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ORIGINAL ARTICLE

The application of genetics methods to differentiation of three *Lactobacillus* species of human origin

Tomasz Gosiewski · Agnieszka Chmielarczyk · Magdalena Strus · Monika Brzychczy-Włoch · Piotr B. Heczko

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Abstract In recent decades, the interest in probiotics as diet supplements or drugs has increased. In order to determine a specific bacterial isolate to be probiotic, it is necessary to describe precisely its probiotic characteristics and taxonomic properties, including the strain level. Most of the well-known genotyping methods were designed for the commonly-found pathogenic bacteria. The objective of this study is to undertake an attempt at standardization of FISH, RAPD and PFGE methods to genotype and identify the bacteria belonging to *Lactobacillus fermentum, L. gasseri* and *L. plantarum* species. The FISH probes have been designed and tested for *Lactobacillus fermentum, L. gasseri* and *L. plantarum* species and an endeavor has been made at standardization of RAPD and PFGE methods for these bacterial species. Moreover, the MLST method was applied to differentiate *Lactobacillus plantarum* strains. *L. plantarum* isolated from humans could not be genetically diversified with the use of RAPD, PFGE or MLST methods; only the strains originating from plants have displayed diversification among themselves and have been different from the strains of human origin.

Keywords *Lactobacillus* · FISH · RAPD · PFGE · MLST

T. Gosiewski (✉) · A. Chmielarczyk · M. Strus · M. Brzychczy-Włoch · P. B. Heczko
Chair of Microbiology, Jagiellonian University Medical College, 18 Czysta Str., 31-121 Cracow, Poland
e-mail: tomasz.gosiewski@uj.edu.pl

Introduction

In the last recent decades, the interest in probiotics as diet supplements or drugs has increased. According to their definition, probiotics are live microorganisms which have a beneficial influence on the health of the host when administered in adequate amounts. They are mostly applied as preparations for restoring the ecological balance of the gastrointestinal and genital tracts. Pursuant to the assumptions of the report of the Joint Working Group of Food and Agriculture Organization of the United Nations concerning preliminary guidelines for the evaluation of probiotics, it has been stated that it has to be a common species and a specific bacterial strain within the species of previously confirmed health properties (Araya et al. 2002). To meet these requirements, it is fundamental to draw up a methodology of the molecular typing of the bacterial isolates belonging to the *Lactobacillus* genus since most strains with probiotic properties belong within this bacterial group.

The objective of this study is an attempt at standardization of the molecular methods of RAPD (Random Amplification of Polymorphic DNA), PFGE (Pulsed Field Gel Electrophoresis) and FISH (Fluorescence In Situ Hybridization) in the process of identification of the strains belonging to three species: *Lactobacillus fermentum*, *L. gasseri* and *L. plantarum* isolated from the human genital tract. Additionally, the MLST (Multilocus Sequence Typing) method was used for differentiation of *L. plantarum* strains. These three species are most often applied in probiotics preparations in combination with other bacteria or separately. The research has been conducted to enable the evaluation of the level of colonization of the genital tract by the examined probiotic strains belonging to the above-mentioned species in the

physiological conditions as well as during clinical trials of the probiotic preparations.

Materials and methods

Samples

The examined strains came from a collection gathered during the period 2000 to 2005 at the Chair of Microbiology of the Jagiellonian University Medical College derived from the routine microbiological diagnostics of the female genital tract in 111 healthy women aged 18–45, carried out in our microbiology laboratory. The patients reported no infection that required treatment with antibiotics.

Bacterial cultures

Vaginal swabs were immediately cultured on Columbia blood agar (Oxoid) with 5% sheep blood, de Man–Rogosa–Sharpe MRS agar (Oxoid), McConkey agar (Oxoid) and Schaedler agar (Oxoid). Aerobic bacteria were cultured in aerobic conditions at the temperature of 37°C for 24 h and anaerobes were cultured in an anaerobic chamber (MACS – MG 500 Work Station; DW Scientific) for 72 h.

The cultivated colonies underwent routine identification for genus or species using Gram staining and API tests (bioMerieux). To identify *Lactobacillus* in the samples, the bacterial colonies were isolated from solid MRS agar and Schaedler agar. The single colonies of *Lactobacillus* morphology were isolated and were subsequently classified using phenotypic methods, i.e. Gram staining and API 50CHL (bioMerieux). The initial collection of 235 *Lactobacillus* isolates, which were obtained, was preliminarily classified at the species level. Afterwards, the presence of selected species, i.e. *Lactobacillus fermentum, L. gasseri* and *L. plantarum*, within the collection of isolates was conclusively confirmed with the use of the PCR method.

DNA isolation

The bacterial DNA was isolated with the application of a specialized kit for DNA extraction (Genomic Mini, DNA Gdansk). The isolation was carried out in accordance with the manufacturer's report.

