+	–	–	+	+
Sorbitol	–	–	+	–	–	+	+
α-Methyl-D-mannoside	–	–	–	–	–	–	–
α-Methyl-D-glucoside	V	–	–	–	–	–	–
N-Acetyl-glucosamine	V	–	+	–	–	–	+
Amygdalin	–	–	–	–	–	–	+
Arbutin	–	–	–	–	–	–	+
Esculin	–	–	–	+	–	+	+
Salicin	–	–	–	V	–	–	+
Cellobiose	–	–	–	+	–	+	+
Maltose	–	+	+	–	–	+	+
Lactose	+	+	+	–	–	+	+
Melibiose	–	+	+	–	–	–	–
Saccharose	+	+	+	+	–	+	+
Trehalose	–	–	+	–	–	+	+
Inulin	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	+	+
D-Raffinose	–	+	+	–	–	–	–
Amidon	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–
β-Gentiobiose	–	–	–	–	–	–	+
D-Turanose	–	–	–	–	–	–	–
D-Lyxose	–	–	–	–	–	–	–
D-Tagatose	–	–	–	–	–	+	+
D-Fucose	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	–	–	–
L-Arabitol	–	–	–	–	–	–	–
Gluconate	V	V	–	–	–	–	–
2-Keto-gluconate	–	–	–	–	–	–	–
5-Keto-gluconate	–	–	–	–	+	–	–
Gas production from glucose	+	+	–	–	+	–	–
Species affiliation	*Lactobacillus fermentum*	*Lactobacillus fermentum*	*Lactobacillus salivarius*	*Lactobacillus acidophilus*	*Lactobacillus brevis*	*Lactobacillus curvatus*	*Lactobacillus paracasei* ssp. *paracasei*
Confidence	99.9%	97.0%	100%	71.1%	99.8%	Unacceptable	97.7%

Abbreviations: α = alpha; β = beta; – = negative reaction; + = positive reaction; V = variable between the strains of the group

[a]Sugar utilization profile.

50 CHL software) for the species identification (confidence interval, 71.1-100.0%) [Table 1]. Despite differences in sugar fermentation profiles of the strains from groups A and B, they were identified as *L. fermentum*.

Molecular identification and 16S rRNA gene sequences

Primary affiliation based on the biochemical results facilitated the choice of appropriate molecular methods for further species identification. The 7 vaginal strains identified as *L. fermentum* (A and B groups) were subjected to *L. fermentum*-specific PCR amplification, according to Walter et al [17]. Positive signals were detected for the isolates 301, 302, 303, 304, 361, 362, and 364, as well as for the type strain *L. fermentum* ATCC 14931^T. Only strain 303, which possesses a broad spectrum of activity [12], was subjected to 16S rDNA analyses. The partial 16S rDNA sequence (GenBank accession number, EU402395) showed 98% similarity with the species *L. fermentum*. Additional confirmation of genetic relatedness of these isolates and strain typing were done by BOX-PCR analysis (Fig. 1A). The inter-relationships among the 7 strains were evaluated based on UPGMA analysis and the

