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Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species

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Abstract

PCR amplification of repetitive bacterial DNA elements fingerprinting using the $(GTG)_5$ primer ($(GTG)_5$-PCR) was proven to be useful for differentiation of a wide range of lactobacilli (i.e. 26 different (sub)species) at the species, subspecies and potentially up to the strain level. Using this rapid and reproducible genotypic technique, new *Lactobacillus* isolates recovered from different types of fermented dry sausage could be reliable identified at the (sub)species level. In conclusion, $(GTG)_5$-PCR was found to be a promising genotypic tool for rapid and reliable speciation and typing of lactobacilli and other lactic acid bacteria important in food-fermentation industries.

Keywords: Identification; Fermented dry sausage; $(GTG)_5$-PCR; DNA extraction; *Lactobacillus*

1. Introduction

Lactic acid bacteria (LAB) are of great economical importance to the dairy industry and for the production of other fermented foods and food supplements. Next to their functional characteristics, reliable identification and high-resolution typing of LAB strains is essential in nutritional sciences and fundamental food research. Traditionally, LAB have been classified on the basis of phenotypic properties including physiological parameters and sugar-fermentation patterns [1,2]. However, as witnessed within the *Lactobacillus acidophilus* complex and the *Lactobacillus casei* complex, a correct classification and identification of LAB is difficult without the support of genotypic techniques [3–5]. The currently used methods for the study of LAB such as protein profiling [6], 16S rRNA sequencing [7], ribotyping [8], and pulsed-field gel electrophoresis (PFGE) [9] are either too laborious, are limited in their resolving power or require a species-specific methodology. Therefore, a method that is universally suitable for the LAB with a high resolving power both on the species and intraspecies level would be a highly valuable tool. In this regard, PCR-based genomic fingerprinting techniques are believed to have the most potential, and are easy to perform [10].

So far, randomly amplified polymorphic DNA (RAPD) fingerprinting is by far the most used PCR-based genomic technique for identification of LAB [11–15]. However, primers with a high discriminatory power and a broad applicability within a large group of LAB species have not been described. Moreover, because RAPD primers are not directed against a particular genetic locus, the resulting band patterns often exhibit a poor reproducibility [10,16]. Alternatively, PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the following characteristics: (i) a high discriminatory power, (ii) low cost, (iii) suitable for a high-throughput of strains, and (iv) considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria [10,17]. To our knowledge, the use of the rep-PCR fingerprinting technique on LAB has been described in three papers. De Urraza and co-workers [18] demonstrated the usefulness of the BOXA1R primer for typing thermophilic LAB associated with dairy products. Hyytiä-Trees and co-workers [19] suggest that an adequate level of discrimination among *Lactobacillus sakei* strains can be

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achieved by using a combination of rep-PCR using BOX and REP primers and RAPD. Sohier and co-workers [20] reported on the applicability of rep-PCR to differentiate the species *Lactobacillus hilgardii* and *Lactobacillus brevis*.

The aim of the current study was to assess the applicability of rep-PCR fingerprinting for the genotypic differentiation of a broad range of *Lactobacillus* species. For this purpose, a set of oligonucleotide primers targeting various repetitive DNA elements was evaluated. The method was tested on both reference strains and newly isolated lactobacilli from different types of fermented dry sausage.

2. Materials and methods

2.1. Strains and growth conditions

The taxonomical framework of reference strains consisted of facultatively heterofermentative lactobacilli (39 strains representing 20 species), obligately homo- and heterofermentative lactobacilli (15 strains representing six species), other LAB (seven strains including *Enterococcus faecalis*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Weissella viridescens*, *Weissella halotolerans*). All reference strains were obtained from the BCCM™/LMG bacteria collection (http://www.belspo.be/bccm/lmg) (Fig. 1). Two sets of *Lactobacillus* isolates, recovered from different types of fermented dry sausages, were investigated in this study. For the first set of isolates ($n = 52$), the isolation and the identification at the (sub)-species level by means of protein profiling were described in a previous paper [21]. The isolates of the second set ($n = 42$) were obtained in a similar way, but were not identified prior to this study. All LAB strains were grown overnight at 30°C on MRS agar (Difco, 0882-17-0), except for the *Lc. lactis* strains, which were grown overnight at 30°C on M17 agar (Oxoid, CM785).

