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Use of Group-Specific and RAPD-PCR Analyses for Rapid Differentiation of *Lactobacillus* Strains from Probiotic Yogurts

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Abstract. The increasing interest in probiotic lactobacilli implicates the requirement of techniques that allow a rapid and reliable identification of these organisms. In this study, group-specific PCR and RAPD-PCR analyses were used to identify strains of the *Lactobacillus casei* and *Lactobacillus acidophilus* groups most commonly used in probiotic yogurts. Group-specific PCR with primers for the *L. casei* and *L. acidophilus* groups, as well as *L. gasseri/johnsonii,* could differentiate between 20 *Lactobacillus* strains isolated from probiotic yogurts and assign these into the corresponding groups. For identification of these strains to species or strain level, RAPD profiles of the 20 *Lactobacillus* strains were compared with 11 reference strains of the *L. acidophilus* and *L. casei* group. All except one strain could be attributed unambigously to the species *L. acidophilus, L. johnsonii, L. crispatus, L. casei,* and *L. paracasei.* DNA reassociation analysis confirmed the classification resulting from the RAPD-PCR.

Strains of *L. acidophilus* and *L. casei* are increasingly being used in the manufacture of probiotic yogurts [12]. Taxonomically, those strains may not always be members of these two species, but of closely related lactobacilli grouped together in the *L. acidophilus* and *L. casei* complexes. The *L. acidophilus* group comprises six species (*L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri, L. johnsonii*) that resemble each other in their phenotypic features and which, therefore, can often not be unequivocally differentiated by physiological or biochemical properties, such as sugar fermentation profiles [6]. Similarily, the *L. casei* group includes a number of species which cannot unambigously be distinguished by phenotypic properties [6]. These difficulties in the correct identification of probiotic lactobacilli have led to the misclassification of *Lactobacillus* strains in the past. For example, two human isolates selected for probiotic products were originally identified as *L. acidophilus* by Gilliland and coworkers [3, 5]. However, DNA–DNA hybridization studies revealed that one strain was *L. crispatus* and the other *L. johnsonii* [12]. DNA reassociation studies and molecular typing methods like PFGE or 16S rDNA sequencing are laborious, time-consuming, and are nonroutine techniques for the differentiation of closely related species. RAPD-PCR, on the other hand, is a rapid fingerprinting method that has already been used by several workers for *Lactobacillus* differentiation [4, 10, 13], and thus may represent a good technique for differentiation of probiotic yogurt strains. In this study, 18 *Lactobacillus* strains isolated from 15 different probiotic yogurts and two strains from a previous investigation identified as members of the *L. acidophilus* or *L. casei* group were investigated by group-specific PCR, RAPD-PCR, and DNA reassociation analysis.

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Materials and Methods

Bacterial strains. Eighteen *Lactobacillus* strains were isolated from 15 different probiotic yogurts, using the methods described previously [12]—they are listed in Figs. 1 and 2. *L. crispatus* BFE 693 and *L. casei* BFE 728 identified during a previous study [12] and reference strains obtained from the German collection of microorganisms and cell cultures (DSMZ), were also used in this study (Figs. 1, 2).

For DNA reassociation analysis, *L. acidophilus* BFE 665 and BFE 682, *L. johnsonii* BFE 654, *L. paracasei* BFE 675 and BFE 687, and *L. rhamnosus* BFE 659 were used as reference strains. All strains were propagated in MRS broth at 37°C. All strains were also assessed for their ability to grow in MRS broth at 15°C. Stock cultures were kept in MRS broth at −20°C with 15% glycerol added.

