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IDENTIFICATION OF *LACTOBACILLUS* STRAINS PRESENT IN FERMENTED DAIRY PRODUCTS AND THEIR DIFFERENTIATION USING MOLECULAR METHODS

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Key words: *Lactobacillus*, identification, differentiation, PCR, ITS-PCR, PFGE

The aim of the study was to identify and differentiate five strains isolated from fermented dairy products using species-specific polymerase chain reaction (PCR) as well as PCR of internal transcribed spacer (ITS-PCR) and pulsed field gel electrophoresis (PFGE) in reference to type strains. Results of species-specific PCRs showed that three strains belonged to the species of *L. helveticus* and two strains to the species of *L. casei*. Results obtained with both IST-PCR and PFGE method showed low diversity of the isolates since only three different ITS-PCR and PFGE profiles were obtained. Moreover, differentiation conducted merely with the PFGE method allowed distinguishing the type *L. casei* DSMZ20011, *L. rhamnosus* DSMZ20021 and *L. paracasei* subsp. *paracasei* DSMZ5622 strains.

Results of this study confirmed that, although time-consuming and expensive, the PFGE method was characterised by the highest discriminatory power in strain differentiation. The ITS-PCR method even though fast, easy and relatively inexpensive, showed to be more suitable for the pre-selection of strains.

INTRODUCTION

Lactobacilli constitute an important group of non-pathogenic Gram-positive bacteria commonly found in foods like dairy products, beverages, fruits, vegetables and fermented meat as well as used in the preservation of plant-originated foods and feeds. Members of the genus *Lactobacillus* are also present in human and animal gastrointestinal (GI) and urogenital tract [Tannock 1995]. Lactobacilli, as beneficially affecting health and functions of GI tract as well as because of their technological properties, have been widely used in dairy industry and in probiotic diet supplement production. *Lactobacillus* species most frequently found in dairy products are *L. acidophilus*, *L. casei*/*L. paracasei*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis* and *L. helveticus* [Andrighetto *et al*., 1998; Couret *et al.*, 2004; Guidemonde *et al.*, 2004; Tannock *et al*., 1999].

Conventional biochemical and physiological tests have some limitations in discriminating large numbers of isolates showing similar physiological characteristics [Berthier & Ehrlich, 1999]. Therefore, many studies have focused on the application of molecular biology techniques for the rapid identification and differentiation of lactobacilli [Andrighetto *et al.*, 1998; Zhong *et al.*, 1998]. Nowadays the rRNA genes have been generally accepted as the potential tools for the identification and phylogenetic analysis of the bacteria [Song *et al.*, 2000; Ward *et al.*, 2005].

Investigations aiming to get new strains of outstanding probiotic features result in a large number of isolates. In this context, some important points remain to be considered for an objective selection of strains of interest: (i) rapid classification and identification of unknown isolates; (ii) evaluation of genetic diversity among strains and the impact of diversity on the relevant properties of microorganisms; and (iii) strain typing to assess genetic stability over time [Berthier & Ehrlich, 1999]. Moreover, identification of bacteria at a strain level is necessary in the case of applying selected strains in industry for the protection of patented strains property. In turn, strains incorrectly classified and without proper genetic discrimination make standardization of the production and the classification of products as probiotic impossible.

In the present study we applied molecular techniques in order to verify phenotypic identification and differentiate *Lactobacillus* isolates in reference to type strains. Moreover, a preliminary assessment of the usefulness of typing methods for rapid strain discrimination has been made.

MATERIALS AND METHODS

Strains and culture conditions

Type strains used in this study were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and are listed in Table 1. *Lactobacillus* strains: b9, K1, K2, 906 and 907 were isolated from fermented milk products. The isolates were deposited

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TABLE 1. Type strains used for the evaluation of specificity of Lhel-1N and Lhel-2N primers and PCR results obtained with the primers.