PCR species confirmation

DNA amplification was conducted on the basis of the isolated DNA in order to determine the species affiliation of the tested bacterial isolates (Table 1) (Walter et al. 2000).

Reference strains and *Lactobacillus* isolates

The reference strains of the representatives of various bacterial species, which can appear in the genital tract, were used to conduct the specificity test of the FISH probes (Table 2). The reference strains belonging to the tested *Lactobacillus* species were used to verify whether the RAPD and PFGE methods allow differentiation among strains within each of the three examined bacterial species.

For FISH, RAPD and PFGE, 60 isolates of *Lactobacillus*, i.e. 20 isolates of each of the three studied species: *L. fermentum*, *L. gasseri* and *L. plantarum*, were used.

Fluorescence in situ hybridization (FISH)

Standardization of the FISH method was conducted on 60 isolates of lactobacilli. DNA LbpV3 probe sequences, specific for *Lactobacillus plantarum*, have been described in the literature (Ercolini et al. 2006). Because of this, only two probes for *Lactobacillus fermentum* and *L. gasseri* were designed in this study. The probe sequences were selected in such a way as to enable hybridization at the same temperature for all three probes, which allows typing of the examined *Lactobacillus* species in a single hybridization reaction with the use of a mixture of two or three probes. Designing the sequence of DNA probes consisted in the isolation of specific multiple nucleotide sequences located within the DNA coding of the bacterial 16S rRNA. Ribosomal Database Project II was used for this purpose (Loy et al. 2008). The probe sequences were selected in such a way that their species specificity should be preserved whereas the quantitative ratio of specific nucleotides should be at the level enabling the conducting of hybridization at the same temperature for both designed probes. The initially selected nucleotide sequences were checked for the species specificity for *Lactobacillus gasseri* and *L. fermentum* in the National Center for Biotechnology Information (NCBI) database with BLASTN software (Loy et al. 2008). The determined probe sequences were sent to the Eurobiotech Company for the synthesis of oligonucleotides which were additionally marked at the 5′ end with a red fluorescent (CY3) or green (Alexa488) dye (Eurobiotech). Afterwards, the specificity of the designed probes was examined on the reference strains and the gathered collection of isolates belonging to the three examined bacterial species was checked (Table 2) with the application of the procedure by Gosiewski et al. (2005). Bacterial cultures in 5 ml liquid MRS were incubated during 24 h in the anaerobic conditions at 37°C. Then, 0.5 ml of cultures were transferred into 1 ml of saline and mixed. The samples were centrifuged for 5 min at 8,000 *g*. The pellet was carefully diluted in 20 μl of sterile distilled water from which 10 μl was transferred onto a SuperFrost®Plus (Menzel–Glaser) slide with a pipette for

Table 1 Primers and PCR products for the three studied *Lactobacillus* species

Species	Primer sequences	Primer	Target	PCR products
L. fermentum	5′ GCC GCC TAA GGT GGG ACA GAT 3′ 5′ CTG ATC GTA GAT CAG TCA AG 3′	Lfpr FermII	16S-23S spacer	200 and 400 bp
L. gasseri	5′ GAG TGC GAG AGC ACT AAA G 3′ 5′ CTA TTT CAA GTT GAG TTT CTC T 3′	Gas I Gas II	16S-23S spacer	300 and 500 bp
L. plantarum	5′ GCC GCC TAA GGT GGG ACA GAT 3′ 5′ TTA CCT AAC GGT AAA TGC GA 3′	Lfpr PlanII	16S-23S spacer	200 and 400 bp

hybridization so as to obtain an examination area approximately 10 mm in diameter. The slide was dried under laminar flow. Subsequently, it was poured with 500 μl of 4% paraformaldehyde (Sigma) solution and incubated for 20 min at 4°C. The specimen was then washed with PBS and 1–2 ml of 96% methanol (POCh) was poured over the slide. The whole specimen was incubated under the cover for 30 min at –20°C. On completion of the fixation process, methanol was washed off with warm (50°C) PBS and 20 μl of diluted lysozyme (1 mg/ml) (Sigma) was placed on the slide. It was incubated for 5 min at 37°C and washed with warm PBS. Hybridization was performed with the use of the designed probes directed at the conservative fragment of 16S rRNA typical for *Lactobacillus fermentum, L. gasseri* and *L. plantarum*. To perform hybridization, 5 μl of the examined probe solution (50 ng/μl) was mixed with 45 μl of hybridization buffer (20 mM Tris HCl; 0.9 M NaCl; 0.1% SDS; pH =7.2), and heated to the temperature of approximately 50°C. The prepared hybridization solution (50 μl) was transferred onto the slide using an automatic pipette, and the whole preparation was placed in a moist chamber covered with an aluminium foil at the given temperature for 2 h. Hybridization was conducted at temperatures ranging from 46 to 52°C in order to select the optimal one for all the three examined probes. Consequently, the specimen was washed with warm washing buffer (of the same composition as the hybridization buffer except for 0.1% SDS). The specimen was stained with DAPI at the concentration of 0.5 μl/ml (Sigma) for 5 min as well as carefully washed with sterile distilled water and dried in the dark. The slide was analyzed with the use of the Olympus BX51 fluorescence microscope and AnalySYS (Soft Imaging) software.

