Differentiation of *Lactobacillus* Species by ARDRA

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Abstract

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The *Lactobacillus* species by 16S Amplified Ribosomal DNA Restriction Analysis (16S-ARDRA) was identified. Lactobacilli are bacteria often found in foodstuffs of both animal and vegetable origins. On one hand, they play an important role in the food spoilage and, on the other hand, they are used as starter cultures in food fermentation processes. The species-specific identification by traditional biochemical methods is time consuming and not always fully effective. Therefore, more efficient techniques are searched for. We focused on rapid identification of *Lactobacillus* isolates from different habitats. Forty-nine collection strains and isolates belonging to the genus *Lactobacillus* were discriminated. ARDRA was carried out with two restriction endonucleases. For the comparison of similarity, the Jaccard coefficient and clustering by the unweighted pair group method with arithmetic averages (UPGMA) were used. The percentages of similarity between profiles varied from 22% to 100% (*Alu*I) and from 27% to 100% (*Msp*I). This method proved applicable to the differentiation of 10 species.

Keywords: restriction analysis of the 16S rDNA gene; *Alu*I; *Msp*I; *in silico* fragmentation; species identification

Several species of the genus *Lactobacillus* have been widely used as adjunct cultures for the production of various commodities, which can influence human health (Klaenhammer *et al.* 2005). They are often used for their bioprocessing role as starters in dairy products where they play an important role in human nutrition. The exogenous administration of certain strains of lactobacilli has reduced the risk of infection and eradicated bacterial vaginosis (Soledad & Covadonga 2000; Saunders *et al.* 2007). The incorporation of bacteria in commercial products such as dairy products or vaginal tablets necessitates correct identification of bacterial species and strains including their characteristics. Due to the accumulation of new scientific knowledge, it is necessary and important to identify bacteria not only at the genus level but also at the species and strain levels (Holzapfel *et al.* 2001). The accurate identification of the genus *Lactobacillus* is not an easy task as currently 154 *Lactobacillus* species are known (Kant 2011). It is possible to identify only a small part of isolates by phenotyping methods which are time consuming and of a low discriminatory level (Coeuret *et al.* 2003).

Many authors have shown that Amplified Ribosomal DNA Restriction Analysis (ARDRA) discriminates very well between the species of the genus *Lactobacillus* (Kim & Chua 2005; Sklarz *et al.* 2009; Soto *et al.* 2010). ARDRA is based on PCR amplification and digestion of PCR products using restriction enzymes. The discriminatory power of

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ARDRA depends on the correct choice of restriction endonucleases. The aim of this study was to identify the *Lactobacillus* species by ARDRA. The study was divided into two parts. The first step was to select suitable high resolution restriction endonucleases for the *Lactobacillus* species, namely for *L. brevis*, *L. fermentum*, *L. reuteri*, *L. plantarum*, *L. delbrueckii*, *L. sakei*, *L. amylovorus*, *L. helveticus*, *L. salivarius*, *L. acidophilus*, *L. crispatus*, *L. johnsonii*, *L. gasseri*, and the group of *L. casei*, *L. paracasei*, *L. rhamnosus*, and *L. zeae* on the basis of *in silico* prediction. And the second step was to apply the selected types of restriction endonucleases to forty-nine collection strains and isolates belonging to the genus *Lactobacillus* and to test their discriminatory power.

MATERIAL AND METHODS

Twenty-one collection strains (Table 1) were obtained from the Czech Collection of Microorganisms, Brno, Czech Republic (CCM) and 28 isolates were isolated from vaginal tablets, probiotic drops, and from dairy and meat products. All strains were cultured aerobically in MRS broth (Oxoid, Basingstoke, UK) at 37°C for 24 hours. A 1.5 ml aliquot of each culture was centrifuged and the sediment, after washing, was resuspended in 500 µl lysis buffer (10mM Tris-HCl, 5mM EDTA, pH 7.8) with lysozyme (3 mg/ml). After incubation at 37°C for 1 h, 12.5 µl SDS (20%) and 5 µl proteinase K (10 mg/ml) were added and the mixture was incubated at room temperature for 16 hours. DNA was isolated from crude lysates of cells by phenol extraction (Sambrock & Russell 2001) and resuspended in 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.8). The obtained DNA was quantified by UV spectrum (260 nm) and DNA integrity was verified by 0.8% agarose gel electrophoresis.