Strain	PCR results
L. acidophilus DSMZ20079	-
L. casei DSMZ20011	-
L. delbrueckii subsp. *bulgaricus* DSMZ20081	-
L. fermentum DSMZ20052	-
L. gasseri DSMZ20243	-
L. helveticus DSMZ20075	+
L. johnsonii DSMZ10533	-
L. paracasei subsp. *paracasei* DSMZ5622	-
L. plantarum subsp. *plantarum* DSMZ20174	-
L. reuteri DSMZ20016	-
L. rhamnosus DSMZ20021	-
L. salivarius subsp. *salicinius* DSMZ20554	-
L. salivarius subsp. *salivarius* DSMZ20555	-

in the collection of the Department of Food Microbiology (IAR&FR PAS, Olsztyn, Poland) under species names determined on the basis of morphological and phenotypic features (Table 3). Lactobacilli were cultured in MRS broth [De Man *et al.*, 1960] under semi-aerobic conditions at 37°C for 18-20 h.

Genetic identification of *Lactobacillus* isolates with PCR method

Bacterial DNA was extracted from 0.5 mL of active cultures that after washing in PBS buffer (phosphate-buffered saline, pH 7.4) and centrifuging (14,500 × *g*, 3 min) were suspended in 150 μL of a lysozyme solution (10 mg/mL (Sigma) in TE buffer (pH 8.0, 1 mmol/L Tris-Cl and 10 mmol/L EDTA)) and incubated at 37°C for 30 min. Next, 75 μL of 2xT&C solution containing proteinase K (Epicentre Technologies, USA) were added and incubated at 65°C for 20 min. Then, the samples were cooled and proteins were precipitated using 80 μL of MPC solution (Epicentre Technologies, USA). The samples were subsequently centrifuged (4°C, 10 min, 20000 × *g*) and the supernatant was transferred into a clean 1.5 mL Eppendorf tube containing 600 μL of isopropanol. Precipitated DNA was centrifuged (4°C, 10 min, 20,000 ×*g*), washed twice with 300 μL of 75% ethanol and after drying resuspended in 50 μL of TE buffer. The DNA solution was store at -20°C until further studies.

Identification to the genus and to the species was carried out by PCR with primers showed in Table 2. The primers Lhel-1N/Lhel-2N were designed on the basis of the 16S rRNA gene partial sequence deposited in GenBank (Acc. No. AF213704) and using Primer3 software [http://frodo.wi.mit.edu/]. Amplifications were carried out in Mastercycler Gradient (Eppendorf, Germany) in a total volume of 15 μL, containing: 1.5 μL of 10x PCR buffer, 250 μmol/L of each deoxynucleoside triphosphate, 0.25 μmol/L of each primer, 5 mmol/L of $MgCl_2$, 0.4 U of *Taq* polymerase (Fermentas, Lithuania) and 0.5 μL of the template DNA. Temperature profiles of the reactions were as follows: 1 cycle of 94°C for 4 min, 30 cycles of 94°C for 15 s, 58°-66°C (see Table 2) for 15 s, 72°C for 15 s; and 1 cycle of 72°C for 4 min. Amplicons were separated in 1.5% (wt/v) agarose and stained with ethidium bromide (0.5 μg/mL) in 0.5x TAE buffer, at 100 V for *ca.* 40 min.

Analysis of ITS-PCR

The analysis of internal transcribed spacer (ITS) rRNA region was conducted according to Jacobsen *et al.* [1999]. Amplifications were performed with 23S-32 F and 16S-1500 R primers under the following conditions: 3 μL of 10x PCR buffer, 250 μmol/L of each deoxynucleoside triphosphate, 0.25 μmol/L of each primer, 2 mmol/L of $MgCl_2$, 0.6 U of *Taq* polymerase (Fermentas, Lithuania) and 0.5 μL of the template DNA. The following temperature program was applied: 1 cycle of 94°C for 4 min, 10 cycles of 94°C for 15 s, 48°C for 15 s, 72°C for 15 s; 20 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 15 s, and 1 cycle of 72°C for 4 min. ITS-PCR fragments were separated in 2.5% (w/v) agarose in 0.5x TAE buffer, at 100 V for 2 h.

TABLE 2. Primers applied for identification at the genus and species level.