Group-specific PCR. For group-specific PCR, specific regions of the

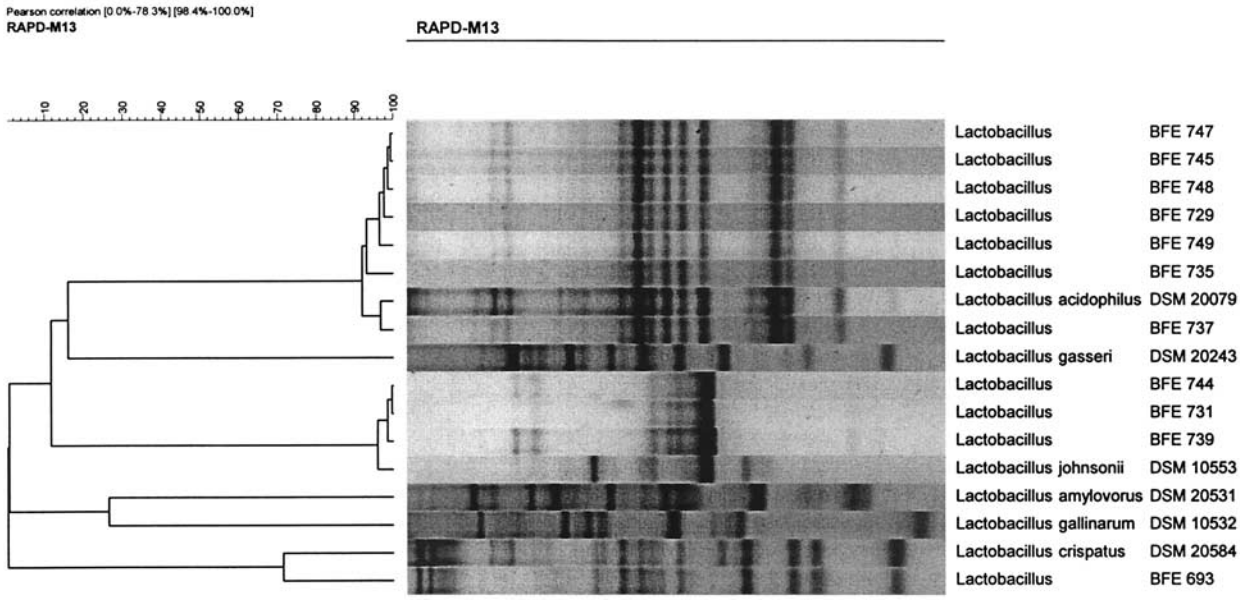

Fig. 1. Clustering of RAPD patterns generated by primer M13 with strains of the *L. acidophilus* group.

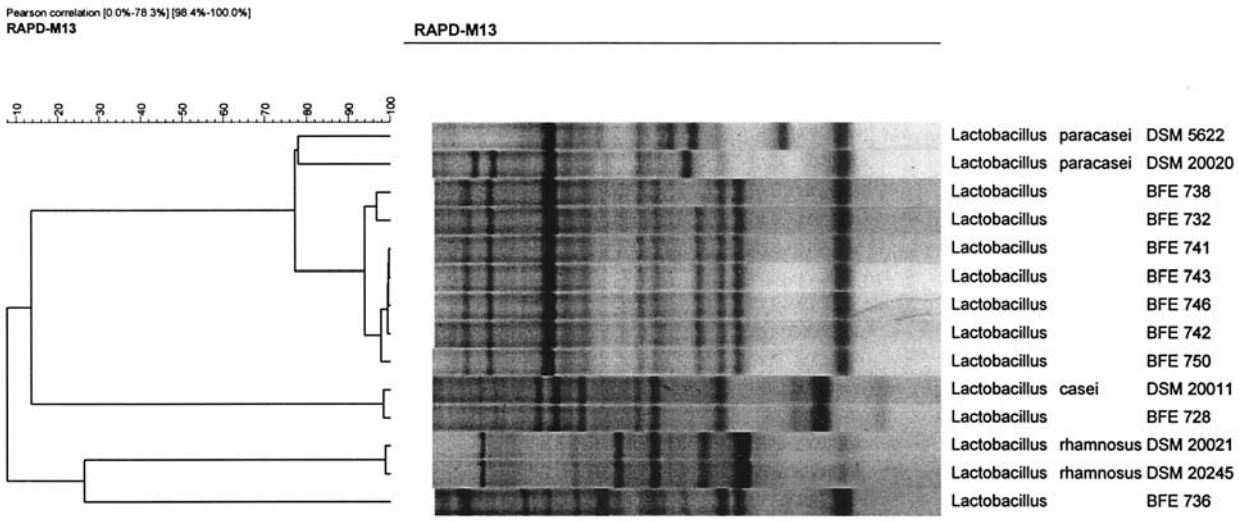

Fig. 2. Clustering of RAPD patterns generated by primer M13 with strains of the *L. casei* group.

16S rDNA gene were amplified using primers and amplification conditions as reported by Roy et al. [11]. Total genomic DNA was isolated according to the method of Pitcher et al. [9] and was used as a template. Primers Lho and LBL R1 were used to identify members of the *L. acidophilus* group (except *L. gasseri* and *L. johnsonii*), primers LCS and LBL R2 were used to identify members of the *L. casei* group, while primers Lgj and LBL R1 were used to identify *L. gasseri* and *L. johnsonii* strains [11].