Random amplification of polymorphic DNA (RAPD)

Standardization of the RAPD method was performed on 60 bacterial isolates belonging to the above-mentioned three species, i.e. *Lactobacillus fermentum, L. gasseri* and *L. plantarum*, and confirmed with the PCR method. The test was performed with the application of Ready–To–Go RAPD

Table 2 Bacterial strains used in the specificity testing of the designed FISH probes

Strains	Tested probes		
	LFERM	LGASS	LbpV3
Lactobacillus fermentum DSM 20052	+	−	−
L. fermentum isolates (no. 20)	+	−	−
Lactobacillus gasseri DSM 20243	−	+	−
L. gasseri isolates (no. 20)	−	+	−
Lactobacillus plantarum ATCC 14431	−	−	+
L. plantarum NCIMB 8825	−	−	+
L. plantarum NCIMB 1406	−	−	+
L. plantarum isolates (no. 20)	−	−	+
Lactobacillus johnsonii ATCC 33200	−	−	−
Lactobacillus delbruecki subsp. *lactis* DSM 20072	−	−	−
Lactobacillus acidophilus DSM 4356	−	−	−
Streptococcus agalactiae DSM 2134	−	−	−
Gardnerella vaginalis ATCC 14018	−	−	−
Escherichia coli ATCC 25922	−	−	−
Staphylococcus aureus ATCC 29213	−	−	−
Bifidobacterium breve DSM 20213	−	−	−

Analysis Kit (GE Healthcare). This kit contained five standard RAPD primers: primer no. 1: 5′-GGTGCGGGAA-3′; primer no. 2: 5′-GTTTCGCTCC-3′; primer no. 3: 5′-GTAGACCCGT-3′; primer no. 4: 5′-AAGAGCCCGT-3′; primer no. 5: 5′-AACGCGCAAC-3′. Standardization of the RAPD method consisted in testing five primers and selecting the most discriminative one for the strains within the species of *Lactobacillus fermentum, L. gasseri* and *L. plantarum*.

The detailed protocol for RAPD was provided by the manufacturer of the kit. After DNA amplification, high resolution electrophoresis (1.5 h; 5 V/cm) in 8% polyacrylamide gel (Sigma) was applied. Electrophoresis was carried out with the use of PROTEAN II (BioRad) in the presence of ethidium bromide of the concentration of 0.25 μl/ml (Sigma). The obtained image was processed with the application of QuantityOne (BioRad) software and GelDoc 2000 (BioRad) apparatus.

Pulsed field gel electrophoresis (PFGE)

Standardization of the method was conducted on 60 isolates belonging to the three *Lactobacillus* species. Standardization of the PFGE method consisted in selecting the appropriate restriction enzyme as well as establishing the optimal parameters of electrophoretic separation, such as duration and the electrical pulse which were the most discriminative for strains within the three species.