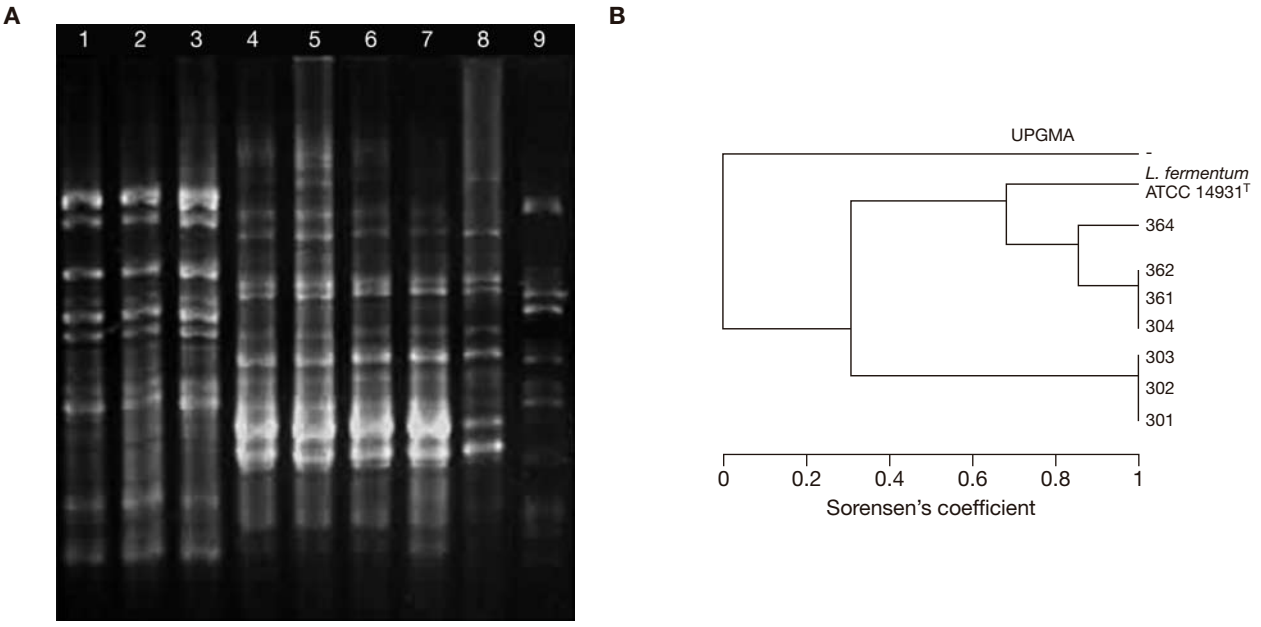

Fig. 1. BOX-polymerase chain reaction analysis of vaginal isolates from groups A and B, identified as *Lactobacillus fermentum*. BOX-electrophoresis band patterns of isolates and type strain in 2% agarose gel (A) and dendrogram derived from BOX-DNA patterns showing clustering of the type strain *L. fermentum* American Type Culture Collection (ATCC) 14931^{T} and identified isolates (B). Lane 1, 301; lane 2, 302; lane 3, 303; lane 4, 304; lane 5, 361; lane 6, 362; lane 7, 364; lane 8, *L. fermentum* ATCC 14931^{T}; lane 9, pUC19 DNA-digested *Sau*3A molecular mass marker. UPGMA = unweighted pair-group method with arithmetic averaging.

