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Molecular Methods Used for the Identification of Potentially Probiotic *Lactobacillus reuteri* Strains**

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Summary

Forty potentially probiotic *Lactobacillus* strains as well as reference strains of different genera were grown under standardised conditions. Cell masses were harvested and DNA was isolated. For identification, all strains were subjected to genus-specific polymerase chain reaction (PCR), and the affiliation with the genus *Lactobacillus* was confirmed for all isolates. Using two species-specific primer-pairs for *Lactobacillus reuteri*, specific amplicons were observed for eight of the forty investigated strains. For differentiation, these eight strains as well as the reference strains of the species *L. reuteri* and closely related species were subjected to randomly amplified polymorphic DNA (RAPD)-PCR using fourteen arbitrary primers. Two selected strains as well as probiotic and common reference strains were further investigated applying pulsed field gel electrophoresis (PFGE). With the latter two methods, individual profiles were found for most strains, but no difference between probiotic and common strains could be made out.

Key words: *Lactobacillus reuteri*, probiotics, identification, molecular methods, PCR, RAPD-PCR, PFGE

Introduction

Several strains of probiotic lactobacilli have been incorporated in a wide range of products for human (*1–8*) and animal (*4,9*) nutrition over the past decades. As the probiotic capacities are strain-dependent, methods for reliable identification of lactobacilli at the strain level are of great importance, especially for the quality control of approved strains – to avoid health risks and misleadings – and for the description of new strains. Nowadays, the main focus for the identification has moved from phenotypic to genotypic methods as they yield more sensitive and accurate results, as reported for lactic acid bacteria by several authors (*10,11*). These methods were to be applied to a set of *Lactobacillus* isolates to be incorporated into probiotic feeds.

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Material and Methods

Microorganisms

Thirty-nine potentially probiotic *Lactobacillus* strains were isolated from samples from the intestines and the vagina of living, healthy animals, the intestines of just slaughtered animals and the diapers of human babies. For details see Table 1. Strains isolated from silage were included in this study for identification. For reference purposes, a strain collection of *Lactobacillus* strains (both common and probiotic) and of type strains of related genera was compiled and is shown in Table 2.

Growth conditions and harvesting of cell mass

Lactobacilli and *Streptococcus thermophilus* were grown in MRS broth (Merck, Darmstadt, Germany) under anaerobic conditions at 37 °C for 24 h, and *Weissella confusa* at 30 °C. *Lactococcus lactis* subsp. *lactis* and *Pediococcus acidilactici* were grown in MRS broth under aerobic conditions at 30 °C. *Enterococcus faecium* and *Bifidobacterium animalis* were grown in brain heart infusion under anaerobic conditions at 37 °C for 24 and 48 h, respectively.

The cells were harvested by centrifugation at 9300 × *g* and 4 °C for 10 min. The pellets were washed twice with physiological saline. The cells were resuspended in

Table 1. Sources of *Lactobacillus* isolates

Source	Isolates
Mammals (alive):	
faeces of sucking pigs	L25, L86, L264, L268, L326, L457, L461, L479, L480, L522, L524, L533, L539, L550
faeces of weaning pigs	L281, L305, L404, L529
faeces of calves	L41, L47, L50, L103, L104
vaginal swabs of sows	L443, L452, L454, L455, L456, L547
faeces of human babies	L720, L722, L865
Mammals (instantly after slaughter):	
intestinal contents of sows	L627, L654, L655, L657, L662, L665, L674
Silage	R7, R8, R9, R10, R11, R12, R13, R14

Table 2. *Lactobacillus* type, and reference strains and strains of related genera