RAPD-PCR. RAPD analysis was performed using primer M13 (5′-GAG GGT GGC GGT TCT-3′) [7] in a 50-μL reaction volume. The PCR reaction mixture contained 100 ng of total genomic DNA, 200 μM of dNTPs, 50 pM of primer M13, 1 U *Taq* polymerase (Amersham Pharmacia, Freiburg, Germany), and 1 × *Taq* polymerase buffer (Amersham Pharamacia) containing 3 mM $MgCl_2$. DNA was amplified in 40 cycles (initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min; annealing at 40°C for 20 s followed by ramping to 72°C at 0.6°C/s; extension at 72°C for 2 min). Amplification products were separated on a 1.5% agarose gel. Photographs of RAPD patterns were scanned and conversion, normalization, and further analysis of the scanned patterns were carried out using the Bionumerics software (Applied Maths, Kortrijk, Belgium). Similarity coefficients were calculated by using Pearson's product-moment correlation coefficient, and strains were grouped by using the unweighted pair group method with arimethic averages (UPGMA).

DNA reassociation analysis. Total genomic DNA was isolated and purified according to the guanidium thiocyanate method of Pitcher et al. [9], as described by Björkroth et al. [1]. DNA–DNA renaturation analysis was performed using a Gilford response spectrophotometer

(Ciba-Corning, Giessen, Germany). DNA homology values were calculated from renaturation rates according to standard methods [8].

Results and Discussion

Lactobacilli used for the manufacture of probiotic yogurts generally belong either to the so-called *L. acidophilus* or the *L. casei* groups. These two groups of unrelated lactobacilli can be separated by their growth behavior at 15°C, since only strains of the *L. casei* group are able to grow at this temperature. Consequently, 10 of the *Lactobacillus* isolates from yogurts (BFE 729, BFE 731, BFE 735, BFE 737, BFE 739, BFE 744, BFE 745, BFE 747, BFE 748, BFE 749) that were unable to grow at 15°C were presumptively assigned to the *L. acidophilus* group. This presumptive identification was confirmed by group-specific PCR, which showed that PCR products were obtained for strains BFE 729, BFE 735, BFE 737, BFE 745, BFE 747, BFE 748, BFE 749 using the *L. acidophilus*-group primers. Similarly, PCR products were also obtained for strains BFE 731, BFE 739, and BFE 744 using the *L. johnsonii/gasseri* primer set (results not shown). Although the use of group specific PCR could accurately designate the lactobacilli strains in this study to the *L. acidophilus* group, they could not provide an accurate identification to the species level.

RAPD-PCR analysis of these lactobacilli and the type strains of the *L. acidophilus* group resulted in a clear distinction between the six related species *L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri,* and *L. johnsonii* (Fig. 1).

This is in agreement with the results of Du Plessis and Dicks [4] and Roy et al. [10], who studied the same *Lactobacillus* species using RAPD-PCR with different primers. Seven of the 10 strains newly isolated from yogurts showed a high degree of similarity and clustered with the type strain of *L. acidophilus* at $r = 91.9\%$, while three (BFE 731, 739, and 744) grouped together into a cluster with the type strain of *L. johnsonii* at $r = 95.9\%$. These results were in good agreement to those obtained for group-specific PCR analyses. Furthermore, strains BFE 745, BFE 747, and BFE 748 showed identical fingerprint patterns ($r = 99.4\%$), indicating that the same strain may have been used in different yogurts. In fact, the yogurts from which BFE 745 and BFE 748 were isolated should contain the same strain (*L. acidophilus* LA 5), because this strain was indicated on the packaging label. *L. crispatus* BFE 693, identified by DNA-DNA hybridization during a previous study [12], clustered with the type strain of this species (Fig. 1). DNA reassociation experiments with the 10 strains confirmed the results of RAPD-PCR. All strains of the *L. acidophilus* cluster (Fig. 1) showed a high degree of DNA relatedness (DNA homology values ranging from 80 to 101%) with the reference strains *L. acidophilus* BFE 665 and 682, indicating that the isolates belong to this species. Similarily, DNA-DNA hybridizations of the strains of the *L. johnsonii* cluster (BFE 731, 739, and 744) resulted in high homology values ranging from 82 to 102% with *L. johnsonii* BFE 654, which also indicated that these isolates were strains of this species.