The examined isolates were prepared according to the Tynkkynen et al. (1999). Single bacterial colonies of *Lactobacillus* were cultured in the liquid MRS (Oxoid) with the addition of 1% glycine (Sigma) and 0.25% threonine (Sigma). Subsequently, the incubation was carried out for 24 h in anaerobic conditions at 37°C. Cultured lactobacilli were centrifuged (approximately 500 μl of culture medium) for 5 min at 8,000 *g*. The pellet was washed with 1 ml of WB1 buffer (10 mM Tris; 20 mM NaCl; 50 mM $EDTA_2Na$; pH=7.2), and afterwards diluted in 150 μl of WB1 buffer. The obtained suspension was heated at the temperature of 50°C in the thermoblock and then 150 μl of 2% LMP agarose (Clean Cut BioRad) was added. The mixture was poured into Plug Mold (BioRad) to form blocks and cooled for 10 min at 4°C to allow the agarose to solidify. The blocks were placed in sterile 2.0-ml Eppendorf tubes containing 500 μl of LB1 lysis buffer (6 mM Tris; 1 M NaCl; 100 mM $EDTA_2Na$; 1% SDS; 0.2% deoxycholate; pH=7.6) with the addition of 2.5 mg/ml of lysozyme (Sigma) and 20 U of mutanolysin (Sigma). The blocks were incubated for 16 h at 37°C. On completion of the incubation, LB1 was removed and blocks were washed with WB1 (1 ml). Afterwards, 500 μl of LB2 (100 mM $EDTA_2Na$; 1% SDS; 0.2% deoxycholate pH=8.0) was added with the addition of 1 mg/ml proteinase K (Applichem). The blocks were incubated for t 16 h at 50°C. On completion of the enzymatic digestion phase, the blocks were washed with 1 ml of WB2 (20 mM Tris; 50 mM $EDTA_2Na$; pH=8.0) for 30 min. The process of washing was repeated three times. Later, the blocks were washed twice with TE buffer (10 mM Tris HCl; 1 mM $EDTA_2Na$; pH=8.0) for 30 min. After TE buffer was removed, the blocks were transferred to new sterile Eppendorf (1.5 ml) tubes containing 500 μl of 1× concentrated buffer for the restriction enzyme selected for the specific *Lactobacillus* species. The blocks were incubated in the buffer at room temperature for 1 h. Afterwards, the buffer was drained off and again 300 μl of the same buffer was added as well as 25 U of one of the tested restriction enzymes: *Sfi*I, *Sma*I, *Not*I, *Sgs*I (Fermentas). Restriction digestion was carried out in the thermoblock for 16 h at the temperature optimal for the applied restriction enzyme (producer's recommendations). On completion of the digestion process, the blocks were placed in the wells in 1% agarose gel for PFGE (BioRad). The gel underwent the pulsed electrophoresis pursuant to the individually tested parameters for the examined *Lactobacillus* species. The electrophoresis was conducted in CHEF-DR III (BioRad) apparatus at 14°C. After the process of electrophoresis, the gel was stained in water for 20 min in the presence of ethidium bromide at the concentration of 0.25 μl/ml (Sigma). The obtained image was processed with the application of QuantityOne (BioRad) software and GelDoc 2000 (BioRad) apparatus. Comparative analysis of the obtained genetic profiles of the examined isolates was performed with the use of Molecular Analyst (Applied Maths) software.

Multilocus sequence typing (MLST)

Analysis of 60 isolates of *Lactobacillus plantarum* strains using the MLST method was carried out in accordance with the methodology developed by de las Rivas et al. (2006). DNA sequencing of six housekeeping genes (*gdh*, glutamate dehydrogenase; *gyrB*, DNA gyrase B subunit; *ddl*, D-alanine ligase; *mutS*, DNA mismatch repair protein; *purK1*, phosphocarboxylase; *pgm*, phosphoglucomutase) was done by GENOMED. Analysis of received DNA sequences was performed using computer programs ChromasPro 1.5 (Digital River). The dendrograms were prepared using Molecular Evolutionary Genetics Analysis MEGA v.3.1 (http://www.megasoftware.net).

Results

In the collection of 235 isolates belonging to *Lactobacillus* genus, their species affiliation was confirmed with the use of API 50CHL tests. Approximately 2.12 isolates representing different species were found in one patient. They belonged to the following species: *Lactobacillus gasseri, L. crispatus, L. acidophilus, L. johnsonii, L. fermentum, L. plantarum, L.*

rhamnosus, L. delbrueckii and *L. brevis*. Detailed results are presented in an earlier publication of Strus et al. (2006).

All *Lactobacillus fermentum, L. gasseri* and *L. plantarum* isolates were selected for further analysis and their taxonomic verification was performed with the application of the PCR method. For standardization of RAPD, PFGE and FISH methods, 60 isolates belonging to the three mentioned *Lactobacillus* species were chosen.

The following two probes: LFERM 5′ CGT CAA CGT ATG AAC AGT 3′, LGASS 5′ TTT TAA ACT CTA GAC ATG 3′ were designed. The specificity of the planned probes and the probe LbpV3 5′ CCG TCA ATA CCT GAA CAG 3′ (Ercolini et al. 2006) was tested on the examined reference strains representing the species appearing in the female genital tract and within the gathered collection of 60 isolates belonging to the three studied *Lactobacillus* species (Table 2). Furthermore, the probes were also tested on the mixtures consisting of two different *Lactobacillus* species to confirm their species affiliation. After the conducted hybridization, it was proved that the designed probes were species specific and the process of hybridization should be carried out at 48°C, which was optimal for the three examined probes (Fig. 1).