The 16S rDNA gene was amplified by PCR in a 25 µl reaction volume. Approximately 1500 bp DNA fragment was amplified using the primers 16S-F1 (Hughes *et al.* 2000) and 16S-R1530 (Coenye *et al.* 1999). Each PCR mixture contained 2.5 µl buffer complete (Top-Bio, Jesenice, Czech Republic), 0.5 µl of each primer (10 pmol/ml) (Generi Biotech, Hradec Králové, Czech Republic), 0.5 µl of each dNTP (10 mmol/ml), 0.25 µl BSA (10 mg/ml), 0.2 µl *Taq* polymerase 1.1 (1 U/µl) (both Top-Bio, Jesenice, Czech Republic), 2.0 µl DNA (50 ng/µl, and PCR water was added to make the volume 25 µl. PCRs were conducted in a Techne Touchgene Gradient Thermal Cycler (Techne, Cambridge, UK). The conditions of the PCR reactions were as follows: 95°C for 5 min, 3 cycles of 95°C for 45 s, 55°C for 2 min, 72°C for 1 min, 30 cycles of 95°C for 20 s, 55°C for 1 min, 72°C for 1 min, and the final extension step at 72°C for 7 min (Švec Pavel, unpublished protocol). The integrity and the size of the PCR products were checked on a 1.2% agarose gel (1 h/70 V) and visualised with ethidium bromide under UV light.

The amplified ribosomal DNA restriction analysis was performed using two restriction enzymes, *Alu*I and *Msp*I (New England Biolabs, Massachusetts, USA). A final volume (15 µl) of each reaction mixture was prepared and the reaction conditions were set according to the manufacturer's instructions. Complete digestion was achieved after four

Table 1. List of *Lactobacillus* strains from the Czech collection of microorganisms

Species	Strain designation
L. acidophilus	CCM 4833^T (ATCC 4356)
L. amylovorus	CCM 4380^T (ATCC 33620)
L. brevis	CCM 3805^T (ATCC 14869)
L. casei	CCM 4791
L. casei subsp. *casei*	CCM 7088^T (ATCC 393)
L. crispatus	CCM 7010^T (ATCC 33820)
L. debrueckii subsp. *delbrueckii*	CCM 7191^T (ATCC 9649)
L. debrueckii subsp. *lactis*	CCM 2772 (ATCC 7830)
L. fermentum	CCM 7192^T (ATCC 14931)
L. gasseri	CCM 7009^T (ATCC 33323)
L. helveticus	CCM 4280
L. johnsonii	CCM 2935 (ATCC 11506)
L. paracasei subsp. *paracasei*	CCM 1753^T (ATCC 25302)
L. paracasei subsp. *paracasei*	CCM 7052
L. paraplantarum	CCM 4613^T
L. plantarum	CCM 7039^T (ATCC 14917)
L. reuteri	CCM 3642
L. rhamnosus	CCM 1825^T (ATCC 7469)
L. sakei subsp. *sakei*	CCM 7203^T (ATCC 15521)
L. salivarius subsp. *salivarius*	CCM 7561^T (ATCC 11741)
L. zeae	CCM 7069^T (ATCC 15820)

CCM – Czech Collection of Microorganisms, Brno, Czech Republic; ATCC – American Type Culture Collection, Manassas, USA

Table 2. *Lactobacillus* strains with reference sequences (NC) used in *in silico* study

Species	Strain determination	NC
L. acidophilus	NCFM	006418
L. amylovorus	GRL 1112	014724
L. brevis	ATCC 367	008497
L. casei	BL 23	010999
L. casei	Zhang	014334
L. casei subsp. *casei*	CCM 7089 (ATCC 334)	008526
L. crispatus	ST 1	014016
L. delbrueckii subsp. *bulgaricus*	CCM 7190T (ATCC 11842)	008054
L. delbrueckii subsp. *bulgaricus*	NDO 2	014727
L. delbrueckii subsp. *delbrueckii*	ATCC 365	008529
L. fermentum	IFO 3956	010610
L. gasseri	CCM 7009^T (ATCC 33323)	008530
L. helveticus	DPC 4571	010080
L. johnsonii	F 19785	013504
L. johnsonii	NCC 533	005362
L. plantarum	JDM 1	012984
L. plantarum subsp. *plantarum*	ST III	14554
L. reuteri	DSM 20016	009513
L. reuteri	JCM 1112	010609
L. rhamnosus	CCM 7091 (GG; ATCC 53103)	013198
L. sakei subsp. *sakei*	23K	007576
L. salivarius	UCC 118	007929