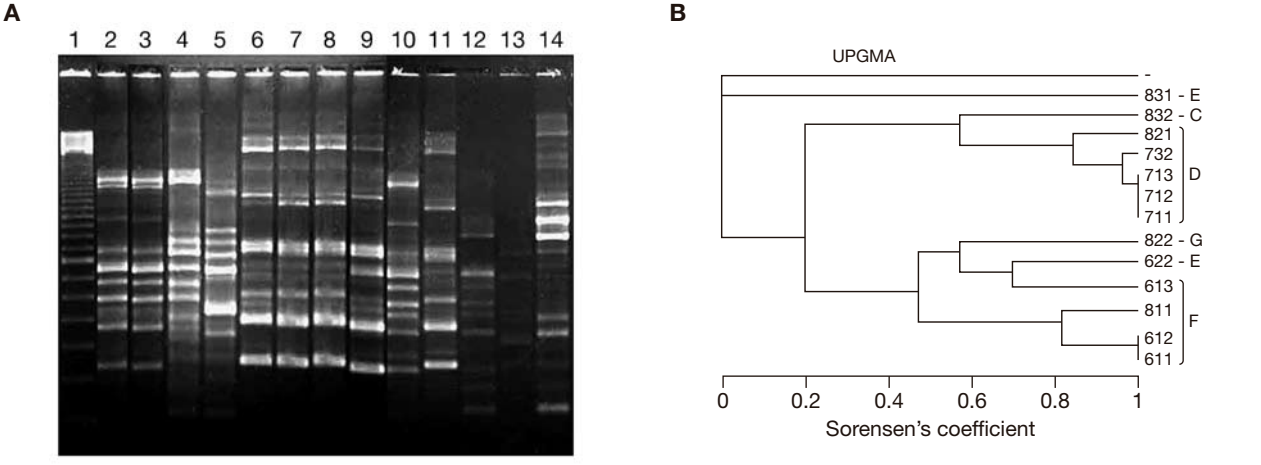

Fig. 2. DNA fingerprints obtained after random amplified polymorphic DNA (RAPD)-polymerase chain reaction analysis of vaginal isolates (A) and cluster analysis of RAPD patterns (B). Lane 1, 100-bp molecular mass marker; lane 2, 611; lane 3, 612; lane 4, 613; lane 5, 622; lane 6, 711; lane 7, 712; lane 8, 713; lane 9, 732; lane 10, 811; lane 11, 821; lane 12, 822; lane 13, 831; lane 14, 832; C to G, vaginal strain groups. UPGMA = unweighted pair-group method with arithmetic averaging.

resulting dendrogram produced (Fig. 1B). Generated BOX-DNA patterns were relatively complex and some genetic diversity among species, expressed in the presence of 2 *L. fermentum* biotypes, was revealed. The strains 622 and 831 (group E) were also clustered by BOX-PCR analysis, with the reference strain *L. brevis* ATCC 14869^{T} (data not shown). Similarly, full 16S rRNA gene sequence of the strain 831 (GenBank accession number EU 401990) showed 99% similarity with *L. brevis*.

The RAPD patterns of 13 of the 20 vaginal strains (Fig. 2A) were generated using M13V primer [15] and 2 non-coherent clusters were visualized on the derived dendrogram (Fig. 2B). Clusters I and II were divided into subgroups. The data from API 50 CHL (Table 1) and RAPD grouping were not entirely in agreement

(Fig. 2A and 2B). Identical RAPD patterns of strains 711, 712 and 713 as well as strains 611 and 612 suggested that they were multiple isolates from a single species or identical strains.

The initial API identification of strain 832 (group C) as *L. salivarius* was confirmed by species-specific PCR analyses (Fig. 3) and partial 16S rRNA gene sequencing (accession number EU391635). In addition, 16S rDNA-amplified ribosomal DNA restriction analysis (ARDRA) was used for species and strain typing. The ARDRA-*Hae*III polymorphic patterns of the strain 832 (generated fragments: <100, ~200, 470 and 600 bp) revealed a high similarity with *L. salivarius* subsp. *salivarius* ATCC 11741^T (generated fragments: <100, 140, 470 and 600 bp).

The sugar fermentation profiles determined a species affiliation with *L. acidophilus* for the strains from group D. However, positive signals from PCR analysis with species-specific primers for *L. acidophilus* and *L. crispatus* were not obtained with target DNAs from these 5 strains. After the 16S rRNA gene from the isolates 712 and 713 were amplified by PCR, the amplicons, which contained the V1 and V2 variable regions, were sequenced (accession numbers EU402394 and DQ456489). The sequences were compared with available data at the National Center for Biotechnology Information (NCBI) Blast Library and a high similarity (range, 90-98%) to the species *L. gasseri* was estimated for the sequenced isolates from group D. According to the identical *Sau*3A-ARDRA restriction fragments obtained (~100 to ~1200 bp), all vaginal isolates from group D formed a coherent cluster identified as *L. gasseri* ATCC 9857 (similarity, 0.9-1.0).

The identification of the strains from the groups F and G was not established by molecular methods and is still in progress.