Strain reference	Genus – species – subspecies	Other name*
Lb 7	*L. amylovorus*^{T}	DSM 20531
Lb 13	*L. reuteri*^{T}	DSM 20016
Lb 21†	*L. reuteri*	Isolate (commercial dairy drink)
Lb 23	*L. plantarum*^{T}	DSM 20174
Lb 32	*L. buchneri*^{T}	DSM 20057
Lb 34	*L. brevis*^{T}	DSM 20054
Lb 87	*L. reuteri*	Isolate (commercial probiotic feed)
Lb 89	*L. reuteri*	Isolate (commercial probiotic feed)
Lb 90	*L. kefiri*^{T}	DSM 20587
Lb 95	*L. fermentum*^{T}	DSM 20052
Lb 145	*L. reuteri*	DSM 20015
Lb 162	*L. reuteri*	LMG 13046
Lb 164	*L. reuteri*	LMG 18238
Lb 168	*L. reuteri*	DSM 12246
R 21†	*L. reuteri*	Isolate (commercial dairy drink)
Lc	*Lactococcus lactis* subsp. *cremoris*^{T}	DSM 20069
En	*Enterococcus faecium*^{T}	DSM 20477
Sc	*Streptococcus thermophilus*^{T}	DSM 20617
Pd	*Pediococcus acidilactici*^{T}	DSM 20284
Ws	*Weissella confusa*^{T}	DSM 20196
Bf	*Bifidobacterium animalis*^{T}	DSM 20104

*DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG – BCCM™/LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium
†Lb 21 and R21 are identical strains isolated at two different occasions
^{T}Type strain of a species

EDTA solution (50 mM, pH=8.0) and portioned into tubes. The tubes were centrifuged, the supernatants were discarded, and the cell masses were stored in the freezer at –20 °C until further use.

Isolation of DNA

DNA was isolated from the bacterial cells according to the standard procedure published by Ausubel *et al.* (*12*).

Genus- and species-specific PCR

PCR primers applied are given in Table 3 and were purchased at MWG Biotech, Ebersberg, Germany. The reaction mixtures (25 μL) contained 10 pmol of each primer, 0.2 mM of each dNTP, 1 × PCR buffer (10 mM Tris--HCl, pH=8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100), 0.5 μL of DNA solution and 0.5 U of DyNAzyme Polymerase (Finnzymes Oy, Espoo, Finland). The mixtures were overlaid with mineral oil. Amplification was carried out in a TRIO-Thermoblock TB-1 thermal cycler (Biometra, Hamburg, Germany) as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing for 1 min, and extension at 72 °C for 1 min, and a final 8--minute extension step at 72 °C. The following annealing temperatures were chosen: Lb1/Lb2 54 °C, LbLMA1-rev/R16-1 56 °C, Lreu-1/Lreu-4 60 °C and Lfpr/Reu 55 °C. The PCR products were stored at 4 °C. Aliquots of the PCR products were separated by horizontal 2 % agarose gel electrophoresis in TBE buffer pH=8.0 (44.5 mM Tris-borate, 1 mM EDTA). A 100-bp DNA ladder was used as a reference. Gels were stained with ethidium bromide (1 μg/mL) and visualised under UV light.

Table 3. Primers applied for genus- and species-specific PCR

Name	Sequence 5′-3′	Reference
genus-specific PCR		
Lb1	AGA GTT TGA TCA TGG CTC AG	*13*
Lb2	CGG TAT TAG CAT CTG TTT CC	
LbLMA1-rev	CTC AAA ACT AAA CAA AGT TTC	*14*
R16-1	CTT GTA CAC ACC GCC CGT CA	
species-specific PCR		
Lreu-1	CAG ACA ATC TTT GAT TGT TTA G	*15*
Lreu-4	GCT TGT TGG TTT GGG CTC TTC	
Lfpr	GCC GCC TAA GGT GGG ACA GAT	*16*
Reu	AAC ACT CAA GGA TTG TCT GA	

RAPD-PCR

The primers 1 to 14 as described in Table 4 were applied. The reaction mixtures (25 μL) contained 25 pmol of primer, 0.2 mM of each dNTP, 1 × PCR buffer (10 mM Tris-HCl, pH=8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100), 0.5 μL of DNA solution and 1 U of DyNAzyme Polymerase (Finnzymes Oy). The mixtures were overlaid with mineral oil. The amplification was carried out in the same cycler as described above, as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles consisting of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 minute, and extension at 72 °C for 2 min, and a final 8-minute extension step at 72 °C. The PCR products were stored at 4 °C. Aliquots of the PCR products were separated by horizontal electrophoresis using CleanGels (Amersham Pharmacia, Uppsala, Sweden). A 100-bp DNA ladder was used as a reference. The gels were silver-stained.