ATCC – American Type Culture Collection, Manassas, USA; CCM – Czech Collection of Microorganisms, Brno, Czech Republic; DSM – German Collection of Microorganisms, Braunschweig, Germany; JCM – Japan Collection of Microorganisms, Saitama, Japan

hours of incubation. ARDRA fingerprints were followed using 2% agarose gel with ethidium bromide (5 h/70 V). ARDRA profiles were visualised under UV light and analysed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The similarities were calculated using the Jaccard correlation coefficient. The dendrograms were constructed by means of the unweighted pair group method using arithmetic averages (UPGMA).

The theoretical fragment sizes were obtained by restriction enzyme digestion of the 16S rDNA sequences. After the compilation of nucleotide sequences of the whole 16S rDNA gene of *Lactobacillus* strains, which have been deposited at the GeneBank (Table 2), two restriction enzymes (*Alu*I, *Msp*I) were chosen (*in silico* method). For this study, the Nebcutter tool was used (http://tools.neb.com/NEBcutter2/index.php).

RESULTS AND DISCUSSION

The genus *Lactobacillus* with 154 species (Kant 2011) is the largest of 13 genera, which rank among the lactic acid bacteria (Hugenholtz 1998). The identification of *Lactobacillus* isolates at the species level by phenotypic methods is difficult (Coeuret *et al.* 2003). Therefore, we selected a total of 22 various *Lactobacillus* strains for which complete genomic sequences have been reported.

The *in silico* prediction was made of the restriction patterns of 16S rDNA of 22 *Lactobacillus* strains (Table 2) belonging to 15 species. The parallel restriction with the enzyme *Alu*I distinguished 10 of 15 species, and that with the enzyme *Msp*I differentiated 9 of 15 species (Table 3). Very similar patterns are predicted for the species *L.casei* and *L. rhamnosus* using both *Alu*I and *Msp*I. Almost