The remaining eight *Lactobacillus* isolates were able to grow at 15°C, indicating that the strains may belong to the *L. casei* group. This was confirmed by group-specific PCR, as PCR products were obtained for strains BFE 732, BFE 376, BFE 738, BFE 741, BFE 743, BFE 746, BFE 742, and BFE 750 using the *L. casei*-group specific primers (results not shown). RAPD-PCR with primer M13 showed that most strains of this group showed a very similar fingerprinting pattern ($r = 93.8\%$), indicating that they were probably closely related (Fig. 2). They formed a tight cluster with the two reference strains of *L. paracasei* DSM 20020 and DSM 5622 at $r = 77.2\%$. These findings were confirmed by DNA homology studies using *L. paracasei* BFE 675 and 687 as reference strains. The levels of DNA homology ranged from 87 to 114%, indicating that the isolates indeed could be considered as strains of this species. The other phylogenetically closely related species *L. rhamnosus* and *L. casei* showed RAPD patterns easily distinguishable from *L. paracasei* (Fig. 2). Similar results were obtained by other authors studying the taxonomy of the *L. casei* group using molecular fingerprinting techniques [13, 14, 15]. Strain BFE 728, which was already identified as *L. casei* during a previous study [12], generated a RAPD profile very similar to the type strain of this species. Only strain BFE 736 did not cluster with any other strain of the *L. casei* group at a high similarity level, although at least two bands that may be typical for *L. paracasei* (a strong band of approximately 450 bp and a band of approximately 1.5 kb) were present in the fingerprint pattern of this strain. DNA-DNA hybridizations with *L. paracasei* BFE 675 clarified the taxonomic position of BFE 736. A high degree of DNA relatedness (90%) clearly identified this strain as a member of *L. paracasei*. The dissimilarity of its RAPD pattern from the majority of *L. paracasei* strains may reflect the heterogeneity of this species that formerly comprised several subspecies [3]. It may also show the potential of this method to detect intraspecies diversity.

The results of this study show that group-specific PCR together with RAPD-PCR are valuable techniques for rapid differentiation of strains of the *L. acidophilus* and *L. casei* groups commonly used in probiotic dairy products.

Literature Cited

1. Björkroth KJ, Geisen R, Schillinger U, Weiss N, de Vos W, Holzapfel WH, et al. (2000) Characterization of *Leuconostoc gasicomitatum* sp. nov., associated with spoiled raw tomato-marinated broiler meat strips packaged under modified atmosphere conditions. Appl Environ Microbiol 66:3764–3772
2. Buck LM, Gilliland SE (1994) Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human intestinal origin for ability to assimilate cholesterol during growth. J Dairy Sci 77: 2925–2933
3. Collins MD, Phillips BA, Zanoni P (1989) Deoxyribonucleic acid homology studies of *Lactobacillus casei, Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans,* and *Lactobacillus rhamnosus* sp. nov., comb. nov. Int J System Bacteriol 39:105–108
4. Du Plessis EM, Dicks LMT (1995) Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii.* Curr Microbiol 31:114–118
5. Gilliland SE, Nelson CR, Maxwell C (1985) Assimilation of cholesterol by *Lactobacillus acidophilus.* Appl Environ Microbiol 49:377–381
6. Holzapfel W-H, Schillinger U, du Toit M, Dicks L (1997) Systematics of probiotic lactic acid bacteria with reference to modern phenotypic and genomic methods. Microecol Therapy 26:1–10
7. Huey B, Hall J (1989) Hypervariable DNA fingerprinting in *Escherichia coli.* Minisatellite probe from bacteriophage M13. J Bacteriol 171:2528–2532
8. Huss V, Festl H, Schleifer KH (1983) Studies on the spectrophotometric determination of the DNA hybridization from renaturation rates. System Appl Microbiol 4:184–192
9. Pitcher DG, Saunders NA, Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8:151–156
10. Roy D, Ward P, Vincent D, Mondou F (2000) Molecular identification of potentially probiotic lactobacilli. Curr Microbiol 40: 40–46
11. Roy D, Sirois S, Vincent D (2001) Molecular discrimination of lactobacilli used as starter and probiotic cultures by amplified ribosomal DNA restriction analysis. Curr Microbiol 42:282–289
12. Schillinger U (1999) Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. Int J Food Microbiol 47:79–87
13. Tynkkynen S, Satokari R, Saarela M, Mattila-Sandholm T, Saxelin M (1999) Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. Appl Environ Microbiol 65:3908–3914
14. Vasquez A, Ahrne S, Pettersson B, Molin G (2001) Temporal temperature gradient gel electrophoresis (TTGE) as a tool for identification of *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus zeae* and *Lactobacillus rhamnosus.* Lett Appl Microbiol 32:215–219
15. Ward LJH, Timmins MJ (1999) Differentiation of *Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett Appl Microbiol 29:90–92