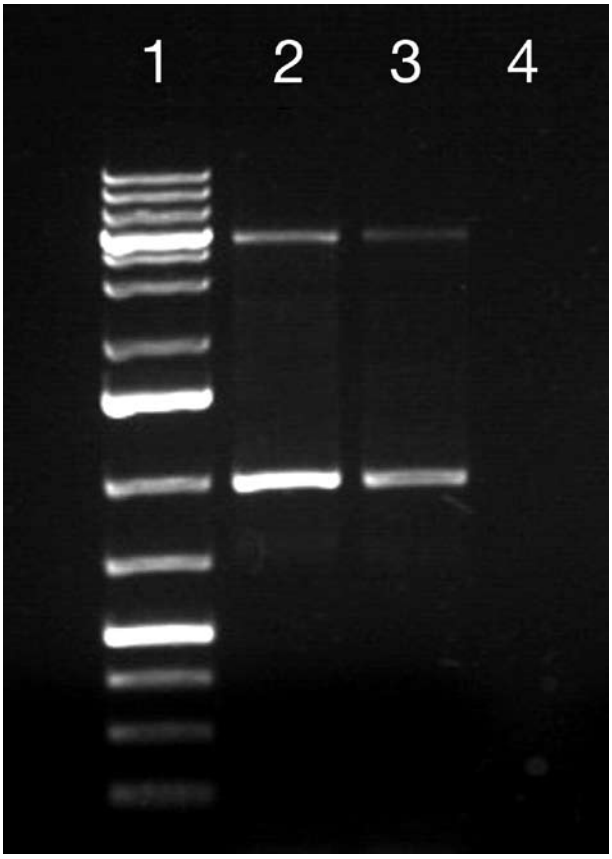

Fig. 3. DNA binding patterns obtained by polymerase chain reaction (PCR) with primers specific for *Lactobacillus salivarius*. Lane 1, O'GeneRuler™ 1-kb DNA ladder; lane 2, *L. salivarius* subsp. *salivarius* American Type Culture Collection 11741^T; lane 3, vaginal strain 832; lane 4, negative PCR control (no DNA).

Discussion

Efficient and reproducible methods for identification and discrimination of vaginal lactobacilli are needed, both for rapid species identification and to facilitate study of the complex microflora of the vaginal ecosystem.

Characterization of 20 selected vaginal isolates was initially conducted by cell morphology, and by physiological and biochemical tests. Analyses using API 50 CH kits gave rapid and not entirely consistent phenotypic identification of vaginal lactobacilli, and these results (Table 1) revealed the limits of this approach. Carbohydrate fermentation tests of vaginal isolates proved difficult, as some genotypically unrelated lactobacilli (from groups D, E, and F) showed the same fermentation results. Chagnaud et al [18] reported that the reference *L. salivarius* UCC 43321 strain was displayed as *Lactobacillus paracasei* with this method. Furthermore, the API database is not updated and some new species are missing [21]. The reliability of these tests in the case of medically important lactobacilli has been questioned and some controversial results were achieved [22-24]. The API system agreed with molecular identification for only 4 of 90 vaginal isolates [24]. In this study, the initial identification by API LAB 50 CHL 5.0 software was confirmed with more discriminative molecular methods for 7 isolates belonging to the species *L. fermentum*, 1 to *L. salivarius* subsp. *salivarius*, 1 to *L. brevis*, but none for *L. acidophilus*.

Results from species-specific PCR and 16S rDNA analysis clearly showed the correct affiliation to *L. fermentum* of all strains from A and B groups.

This species was also reported in vaginal samples by Pavlova et al [8]. Finally, to determine the relatedness between vaginal strains 301, 302, 303, 304, 361, 362, and 364 and *L. fermentum* ATCC 14931^T, we conducted genetic fingerprinting experiments using BOX-PCR, as described by Louws et al [16]. For the other 13 isolates, molecular typing at the strain level was estimated by RAPD-PCR analysis. Our results from RAPD-PCR suggest satisfactory differentiation at the strain and species levels of the examined 13 vaginal lactobacilli. Some correlation with the primary biochemical clustering was shown (Fig. 2).