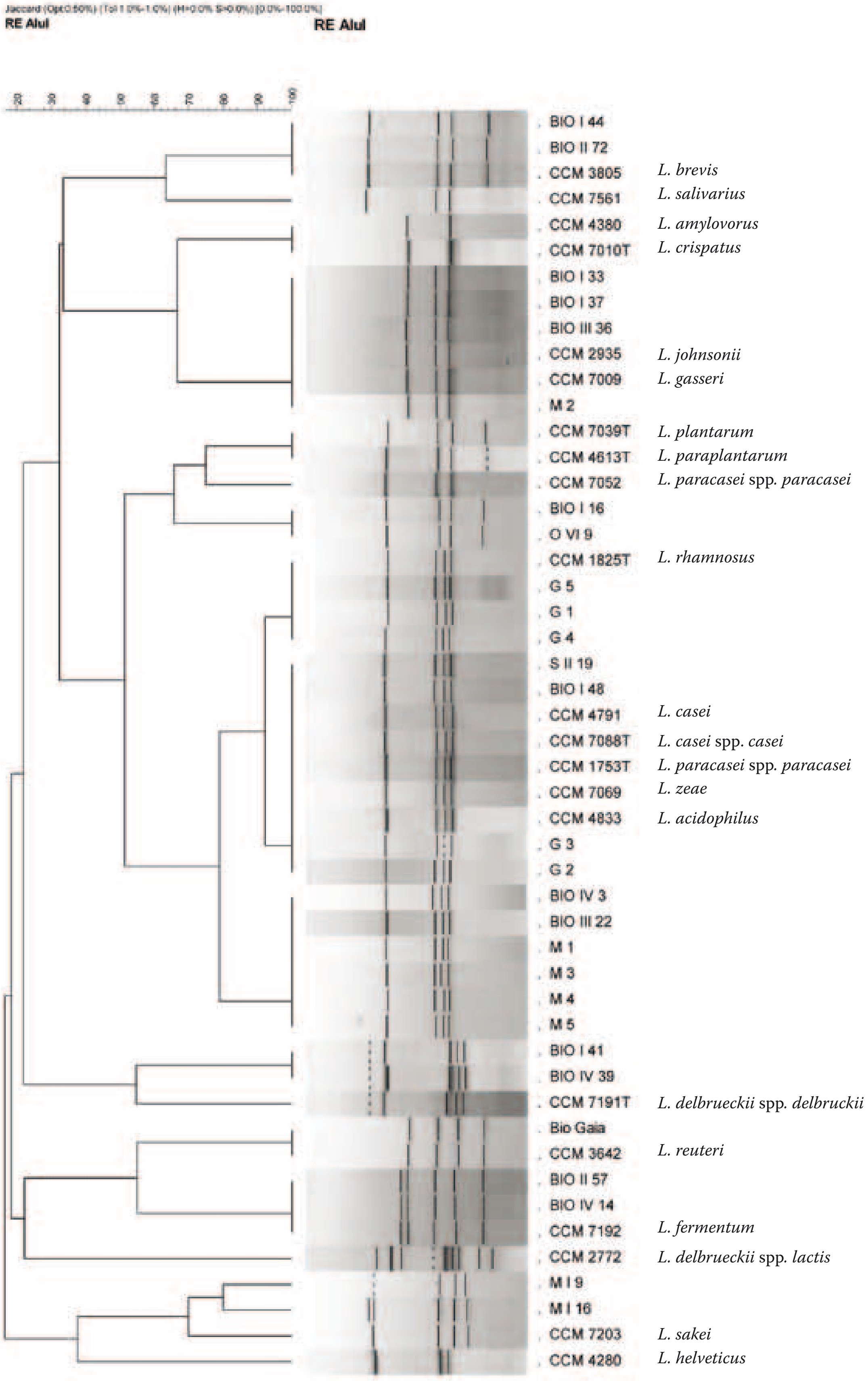

Figure 1. Dendrogram based on the UPGMA clustering analysis and the Jaccard correlation coefficient of *Alu*I ARDRA patterns