Genus/species	Primer name	Sequence 5'→3'	Annealing temp. (°C)	Reference
Lactobacillus	Lac1	agcagtagggaatcttcca	58	Walter *et al*. [2001]
	Lac2	attycaccgctacacatg		
L. acidophilus	Aci I	tctaaggaagcgaaggat	62	Tilsala-Timisjärvi & Alatossava [1997]
	Aci II	ctcttctcggtcgctcta		
L. casei- group	Lcas-1N	gcccttaagtgggggataac	64	Markiewicz & Biedrzycka [2005]
	Lcas-2N	tagagtttgggccgtgtctc		
L. delbrueckii ssp. *bulgaricus/lactis*	LLB1	aagtctgtcctctggctgg	61	Torriani *et al.* [1999]
	LB1	aaaaatgaagttgtttaaagtaggta		
L. helveticus	Lhel-1N	gcagcagaaccagcagattt	66	this study
	Lhel-2N	gcatcattgccttggtaagc		
L. johnsonii	16S II	actaccagggtatctaatcc	58	Walter *et al.* [2000]
	Joh16S I	gagcttgcctagatgatttta		
L. rhamnosus	Pr I	cagactgaaagtctgacgg	60	Walter *et al.* [2000]
	Rha II	gcgatgcgaatttctattatt		

The patterns obtained were analysed with the analysis software package GelComparII version 4.6 (Applied Maths, Belgium). Similarity of band profiles was calculated using the Dice similarity coefficient. A dendrogram was deduced by using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm.

Typing of lactobacilli with PFGE method

Three hundred microliters of active overnight bacterial culture were centrifuged at 14,500 × *g*, washed twice in SE buffer (75 mmol/L NaCl, 25 mmol/L EDTA, pH 7.4) and embedded in the 2% low melting point agarose (CleanCut™ Agarose, Bio-Rad) at a ratio of 1:1 (v/v) and solidified in plug moulds (Bio-Rad). The agarose blocks were then incubated in a buffer containing: 50 mmol/L EDTA pH 8.5, 0.05% N-laurylsarcosine (Sigma), 2 mg/mL lysozyme (Sigma) and 3 U/mL mutanolysine (Sigma) at 37 °C for 16 h. Next, the plugs were incubated in a buffer containing: 10 mmol/L Tris-Cl, 50 mmol/L EDTA pH 8.5, 1% SDS (Fluka) and 2 mg/mL proteinase K (Bio-Rad) at 50°C for 18 h. In order to inactivate the enzymes used, the plugs were washed for 1 h in SE buffer, then in a water solution of 1 mmol/L PMSF (phenylmethylsulphonylfluoride, Sigma) and twice in SE buffer. Plugs were stored at 4°C until further use.

Genomic bacterial DNA was digested with the *Sma*I (5' CCC/GGG 3') restriction enzyme (Fermentas, Lithuania) overnight at 30°C in a buffer supplied by the producer. Forty units of the enzyme were used to digest DNA embedded in agarose blocks of the volume about 100 μL. Macrorestriction fragments were separated in 1% pulsed field certified agarose (Bio-Rad) in 0.5x TBE buffer using CHEF-DR III (Bio-Rad) apparatus and the following program: voltage of 5 V/cm, run time of 20 h, included angle of 120°, switch time of 1-15 s, temperature 10°C. As a molecular mass marker, 5kb Ladder or Lambda Ladder (Bio-Rad) was used. Gels were stained with ethidium bromide (5 μg/mL) for 40 min, then washed in distilled water for 1 h and photographed under UV light. PFGE patterns were analysed using TotalLab 1v1 software (Nonlinear Dynamics, UK) in order to evaluate the size of the bands with reference to molecular mass markers.

RESULTS AND DISCUSSION

Identification of the isolates

Five strains b9, K1, K2, 906 and 907 isolated from fermented milk products were classified to *Lactobacillus* genus on the basis of positive PCR results with Lac1/Lac2 primers (Table 3). Identification to the species was carried out with primer pairs described previously and with the new Lhel-1N/ Lhel-2N primer pair. The sequences of the newly designed oligonucleotides were compared to the sequences in GenBank using Blastn algorithm. The comparison revealed that they had three annealing sites in the *L. helveticus* DPC4571 complete genome sequence (Acc. No. CP000517.1) located in the 16S ribosomal RNA genes at the position between 76283 and 76502, 451006 and 451225, and 468266 and 468504 nucleotide, and can generate PCR products of 219 bp size, which is in agreement with the size of PCR product obtained (Figure 1). Nevertheless, the specificity of the primers was confirmed in PCRs with a template DNA of the type strains (Table 1).