For each of the *Lactobacillus* species examined with the RAPD method, the most discriminative primer from the Ready-To-Go RAPD kit was selected. For *Lactobacillus fermentum* and *L. gasseri* strains, it was primer no. 5, whereas for *L. plantarum* it was primer no. 2 (Fig. 2). The choice of primers was made on the basis of the RAPD typing performed for 20 *Lactobacillus* isolates of the three examined species as well as the reference strains. It was proved that the RAPD method allowed for merely insignificant discrimination of *Lactobacillus fermentum* isolates. Furthermore, no discrimination was possible among *L. gasseri* isolates—only the reference strain DSM 20243 was different from the remaining ones (Fig. 2). In the group of *Lactobacillus plantarum* isolates, it was demonstrated that the applied RAPD primers could not discriminate the examined strains of human origin, only the reference plant strains displayed a small degree of variation of genetic markers (Fig. 2).

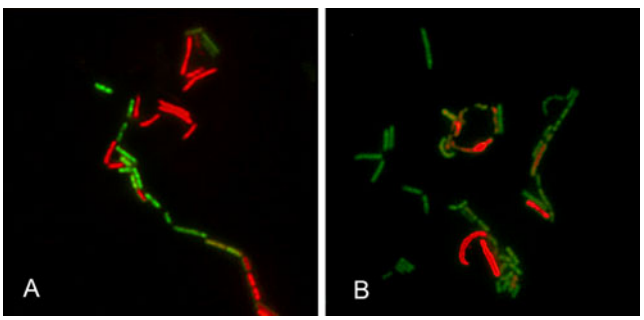

Fig. 1 Application of the FISH method; magnification ×1,000. **a** *Lactobacillus fermentum* labeled with FERM-CY3 probe (*red* fluorescence) and *L. plantarum* labeled with LabV3-Alexa488 probe (*green* fluorescence). **b** *L. gasseri* labeled with LGASS-CY3 probe (*red* fluorescence) and *L. plantarum* labeled with LabV3-Alexa488 probe (*green* fluorescence)

For the purpose of standardization and improvement of the PFGE method, for each *Lactobacillus* species, the appropriate restriction enzyme and the most optimal conditions for electrophoresis were selected (Table 3) in order to obtain readable band profiles, suitable for specifying the level of genetic relatedness and conclusive for differentiation of the examined isolates. For the species belonging to *Lactobacillus fermentum*, out of the four tested restriction enzymes (*Sfi*I, *Sma*I, *Not*I, *Sgs*I), the best-quality restriction profiles were obtained using *SgsI* digestion. For *Lactobacillus gasseri*, it was the *Sma*I restriction enzyme (Fig. 3, Table 3). For collection of isolates belonging to *Lactobacillus plantarum* species, similarly to the RAPD method, it was impossible to display genetic variation among the strains derived from the human genital tract despite testing three restriction enzymes (*Sfi*I, *Sgs*I and *Not*I) and obtaining good quality electrophoretic results (Table 3). Different genotypes were confirmed exclusively for the reference strains originating from plants. Analogous results of *Lactobacillus plantarum* strains genotyping were obtained with the use of the MLST method. The outcome of sequencing six housekeeping genes (*gdh, gyrB, ddl, mutS, purK1, pgm*) indicates 100% genetic similarity of the examined strain collection of human origin. Only the strains of plant origin are heterogeneous (Fig. 4).

Discussion

Methodology of the molecular typing of the bacterial strains and species was developed mainly in order to create more and more effective tools useful in the microbiological diagnostics of pathogens as well as in the molecular epidemiology. Currently, when the interest in probiotics is increasing, there is a need to apply the methods of molecular biology in genotyping of probiotic microorganisms. However, there exists a lack of applications of generally accepted methods, such as RAPD or PFGE, for these bacteria. Standardization of these methods for specific *Lactobacillus* species constituting the reservoir of probiotic strains will allow conducting research on colonization by the above-mentioned species. The application of classical methods of bacterial identification based on the phenotypic characteristics, such as the API 50CHL test, is quite insufficient. Boyd et al. (2005) have demonstrated in their studies that the API 50 CHL test enabled the correct species identification of a mere 4 isolates, taking into account that a total of 90 were isolated from the female genital tracts of the examined patients. The methods based on the phenotypic evaluation of bacteria