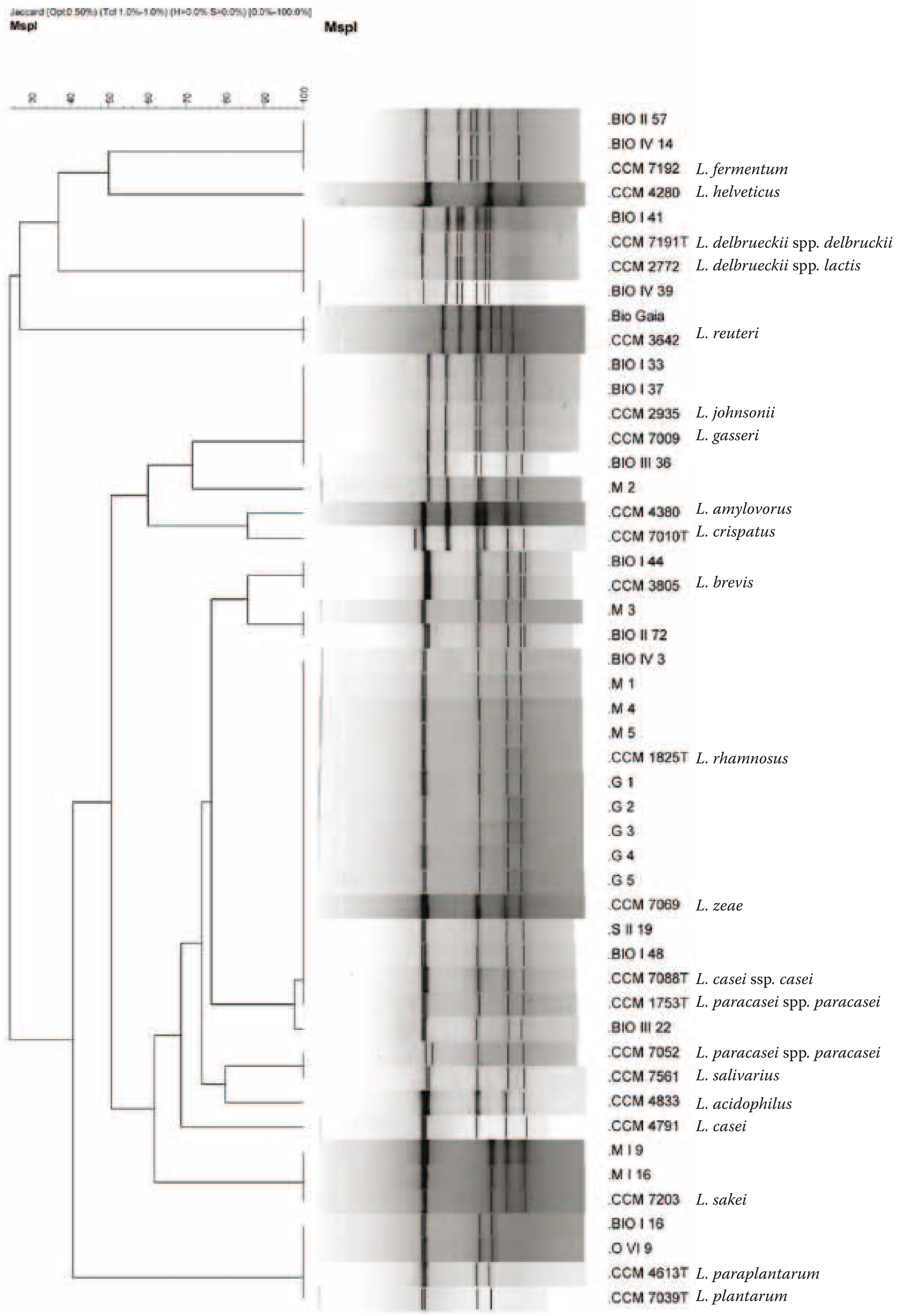

Figure 2. Dendrogram based on the UPGMA clustering analysis and the Jaccard correlation coefficient of *Msp*I ARDRA patterns

Table 3. Species differentiation by *in silico* prediction with restriction enzymes *Alu*I and *Msp*I

Species	Restriction enzyme	
	*Alu*I	*Msp*I
L. acidophilus	D	ND
L. amylovorus	ND	ND
L. brevis	D	D
L. casei	ND	ND
L. crispatus	ND	ND
L. delbrueckii	D	D
L. fermentum	D	D
L. gasseri	D	D
L. helveticus	ND	ND
L. johnsonii	D	D
L. plantarum	D	D
L. reuteri	D	D
L. rhamnosus	ND	ND
L. sakei	D	D
L. salivarius	D	D

D/ND – differentiated/not differentiated by *in silico* analysis

identical patterns are predicted for the species *L. amylovorus, L. helveticus, L. crispatus*, and *L. acidophilus* using *Alu*I and *Msp*I. The strains of the identical species had very similar but not always identical restriction profiles.

The reliability of *in silico* analysis was verified by ARDRA of 21 collection strains and 28 unclassified isolates. The amplified ribosomal DNA restriction analysis with two restriction enzymes was performed to check the theoretical prediction, the similarities of the restriction patterns were compared, and dendrograms were generated (Figures 1 and 2). ARDRA with *Alu*I divided all tested strains into 19 clusters at the similarity levels between 22% and 100%, and ARDRA with *Msp*I into 17 clusters at the similarity levels between 27% and 100%.

The restriction with *Alu*I yielded unique clusters for the species *L. brevis, L. salivarius, L. rhamnosus, L. delbrueckii, L. reuteri, L. fermentum, L. sakei, L. helveticus*, the groups *L. amylovorus* and *L. crispatus, L. johnsonii* and *L. gasseri, L. plantarum* and *L. paraplantarum,* and *L. casei, L. paracasei, L. acidophilus,* and *L. zeae*. Using *in silico* prediction, some discordant results were obtained. We detected identical restriction profiles for the groups *L. acidophilus* and *L. casei* and *L. johnsonii* and *L. gasseri.* We succeeded in demonstrating unique restriction profiles for *L. helveticus, L. casei,* and *L. rhamnosus.*

The restriction with *Msp*I resulted in unique clusters for the species *L. brevis, L. helveticus, L. amylovorus, L. crispatus, L. acidophilus, L. delbrueckii, L. reuteri, L. fermentum, L. sakei*, and the groups *L. plantarum* and *L. paraplantarum*, *L. johnsonii* and *L. gasseri*, *L. salivarius* and *L. paracasei*, and *L. casei, L. paracasei, L. zeae,* and *L. rhamnosus.*