Table 4. Primers applied for RAPD-PCR

Name	Sequence 5′-3′	Other name	Reference
1	GGT GCG GGA A	–	*17*
2	GTT TCG CTC C	–	*17*
3	GTA GAC CCG T	–	*18*
4	AAG AGC CCG T	–	*17*
5	AAC GCG CAA C	–	*17*
6	CCC GTC AGC A	–	*17*
7	AGC AGC GTG G	–	*19*
8	ACG CGC CCT	–	*20*
9	ACG GTC TTG G	SCHAEFER	*21*
10	TGC CGA GCT G	OPA-02	*21*
11	TGG GCG TCA A	OPL-02	*22*
12	ACG CAG GCA C	OPL-05	*22*
13	GCG ATC CCC A	CRA 23	*23*
14	GGG AAC GTC T	OMP-05	*24*

PFGE

Inserts were prepared as described by Mayer *et al.* (*25*) and were stored at 4 °C. Prior to use, they were equilibrated in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and twice in NEB 3 buffer (New England BioLabs, Beverly, MA, USA). The DNAs were restricted with *Not*I in a solution of 1 % BSA in NEB 3 buffer (10 U *Not*I/μL) at 37 °C for at least 18 h. The buffer was discarded, and the inserts were kept in TE buffer at 4 °C for at least 1 h. The inserts were loaded to a 1.1 % agarose gel and were fixed with 0.8 % low-melting agarose solution. As a reference, MidRange I PFG marker (New England BioLabs) was used. The separation was carried out in a Gene Navigator System (Amersham Pharmacia) at 175 V for 24 h at 13 °C using a hexagonal electrode. As initial pulse time 5 s and as final 25 s were chosen. Gels were stained and visualised as described above.

Results and Discussion

Genus- and species-specific PCR

Higher selectivity for the genus *Lactobacillus* was observed for the primer-pair LbLMA1-rev/R16-1 than for Lb1/Lb2, as no false-positive amplicons were found with the former primer-pair for strains of related genera (see Fig. 1). Polymorphisms of the PCR-products, which were of indicative value concerning the species affiliation of the strains, were observed in accordance with literature (*14,26*). Using the primer-pair LbLMA1-rev/R16-1, all forty isolates were assigned to the genus *Lactobacillus*

(data not shown), thus confirming the results of prior studies with these strains.

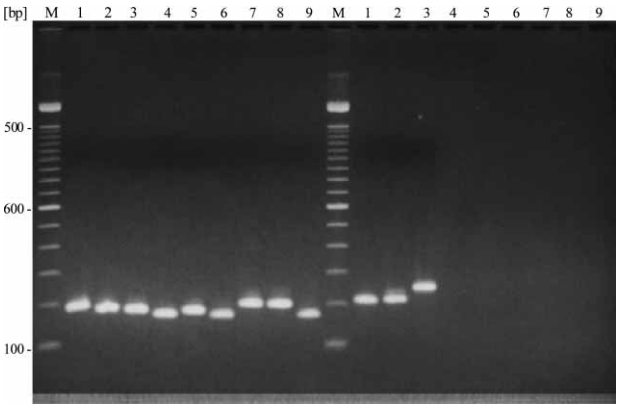

Fig. 1. Patterns of strains of the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Weissella* and *Bifidobacterium* with the primer-pairs Lb1/Lb2 (left) and LbLMA1-rev/R16-1 (right) separated in a 2 % agarose gel and visualised by ethidium bromide staining. M: 100-bp DNA ladder; 1: Lb 13; 2: Lb 23; 3: Lb 7; 4: Lc; 5: En; 6: Sc; 7: Pd; 8: Ws; 9: Bf (for details see Table 2)