Among the isolates tested, strains K1, K2 and b9 gave positive results in *L. helveticus*-specific PCRs, whereas 906 and 907 strains – in the *L. casei*-specific ones (Figure 2). According to the previous studies [Markiewicz & Biedrzycka, 2005], however, in the case of the positive result of Lcas-1N/2N PCR, additional *L. rhamnosus*-specific reaction is needed in order to exclude the identification to *L. rhamnosus* species. None of the isolates gave PCR product in either *L. rhamnosus*-specific amplification or with the other primer pairs applied (Table 3).

Initial phenotypic identification of *L. helveticus* strains to the species of *L. acidophilus* or *L. delbrueckii* subsp. *bulgaricus* could be false due to high similarity of traits included in that analysis. Examination of *Lactobacillus* species by traditional phenotypic tests resulted in dividing these bacteria

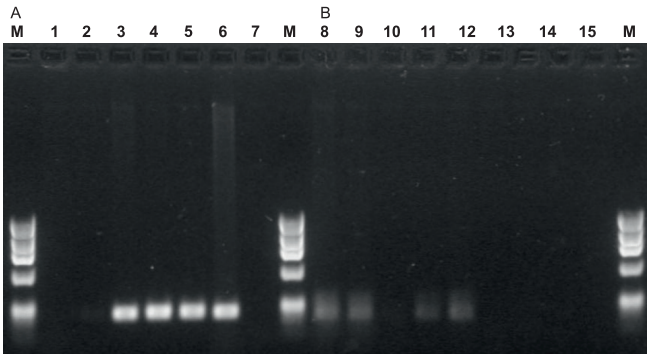

FIGURE 1. Results of species-specific PCR conducted with Lhel-1N/2N (panel A) and Lcas-1N/2N (panel B) primer pairs. Lanes 1 and 8 – strain 906, lanes 2 and 9 – strain 907, lane 3 – *L. helveticus* DSMZ20075, lanes 4 and 12 – strain K1, lanes 5 and 13 – strain K2, lanes 6 and 14 – strain b9, lane 10 – *L. casei* DSMZ20011, lane 12 – *L. paracasei* subsp. *paracasei* DSMZ5622, lanes 7 and 15 – negative controls, M – molecular weight marker ΦX174/BsuRI (Fermentas, Lithuania).

TABLE 3. Identification of isolates to the genus and species with PCR technique.

Strain	Former classification[a]	Results of PCR specific for genus and species						
		Lactobacillus	*L. acido-philus*	*L. casei*	*L. delbrueckii*	*L. helveticus*	*L. johnsonii*	*L. rhamnosus*
K1	*L. acidophilus*	+	-	-	-	+	-	-
K2	*L. acidophilus*	+	-	-	-	+	-	-
b9	*L.del. ssp. bulg.*[b]	+	-	-	-	+	-	-
906	*L. casei*	+	-	+	-	-	-	-
907	*L. casei*	+	-	+	-	-	-	-

[a] Classification based on morphological and phenotypic features; [b] *L. delbrueckii* subsp. *bulgaricus*.