When comparing the theoretical predictions with the experimental data, we found some discordances. We obtained unique fingerprints for the species *L. amylovorus, L. crispatus, L. helveticus, L. acidophilus*, and on the other hand, we got the same profiles for *L. johnsonii* and *L. gasseri* and *L. salivarius* and *L. paracasei.* MORELLI *et al.* (2003) successfully used a set of four restriction enzymes to distinguish *L. paracasei* strains from *L. casei* and *L. rhamnosus* strains. Our findings may be in accordance with theirs. Although *in silico* profiles of the *L. paracasei* strain are missing, the experimental results showed the unique profile of *L. paracasei* subsp. *paracasei* CCM 7052, which differentiates this strain from the *L.casei* group (Figures 1 and 2) and from other strains of *L. paracasei.*

Our data comparison is in line with the results of RODAS *et al.* (2003). Some of the differences between *in silico* and experimental results can be explained by possible punctual errors in the sequences previously reported to the GenBank database and resulting in the disappearance of the restriction sites.

The restriction enzymes *Alu*I and *Msp*I showed a high discriminatory capacity in the identification of isolates. The dendrograms constructed from the restriction digests with both *Alu*I and *Msp*I showed clustering with the same type or collection strains at a similarity level of 100% for the isolates BIO_{II}57 and BIO_{IV}14 (*L. fermentum* CCM 7192$^{\text{T}}$), BG (*L. reuteri* CCM 3642), BIO_{I}44 (*L. brevis* CCM 3805$^{\text{T}}$), BIO_{I}33, BIO_{I}37, and BIO_{III}36 (*L. johnsonii* CCM 2935 and *L. gasseri* CCM 7009$^{\text{T}}$). With the other isolates, we obtained a different discriminatory range of the similarity levels for the collection strains. These isolates were highly related (HR), related (R), or completely unrelated (UR) to the collection strains. The final identification is shown in Table 4. The 80% similarity was defined

Table 4. Identification of lactobacilli isolates by ARDRA with restriction enzymes *Alu*I and *Msp*I

Isolate	Restriction enzyme				Species identification by ARDRA
	*Alu*I		*Msp*I		
	similarity level	strain specification	similarity level	strain specification	
$\text{BIO}_{\text{II}}57$	HR	*L. fermentum* CCM 7192$^{\text{T}}$	HR	*L. fermentum* CCM 7192$^{\text{T}}$	*L. fermentum*
$\text{BIO}_{\text{IV}}14$	HR		HR		*L. fermentum*
$\text{BIO}_{\text{I}}33$	HR	*L. johnsonii* CCM 2935, *L. gasseri* CCM 7009$^{\text{T}}$	HR	*L. johnsonii* CCM 2935, *L. gasseri* CCM 7009$^{\text{T}}$	*
$\text{BIO}_{\text{I}}37$	HR		HR		*
$\text{BIO}_{\text{III}}36$	HR		HR		*
$\text{BIO}_{\text{II}}72$	HR	*L. brevis* CCM 3805$^{\text{T}}$	HR	*L. brevis* CCM 3805$^{\text{T}}$	*L. brevis*
$\text{BIO}_{\text{I}}44$	HR		HR		*L. brevis*
$\text{BIO}_{\text{I}}41$	R	*L. delbrueckii* subsp. *delbrueckii* CCM 7191$^{\text{T}}$	HR	*L. delbrueckii* subsp. *delbrueckii* CCM 7191$^{\text{T}}$, *L. delbrueckii* subsp. *lactis* CCM 2772	*L. delbrueckii*
$\text{BIO}_{\text{IV}}39$	R		HR		*L. delbrueckii*
$\text{BIO}_{\text{I}}16$	R	*L. plantarum* CCM 7039$^{\text{T}}$, *L. paraplantarum* CCM 4613$^{\text{T}}$	HR	*L. plantarum CCM* 7039$^{\text{T}}$, *L. paraplantarum* CCM 4613$^{\text{T}}$	**
$\text{O}_{\text{VI}}9$	R		HR		**
$\text{BIO}_{\text{III}}22$	R	*L. rhamnosus* CCM 1825$^{\text{T}}$, *L. casei* CCM 4791, *L. acidophilus* CCM 4833$^{\text{T}}$, *L. casei* group	HR	*L. casei* group	***
$\text{BIO}_{\text{IV}}3$	R		HR		***
$\text{BIO}_{\text{I}}48$	HR	*L. casei* CCM 4791, *L. acidophilus* CCM 4833$^{\text{T}}$, *L. casei* group	HR		****
$\text{S}_{\text{II}}19$	HR		HR		****
$\text{M}_{\text{I}}16$	R	*L. sakei* subsp. *sakei* CCM 7203$^{\text{T}}$	HR	*L. sakei* subsp. *sakei* CCM 7203$^{\text{T}}$	*L. sakei*
$\text{M}_{\text{I}}9$	R		HR		*L. sakei*
BG	HR	*L. reuteri* CCM 3642	HR	*L. reuteri* CCM 3642	*L. reuteri*
M1	R	*L. rhamnosus* CCM 1825$^{\text{T}}$, *L. casei* CCM 4791, *L. acidophilus* CCM 4833$^{\text{T}}$, *L. casei* group	HR	*L. casei* group	***
M2	HR	*L. johnsonii* CCM 2935, *L. gasseri* CCM 7009$^{\text{T}}$	R	*L. johnsonii* CCM 2935, *L. gasseri* CCM 7009$^{\text{T}}$	*
M3	R	*L. rhamnosus* CCM 1825$^{\text{T}}$, *L. casei* CCM 4791, *L. acidophilus* CCM 4833$^{\text{T}}$, *L. casei* group	HR	*L. brevis* CCM 3805$^{\text{T}}$	***
M4	R		HR	*L. casei* group	***
M5	R		HR		***
G1	HR	*L. rhamnosus* CCM 1825$^{\text{T}}$	HR		*L. rhamnosus*
G2	HR	*L. casei* CCM 4791, *L. acidophilus* CCM 4833$^{\text{T}}$, *L. casei* group	HR		****
G3	HR		HR		****
G4	HR	*L. rhamnosus* CCM 1825$^{\text{T}}$	HR		*L. rhamnosus*
G5	HR		HR		*L. rhamnosus*