Identical results were obtained for the two species-specific primer-pairs (see Figs. 2 and 3), supporting the correctness of the method. For strains of the related species *L. buchneri*, *L. brevis*, *L. kefiri* and *L. fermentum* neither of the primer-pairs gave amplicons, while all the positive controls (lanes 48 to 50) did. Eight of the forty isolates, namely L41, L103, L104, L281, L305, L457, L461 and L722, were thus identified as *L. reuteri*. These strains gave no amplicons when checking for other *Lactobacillus* species by species-specific PCR (data not shown). As it is also evident from Table 1, four strains were isolated from pigs, three from calves and one from a human baby. The results of this study are biased and neither qualitatively nor quantitatively representative for the samples drawn, as the investigated strains were pre-selected from a large number of isolates on account of other criteria. Nevertheless, the results gained are in accordance with literature (*27*,*28*).

RAPD-PCR

This method is fast and easy to perform, but not all strains could be differentiated at once. Selected results of two arbitrary primers are shown in Fig. 4. With pri-

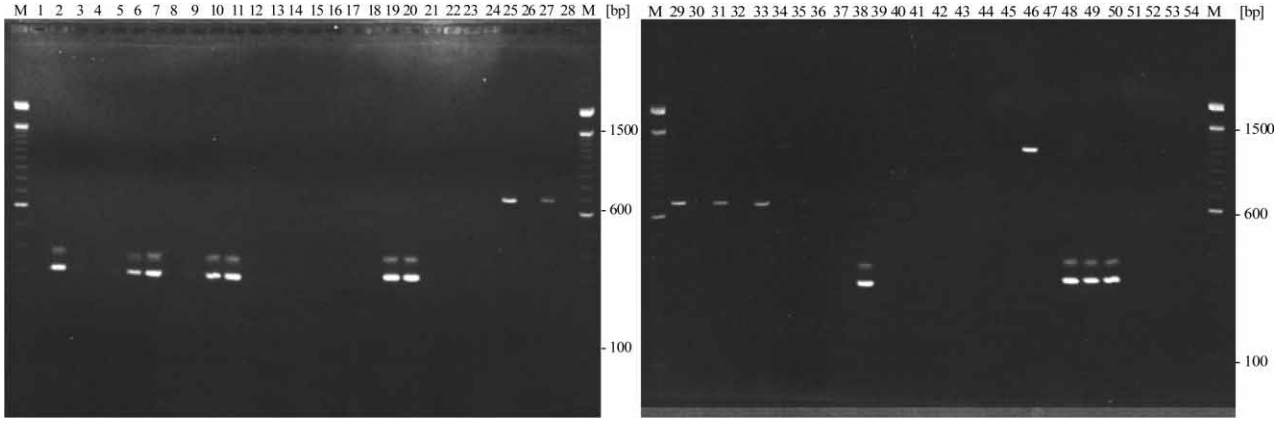

Fig. 2. Patterns of strains of the species *L. reuteri* with the primers Lreu-1/Lreu-4 separated in a 2 % agarose gel and visualised by ethidium bromide staining. M: 100-bp DNA ladder; 1: L25; 2: L41; 3: L47; 4: L50; 5: L86; 6: L103; 7: L104; 8: L264; 9: L268; 10: L281; 11: L305; 12: L326; 13: L404; 14: L443; 15: L452; 16: L454; 17: L455; 18: L456; 19: L457; 20: L461; 21: L479; 22: L480; 23: L522; 24: L524; 25: L529; 26: L533; 27: L539; 28: L547; 29: L550; 30: L627; 31: L654; 32: L655; 33: L657; 34: L662; 35: L665; 36: L674; 37: L720; 38: L722; 39: L865; 40: R7; 41: R8; 42: R9; 43: R10; 44: R11; 45: R12; 46: R13; 47: R14; 48: Lb 13; 49: Lb 21; 50: R21; 51: Lb 32; 52: Lb 34; 53: Lb 90; 54: Lb 95 (for details see Tables 1 and 2)