2.2. Protein profiling

Both reference strains and the first set of isolates were identified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell bacterial proteins. Preparation of protein extracts, SDS–PAGE of whole-cell bacterial proteins and computer processing were done as described previously [21].

2.3. Total DNA preparation

(Personal communication of C. Jerusalem, Department of Microbiology, Chr. Hansen A/S) Total DNA was extracted from 10 ml of cultures harvested in the mid-log phase (OD_{600} of 0.5–1). Cells were collected by centrifugation ($3000 \times g$, 10 min, 4°C) and frozen for at least 1 h at −20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA) and resuspended in 300 μl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA). Seventy-five microliters of lysis buffer (TES containing 1330 U ml^{-1} mutanolysine and 40 mg ml^{-1} lysozyme) was added and the suspension incubated at 37°C for 1 h. After addition of 40 μl preheated (37°C) 20% SDS in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. One hundred microliters of TE buffer was added and the lysate was extracted with 1 volume phenol/chloroform/isoamylalcohol (49:49:1). Phases were separated by centrifugation ($18\,000 \times g$, 5 min) using Phase Lock Gel™ tubes (Eppendorf). The aqueous phase was carefully mixed with 70 μl 5 M NaCl and 1 ml isopropanol, and DNA precipitated on ice for at least 15 min. DNA was collected by centrifugation ($20\,000 \times g$, 30 min, 4°C) and the pellet washed in ice-cold 70% ethanol. DNA was dried by vacuum centrifugation and resuspended in 100 μl TE. One microliter RNase (10 mg ml^{-1}) was added and the solution was incubated at 37°C for 10 min and stored at 4°C.

2.4. rep-PCR genomic fingerprinting

The rep-PCR oligonucleotide primers evaluated in this study were REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-IIICGNCGNCATCNGGC-3′), BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′), each with its own optimal PCR program [17]. PCR amplifications were performed with a DNA thermal cycler (Perkin Elmer 9600) as described before [17], using Goldstar DNA polymerase (Eurogentec, Belgium).

The PCR products were electrophoresed in a 1.5% agarose gel (15 × 20 cm) for 16 h at a constant voltage of 2 V cm^{-1} in 1 × TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) at 4°C. The rep-PCR profiles were visualized after staining with ethidium bromide under ultraviolet light, followed by digital image capturing using a CCD camera. The resulting fingerprints were analyzed by the BioNumerics V2.0 software package (Applied Maths, Ghent, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA) dendrogram was derived from the profiles.

3. Results and discussion

3.1. Evaluation of different primer sets

For the evaluation of the rep-PCR fingerprinting technique, two single oligonucleotide primers (BOXA1R and $(GTG)_5$) and one primer pair (REP1R-I and REP2-I) were initially tested for their ability to type a subset of 30 LAB