L. casei group – *L. casei* subsp. *casei* CCM 7088$^{\text{T}}$, *L. paracasei* subsp. *paracasei* CCM 1753$^{\text{T}}$, and *L. zeae* CCM 7069$^{\text{T}}$; HR – highly related to the collection strains; R – related

**L. johnsonii* and *L. gasseri*; ***L. plantarum* and *L. paraplantarum*; ****L. casei*, *L. paracasei*, *L. acidophilus*, *L. zeae*, and *L. rhamnosus*; *****L. casei*, *L. paracasei*, *L. acidophilus*, and *L. zeae* as the breakpoint beyond which the strains were considered as highly related, the 50% similarity was considered as the threshold of the relationship below which the strains were classified as completely unrelated within the genus *Lactobacillus*. Collins *et al.* (1991) assumed that the analysis of rRNA sequences can not discriminate between the *L. delbrueckii* subspecies. On the other hand, Giraffa *et al.* (1998) differentiated these subspecies with ARDRA with *Eco*RI even if *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *lactis* showed high DNA-DNA homology. We confirmed the results of Giraffa and discriminated the subspecies with *Alu*I into two unrelated clusters (Figure 1).

In summary, although ARDRA proved to be suitable for rapid and highly reliable identification of the species *L. fermentum, L. brevis, L. sakei,* and *L. reuteri* and is able to discriminate between the *L. delbrueckii* subspecies, it should be used with certain limitations. The unclear identification of isolates, which clustered together in both dendrograms with the collection strains of the *L. casei* group, reflects the contentious taxonomic position of *L. casei* and related species. Our findings are in line with the results reported by Rodas *et al.* (2003). Another limitation of our method is that we were not able to distinguish *L. johnsonii* from *L. gasseri* and *L. plantarum* from *L. paraplantarum.* This might be due to the same size of 16S-rDNA amplicons and high homology in the restriction sites for the restriction enzymes used. The latter is the reason why we propose to use two or more restriction enzymes at the same time to verify the discriminatory sensitivity of ARDRA for the identification of *Lactobacillus* at the species level, contrary to the recommendation of Collado and Hernandes (2007).

CONCLUSION

We established ARDRA assay for discriminating *Lactobacillus* species. Although not all isolates could be discriminated using *Alu*I and *Msp*I enzymes, we managed to unambiguously identify 12 isolates out of 28. To enhance the discriminatory power of the ARDRA method, different enzymes or their combinations can be used for the restriction step. When a wide set of collection strains is used, this method allows specific identification of isolates from different habitats.

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