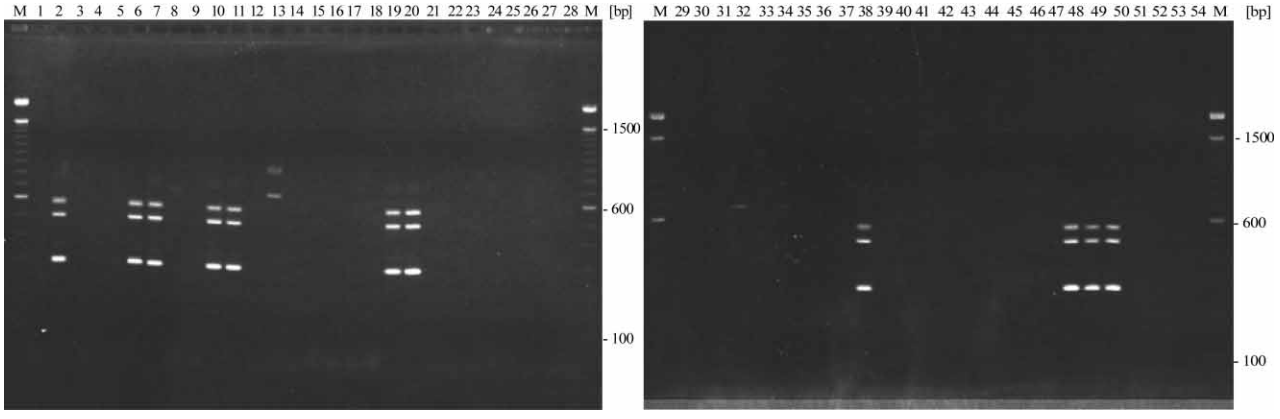

Fig. 3. Patterns of strains of the species *L. reuteri* with the primers Lfpr/Reu separated in a 2 % agarose gel and visualised by ethidium bromide staining. Samples were loaded to the gel in the same order as described in Fig. 2

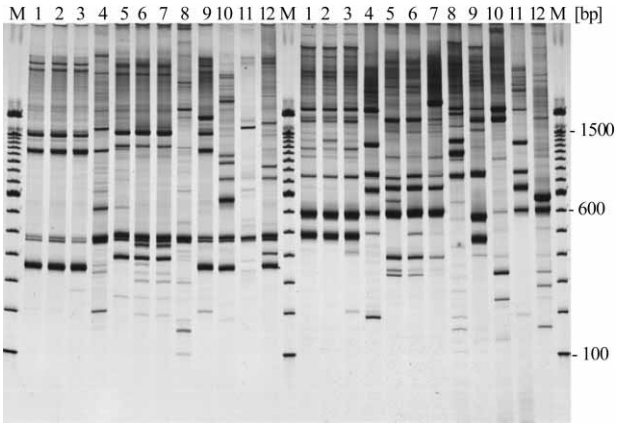

Fig. 4. RAPD-PCR patterns of strains of the species *L. reuteri* with the primers 3 (left) and 4 (right) separated in a CleanGel and visualised by silver-staining. M: 100-bp DNA ladder; 1: L41; 2: L103; 3: L104; 4: L281; 5: L305; 6: L457; 7: L461; 8: L722; 9: Lb 13; 10: R21; 11: Lb 87; 12: Lb 89 (for details see Tables 1 and 2)

mer 3, the strains isolated from calves (lanes 1, 2 and 3) showed similar patterns, as did the strains isolated from sucking pigs (lanes 6 and 7). The strains isolated from weaning pigs and from the human baby gave individual products. The profiles of the isolates were discernible and distinct from the type (lane 9) and reference strains (lanes 10 to 12). With primer 4, similar results were found for the isolates from calves, while those from sucking pigs displayed distinct patterns. Gänzle and Vogel (*29*) had described previously that not all *L. reuteri* strains from different sources could be discriminated on account of their RAPD-PCR patterns with one arbitrary primer. Thus, only with a combination of the results of these and further primers the isolates were distinguished.