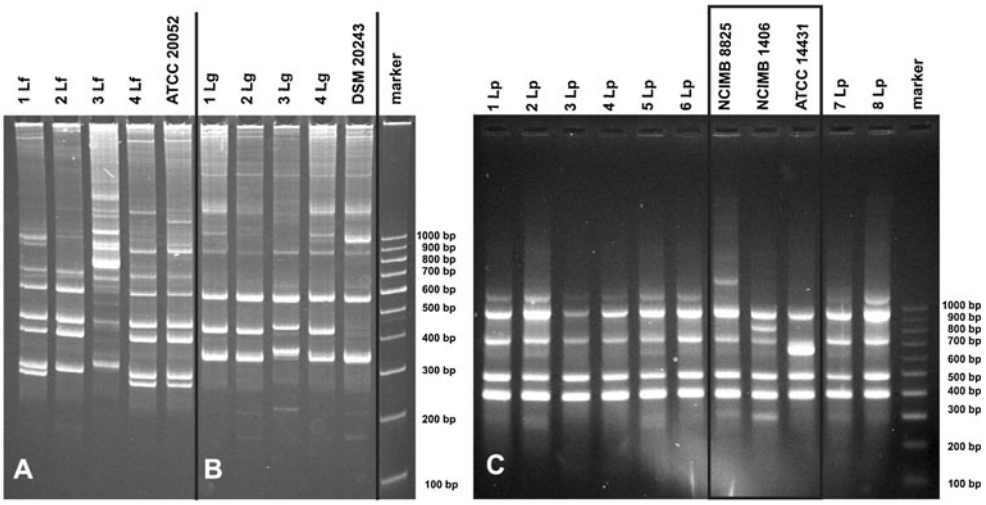

Fig. 2 Results of genotyping the bacterial isolates obtained after the reaction of RAPD with primer no. 5, for isolates of *Lactobacillus fermentum* (*Lf*) (**a**), and *L. gasseri* (*Lg*) (**b**) species, as well as with primer no. 2 for *L. plantarum* (*Lp*) (**c**) species. The identical band profiles for *L. plantarum* of human origin are visible on the gel; the reference strains of plant origin with various genetic profiles are displayed in the frame

depend on numerous external factors, such as changes of the environmental factors in the culture (Nigatu 2000; van Belkum et al. 2007). The application of methods based on the analysis of nucleic acids was necessary since this allowed the precise identification of *Lactobacillus* species supported by stable genetic markers, such as the conservative sequences of 16S rRNA (Walter et al. 2000; Dimitonova et al. 2008).

LFERM, LGASS and LPLAN probes enabled the specific detection of *Lactobacillus fermentum*, *L. gasseri* and *L. plantarum* species with the use of the FISH method on the bacterial cultures. Probe sequences were designed in such a way as to allow hybridization at the same temperature (48°C) for all three probes, which enabled the detection of the bacteria belonging to the three examined species in a single sample. The designed probes may also be used to detect the bacteria using the direct samples collected from the female genital tract. Such an application of the FISH method has been presented by Strus et al. (2009) as well as by Fredricks et al. (2005) for detection of pathogenic vaginal bacteria. Moreover, it has also been described in microbiology for identification of closely related species of pathogenic microorganisms (Burrell et al. 2004; Brzychczy-Włoch et al. 2008).

Within the specific *Lactobacillus* species, one can differentiate the strains possessing their characteristic genetic markers. This study presents an endeavor at working out the technique for genotyping the bacterial strains of *Lactobacillus fermentum, L. gasseri* and *L. plantarum* isolated from the female genital tract with the use of the RAPD and PFGE methods. The RAPD method, which was cheaper and easier to apply, did not provide repeatable results. Additionally, it demonstrated a lower degree of differentiation among the examined strains of *Lactobacillus fermentum* and *L. gasseri* than the PFGE method. For *Lactobacillus plantarum* isolates, it was impossible to demonstrate genetic differences among the strains isolated from the human genital tract. The obtained results confirm the low efficiency of the RAPD method for differentiating the strains belonging to the three examined species, particularly *Lactobacillus plantarum*. However, the *L. plantarum* strains of plant origin demonstrate effective differentiation (Elegado et al. 2004).

Table 3 PFGE standardization for the three studied *Lactobacillus* species

Species	Restriction enzymes	Optimal conditions for electrophoretic separation	Result
L. fermentum	*Sfi*I	pulses: 5.0–45 s; 5.0 V/cm; 22 h	Very small restriction fragments below 97 kb
	*Not*I	pulses: 1.0–40 s; 6.0 V/cm; 22 h	Very small restriction fragments below 97 kb
	*Sma*I	pulses: 0.1–6.0 s; 4.5 V/cm; 22 h	Very small restriction fragments below 97 kb
	*Sgs*I	pulses: 1.0–25 s; 5.5 V/cm; 24 h	Optimal bands distribution on gel
L. gasseri	*Sma*I	pulses: 3.0–13 s; 5.0 V/cm; 22 h	Optimal bands distribution on gel
L. plantarum	*Sfi*I	pulses: 1.0–20 s; 4.5 V/cm; 26 h	Optimal bands distribution on gel
	*Not*I	pulses: 1.0–15 s; 6.0 V/cm; 24 h	Small restriction fragments below 145 kb
	*Sgs*I	pulses: 1.0–25 s; 5.5 V/cm; 24 h	Optimal bands distribution on gel