Dicks et al confirmed the grouping into 6 protein profile clusters of 21 vaginal isolates by the results from numerical analysis of RAPD-PCR binding patterns [25]. A lack of agreement between the API 50 CH and RAPD grouping was observed for strains from group E, and particularly from groups D and F. A similar incongruity of RAPD and carbohydrate clustering was reported in an analysis of food isolates identified as *Lactobacillus plantarum* [23]. Despite the unrelated RAPD profiles of strains 831 and 622 generated by BOX-PCR fingerprints (Fig. 2), we observed a high similarity of patterns with *L. brevis* ATCC 14869^T, which were confirmed by 16S rRNA gene sequencing (GenBank accession number EU401990). The RAPD techniques have been extensively used in the strain and intraspecies typing of lactic acid bacteria. In this study, RAPD proved a fast and relatively inexpensive technique, providing good levels of discrimination of *Lactobacillus* and subtyping of newly isolated strains. Moreover, this method exposed the multiple isolates among the group of vaginal strains (strains 711, 712 and 713 from group D and strains 611 and 612 from group F), and facilitated the selection of appropriate strains for sequencing analysis. Following 16S DNA analysis, strains 712 and 713 were identified as *L. gasseri*.

The phenotypically characterized strains, even those with a good level of identification, could be misidentified. Song et al reported that strains identified as *L. acidophilus* by API tests often belong to the species *L. crispatus* [22]. For that reason, the 5 strains in group D were subjected simultaneously to species-specific PCR analysis with primers for both species. The negative PCR results obtained, as well as the additional *Sau*3A-ARDRA clustering with *L. johnsonii* and *L. gasseri* did not allow their exact species identification. Thus, 16S rRNA gene analysis was applied and successfully identified for 2 isolates from this group.

Simultaneous species-specific PCR, *Sau*3A-ARDRA fingerprint and 16S rRNA sequencing confirmed strain 832 as *L. salivarius* subsp. *salivarius*. This strain is rhamnose-positive (Table 1), which is one of its phenotypic differences from *L. salivarius* subsp. *salicinius* [26]. New taxonomic data has indicated that *L. salivarius* subsp. *salivarius* and *Lactobacillus salivarius* subsp. *salicinius* do not merit separate subspecies status [27]. To the authors' knowledge, this is the first report of molecular identification of *L. salivarius* from healthy Bulgarian women.

The results from polyphasic characterization of 5 strains — 611, 612, 613, 811, and 822 (groups F and G) — exhibited some discrepancies and these strains could not be accurately assigned to the species level from metabolic data alone. However, the isolates from group F not identified by API tests were discriminated as genotypically unrelated strains (Fig. 2) and will be subjected to 16S rRNA analyses. Although phenotypic techniques such as the API system are still regarded as powerful tools capable of discriminating between the species of *Lactobacillus*, the use of genetic methods for *Lactobacillus* taxonomy has become the backbone of reliable identification [28].

Our results revealed the presence of *L. fermentum*, *L. gasseri*, *L. salivarius*, *L. brevis*, and probably *L. paracasei* in vaginal samples. The most commonly identified strains belonged to *L. fermentum*, followed by *L. gasseri*. With 3 different genotypic methods, no strains of *L. acidophilus* and *L. crispatus* were found; both species are reported in vaginal microflora of women from Nigeria, Sweden, Caucasus, and other countries [2,4,8,11]. Nevertheless, other members of *L. acidophilus* group from the species *L. gasseri* were identified. This result confirmed the DNA homology study of vaginal lactobacilli from 27 women [29] and the study of Antonio et al [30]. Thus, the new vaginal isolates from Bulgarian women represent species of the phylogenetic groups *Lactobacillus reuteri*, *L. salivarius*, and *L. acidophilus* [31]. The correct polyphasic characterization of vaginal *Lactobacillus* strains is a precondition for more stable classification schemes of vaginal lactobacilli and for their further evaluation and possible clinical application.

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