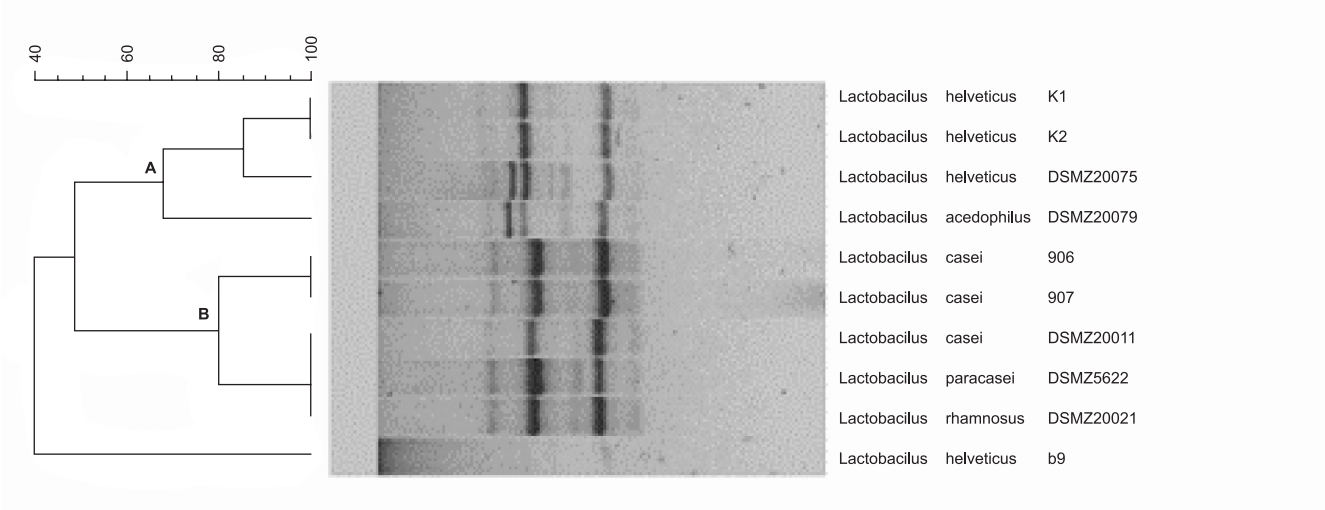

FIGURE 2. Dendrogram of ITS-PCR band profiles obtained for the isolates and the type strains. The dendrogram was constructed with the unweighted pair group algorithm with arithmetic averages (UPGMA).

into three physiological groups: (A) the obligatory homofermentative lactobacilli, (B) the facultatively heterofermentative species and (C) the obligatory heterofermentative species [Vandamme *et al.*, 1996]. The first group consisted, among others, of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. helveticus* species, whereas the second group consisted of *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *L. plantarum*. Problems with distinguishing closely related species by only phenotypic methods were reported by Klein *et al.* [1998], Chagnaud *et al.* [2001] or Ventura *et al.* [2001]. Therefore, works on developing species- and group-specific primers have been undertaken [Tilsala-Timisjärvi & Alatossava, 1997; Kullen *et al.*, 2000; Song *et al.*, 2000; Walter *et al.*, 2000; Chagnaud *et al.*, 2001; Dubernet *et al.*, 2002; Markiewicz & Biedrzycka, 2005].

Molecular typing of *Lactobacillus* strains

Type strains of the species to which the isolates were classified as well as *L. acidophilus* DSMZ20079, *L. paracasei* subsp. *paracasei* DSMZ5622 and *L. rhamnosus* DSMZ20021 as the related species were subjected for typing with both PFGE and ITS-PCR methods.

Cluster analysis of ITS-PCR patterns allowed to gather the tested strains in two clusters (Figure 2). The cluster A comprised of *L. helveticus* strains K1, K2 and DSMZ20075 as well as *L. acidophilus* DSMZ20079. The profiles of K1 and K2 strains were identical and similar to *L. helveticus* DSMZ20075 (level of similarity of 85%). The presence of *L. acidophilus* DSMZ20079 strain in this cluster confirms the close relationship between *L. acidophilus* and *L. helveticus* species, that have been described by Song *et al.* [2000] on the basis of 16S-23S intergenic spacer region sequences. The cluster B included strains belonging to *L. casei* group. Within this cluster profiles of 906 and 907 strains were identical. Moreover, the profiles of the type *L. casei* DSMZ20011, *L. paracasei* DSMZ5622 and *L. rhamnosus* DSMZ20021 strains were recognized as identical, too. This could be explained by high similarity level of 16S-23S intergenic spacer region of these species (92.3%), as reported by Song *et al.* [2000]. The only profile that did not fall into any cluster was that of the b9 strain probably due to its poor quality.

PFGE analysis was carried out with *Sma*I endonuclease as the most suitable for typing of lactobacilli [Markiewicz *et al.*, 2006]. Amongst *L. helveticus* strains, K1 and K2 were characterised by an identical macrorestriction pattern, whereas b9 and DSMZ20075 by unique ones (Figure 3). PFGE patterns obtained for both *L. casei* isolates (906 and 907) were identical while genotypes of the type *L. casei* DSMZ20011, *L. rhamnosus* DSMZ20021 and *L. paracasei* subsp. *paracasei* DSMZ5622 strains could be clearly distinguished from each other (Figure 3).