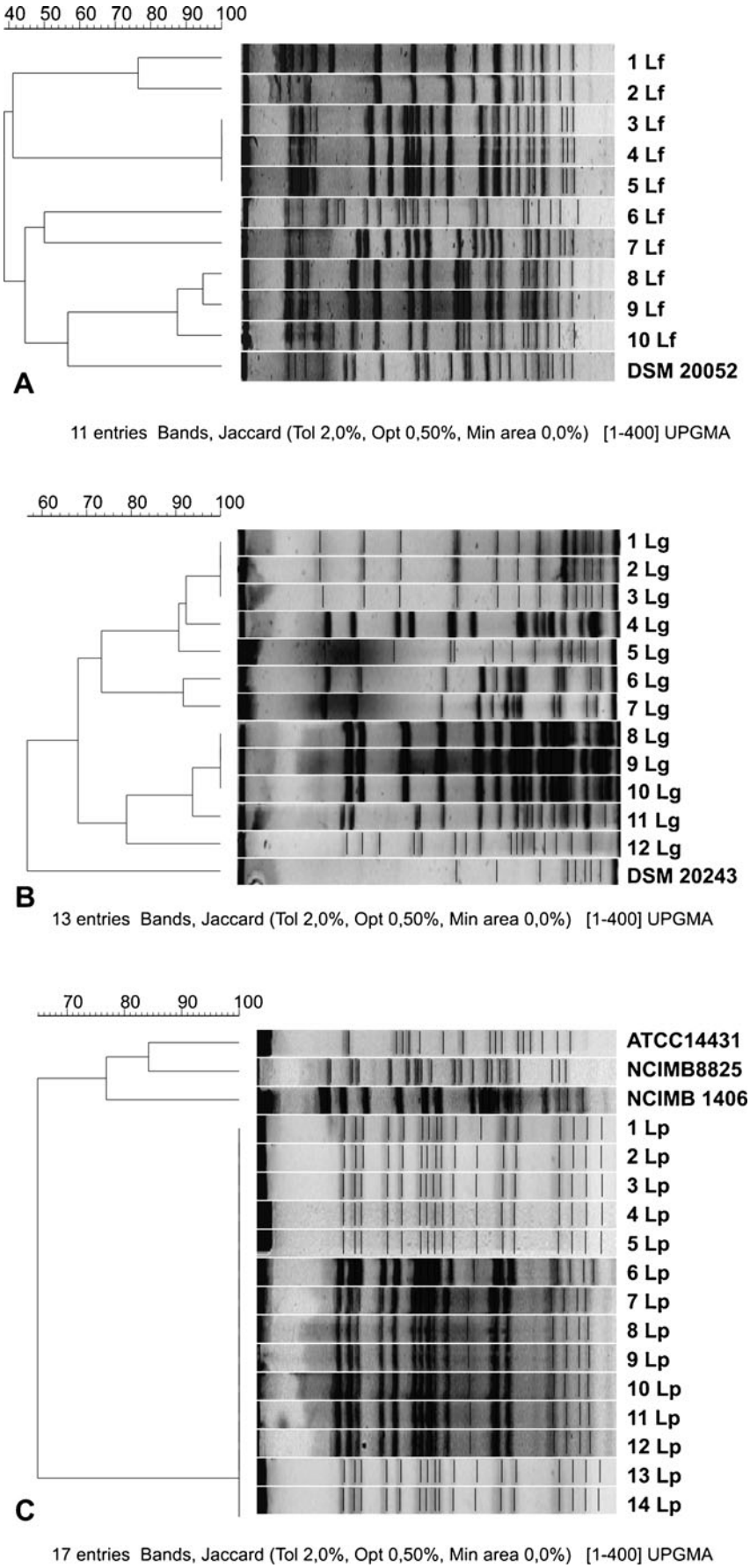

Fig. 3 Results of genotyping the bacterial isolates with the use of the optimized PFGE method. **a** *Lactobacillus fermentum* (*Lf*) (*Sgs*I restriction enzyme); **b** *L. gasseri* (*Lg*) (*Sma*I restriction enzyme) and **c** *L. plantarum* (*Lp*) (*Sgs*I |restriction enzyme)