PFGE

Individual profiles were found for selected *L. reuteri* strains, both isolates and common as well as probiotic reference strains (see Fig. 5), which is in accordance with literature (*30*). For the isolates Lb 21 and R21, the same strain isolated at two different occasions, identical patterns were observed. Thus, despite its complexity and costs, this method proved to be robust and most suitable for the differentiation and identification of *L. reuteri* strains. Similar results as in this study were obtained by Yeung *et al.* (*31*) for four probiotic *L. reuteri* strains digested with *Sma*I and *Not*I. While Sánchez *et al.* (*32*) attest PFGE a better discriminatory power for lactobacilli at the species level than RAPD-PCR, they achieved heightened discrimination by applying combined numerical analysis of the results from both.

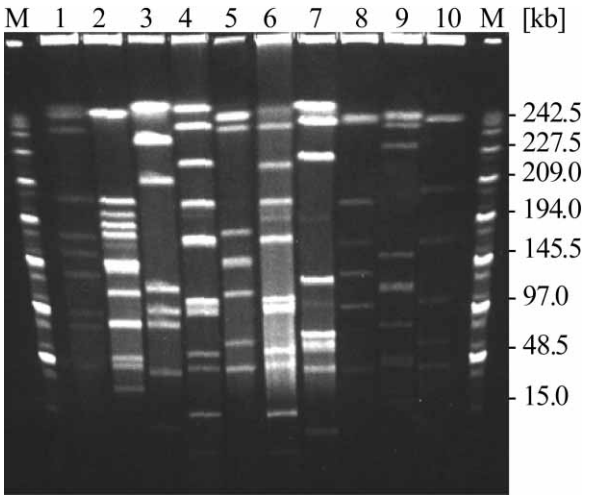

Fig. 5. PFGE patterns of *L. reuteri* strains digested with *Not*I, pulse time gradient 5–25 s. M: MidRange I PFG marker; 1: L461; 2: L722; 3: Lb 13; 4: Lb 21; 5: Lb 87; 6: R21; 7: Lb 145; 8: Lb 162; 9: Lb 164; 10: Lb 168 (for details see Tables 1 and 2)

Conclusions

Genus- and species-specific PCR proved to be powerful methods for identification of *Lactobacillus* strains from intestinal and environmental samples. PFGE posed a more reliable tool for the differentiation at the strain level than RAPD-PCR, as strain-specific patterns were observed.

Acknowledgments

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Molekularni postupci za identifikaciju probiotičkih sojeva *Lactobacillus reuteri*

Sažetak

Četrdeset potencijalnih probiotičkih sojeva roda *Lactobacillus*, uz referentne sojeve različitih rodova, uzgajano je pod standardnim uvjetima. Iz uzgojenih stanica izolirana je DNA. Radi identifikacije svi su sojevi bili podvrgnuti genski specifičnoj polimeraznoj lančanoj reakciji (PCR), a pripadnost rodu *Lactobacillus* potvrđena je u svim izolatima. Koristeći dva, za soj *L. reuteri* specifična, para »primera« u osam od četrdeset ispitanih sojeva nađeni su specifični amplikoni. S ovih osam sojeva, kao i s referentnim sojevima vrste *L. reuteri* i srodnih vrsta, proveden je postupak s nasumce amplificiranom polimorfnom DNA (RAPD)-PCR, koristeći arbitrarne »primere«. Dva odabrana soja, kao i probiotički referentni sojevi bez izrazitih svojstava ispitani su gel-elektroforezom u pulsirajućem polju (PFGE). Postupcima (RAPD)-PCR i PFGE nađene su pojedinačne značajke u većini sojeva, ali se nisu mogle utvrditi razlike između probiotičkih i običnih sojeva.