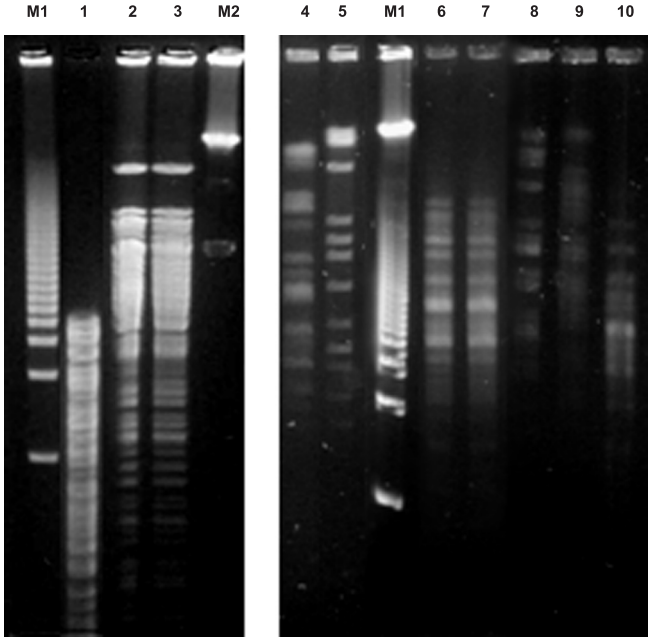

FIGURE 3. PFGE patterns obtained after digestion of genomic DNA with *Sma*I restrictase. Lanes: 1 – *L. rhamnosus* DSMZ20021, 2 – strain 906, 3 – strain 907, 4 – *L. casei* DSMZ20011, 5 – strain b9, 6 – strain K1, 7 – strain K2, 8 – *L. acidophilus* DSMZ20079, 9 – *L. paracasei* subsp. *paracasei* DSMZ5622, 10 – *L. helveticus* DSMZ 20075. M1- 5 kb Ladder (Bio-Rad), M2 – Lambda Ladder (Bio-Rad, USA).

Results obtained with the ITS-PCR and PFGE methods were convergent only partially. None of the methods could distinguish between K1 and K2 as well as between 906 and 907 strains. The discrepancies, however, appeared for the type strains belonging to the *L. casei* group which could be distinguished only with PFGE method. ITS-PCR combined with *Taq*I restriction analysis was applied by Christiansen *et al.* [2005] for identification of strains isolated from semi-hard cheeses. Also in this case no differentiation between *L. paracasei* and *L. casei* was obtained. In our opinion, application of ITS-PCR for identification seems to be pointless as the species-specific PCR is a more precise and unambiguous method while ITS-PCR provides information only on the similarity level of strains and, consequently, does not distinguish species of high level of relatedness. Moreover, results obtained in this study indicate that ITS-PCR should not be recommended for typing but rather for screening of strains in order to limit the number of isolates intended to be differentiated with the PFGE method. An advantage of the PFGE method over other the PCR-based molecular typing methods (*e.g.* RAPD – Randomly Amplified Polymorphic DNA or ARDRA – Amplified rDNA Restriction Analysis) was reported by other authors [Tynkkynen *et al.*, 1999; Simpson *et al.*, 2002; Giraffa *et al.*, 2004].

Already completed and ongoing bacterial genome sequencing projects *e.g. Lactobacillus plantarum* WCFS1 [Kleerebezem *et al*., 2003], *Lactobacillus johnsonii* NCC533 [Pridmore *et al*., 2004], *Lactobacillus acidophilus* [Altermann *et al*., 2005], *Lactobacillus salivarius* subsp. *salivarius* UCC118 [Claesson *et al*., 2006] or *L. helveticus* DPC4571 [Callanan *et al.*, 2008] make a great amount of data available for comparative analysis of sequences at levels of genus, species and intra-species. These could be of great importance for further development of DNA-based identification and differentiation methods.

CONCLUSIONS

The identification method applied in this study appeared to be fast and reliable and allowed to identify isolates tested to the species of *L. helveticus* and *L. casei*, that are commonly found in fermented dairy products. Among methods applied for differentiation of lactobacilli, the ITS-PCR method, even though fast, easy and relatively inexpensive, showed to be more suitable for the pre-selection of strains. There is still a need for a rapid method of discriminatory power comparable to those of the PFGE method.

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