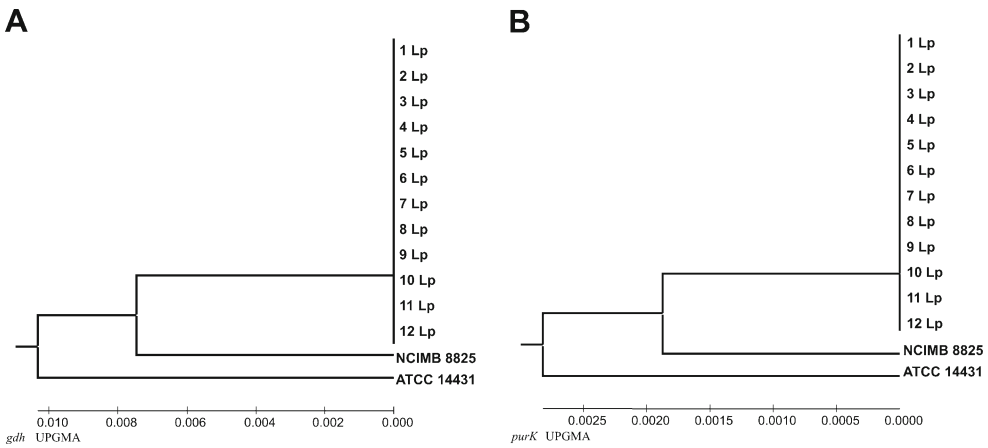

Fig. 4 Results of genotyping of the *Lactobacillus plantarum* (*Lp*) isolates obtained from the MLST method. **a** the genetic similarity within *gdh* housekeeping gene; **b** the genetic similarity within *purK* housekeeping gene

We cannot exclude that the universal primers applied, included in the Ready–To–Go RAPD Analysis Kit, did not allow the obtaining of a higher level of differentiation among the strains. On the other hand, a lower efficiency of the RAPD method as compared to the PFGE method was frequently presented by other authors for numerous bacterial genera and species (Seo et al. 2006; Stakenborg et al. 2006; Pingault et al. 2007). Contrary to the RAPD method, PFGE enabled the obtaining of repeatable results and demonstrated a higher discriminative power than RAPD, which was, as a matter of fact, confirmed during genotyping the pathogenic bacteria by other authors (Löfström et al. 2006; Ostojić 2008). The PFGE method is described as the "golden standard" in bacterial genotyping, yet its applications for probiotic bacteria are still limited. The most significant differences in developing the PFGE methodology were created by *Lactobacillus fermentum* isolates. The starting point was the study by Hautefort et al. (1999) who carried out PFGE genotyping with the use of the *Not*I enzyme. The conducted PFGE genotyping according to Hautefort's procedure revealed that the obtained band profiles consisted of too many small restriction fragments, which effectively prevented differentiation among the examined isolates due to overlapping. Subsequently, three restriction enzymes were selected and tested experimentally determining their electrophoretic parameters. For *Sma*I and *Sfi*I enzymes, similarly to *Not*I, the obtained band patterns were inadequate for analysis. Conclusively, the application of *Sgs*I enzyme and optimized separation parameters enabled obtaining good quality restriction patterns suitable for further analysis of genetic differentiation of *Lactobacillus fermentum* species.

For *Lactobacillus gasseri* isolates, the *Sma*I enzyme was selected which allowed the obtaining of a good quality restriction pattern on the gel. The selection of this restriction enzyme as optimal for *L. gasseri* isolates genotyping was confirmed by Kawase et al. 2011. To conduct the genetic differentiation among *Lactobacillus plantarum* strains with the use of the PFGE method, the procedure by Ventura et al. (2003) as well as the *Sfi*I enzyme were applied with the simultaneous modification of the voltage from 6 to 4.5 V/cm and prolonging the time of electrophoretic separation from 18 to 26 h to obtain a better quality separation on the gel. Consequently, two successive restriction enzymes were selected and tested: *Not*I and *Sgs*I establishing the optimal PFGE parameters in an experimental way. Unfortunately, despite the application of three different restriction enzymes, similarly to the analysis with the use of the RAPD method, it was impossible to provide the genetic differences among the examined isolates derived from the female genital tract, with only the reference strains of plant origin displaying genetic variability. The application of the MLST method also failed to differentiate the studied collection of *Lactobacillus plantarum* isolates of human origin; analogous to PFGE and RAPD methods, only the strains of plant origin were different in terms of genetics. Similar homogeneity of the isolates of *L. plantarum* originating from wine fermentation was presented by Spano et al. (2002) with the application of the RAPD method. Possibly, within the *Lactobacillus plantarum* species, we may deal with a very rare microbiological phenomenon of forming the genetically stable clones, what was described by Tanganurat et al. (2009) and Markiewicz et al. (2010).

This research conducted on the application of genotyping methods will enable the carrying out of studies concerning the colonization of the female genital tract by probiotic bacteria belonging to *Lactobacillus fermentum* and *L. gasseri*. What is more, the research results can be applied to directly detect these species in the vagina with the use of the FISH method. As regards the bacteria belonging to the *Lactobacillus plantarum* species, it is necessary to select a particular strain which possesses specific genetic markers enabling its differentiation from commonly occurring representatives of a genetically stable clone. It is conceivable, too, that one has to make use of other available methods of

genotyping, such as MLVA (Multiple Locus Variable number tandem repeat Analysis) or whole bacteria genome sequencing.

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