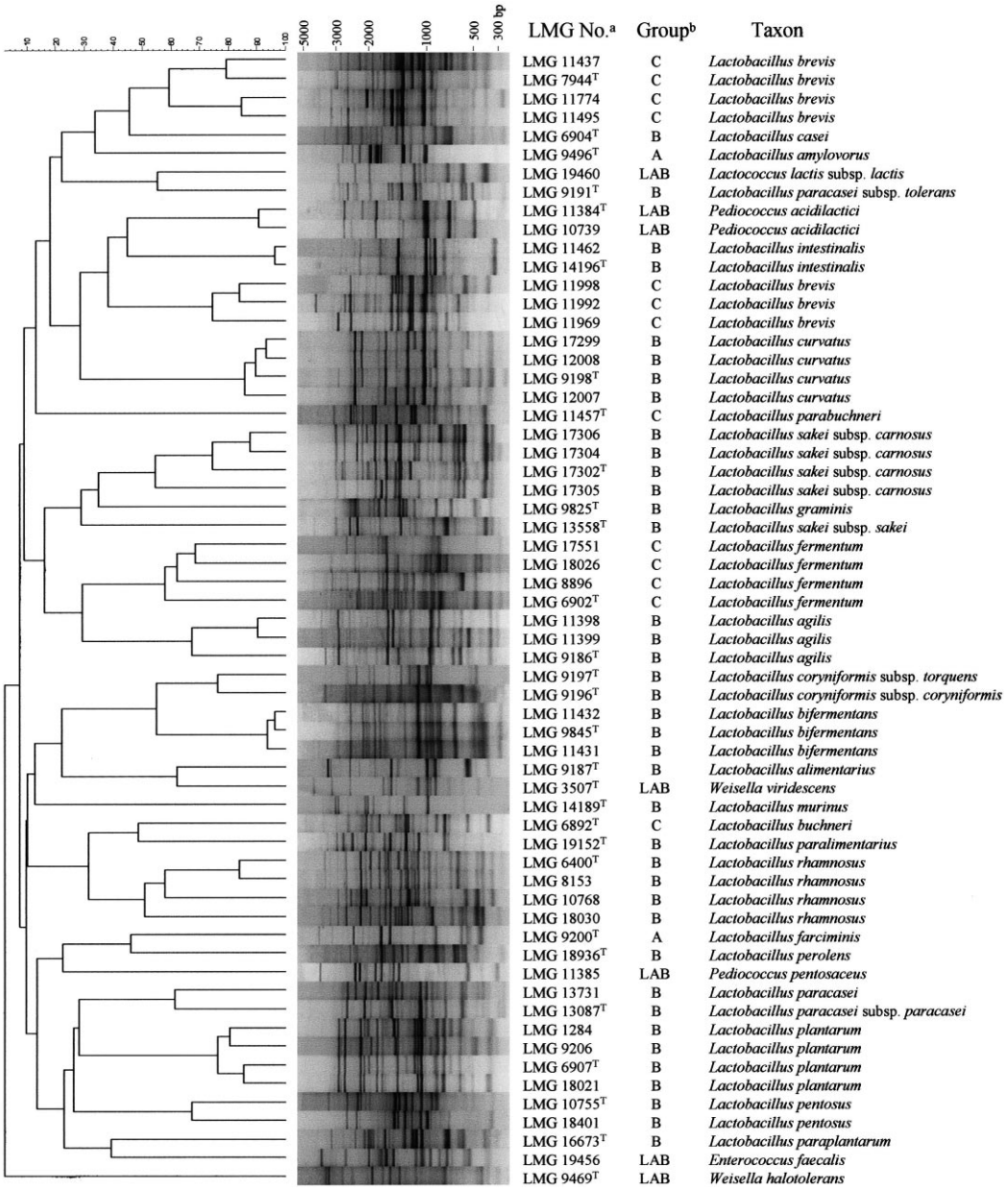

Fig. 1. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of the reference strains. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient. [a]LMG: BCCM/TM/LMG bacteria collection (Laboratory of Microbiology, Ghent University, Belgium, http://www.belspo.be/bccm); T: type strain; [b]Group A: obligately homofermentative lactobacilli, B: facultatively heterofermentative lactobacilli, and C: obligately heterofermentative lactobacilli.

isolates that had already been identified using protein profiling (results not shown). In comparison with the BOXA1R primer and the REP1R-I and REP2-I primer set, the $(GTG)_5$ primer clearly generated banding patterns with the highest complexity. The use of BOX and REP primers resulted in a banding pattern containing approximately 0–6, and 1–10 visualized PCR products respectively, while the $(GTG)_5$ primer generated fingerprints containing between 10 and 20 visualized PCR products with an average of 16.5. The size of the DNA fragments obtained after amplification using the $(GTG)_5$ primer ranged between 300 and 4000 bp. We found that the discriminatory power did not seem to be significantly enhanced when combining BOX, REP and $(GTG)_5$ banding patterns compared to the increase in amount of work. Because it was intended to optimize an easy-to-perform, rapid and reproducible method for cost-efficient speciation and typing of unknown LAB isolates, the use of the $(GTG)_5$ primer was preferred above a PCR assay combining multiple primers. To date, very few studies are available on the use of the $(GTG)_5$ primer for rep-PCR fingerprinting [17]. In this regard, Nick and co-workers [22] recently demonstrated the usefulness of rep-PCR fingerprinting with the $(GTG)_5$ primer in combination with

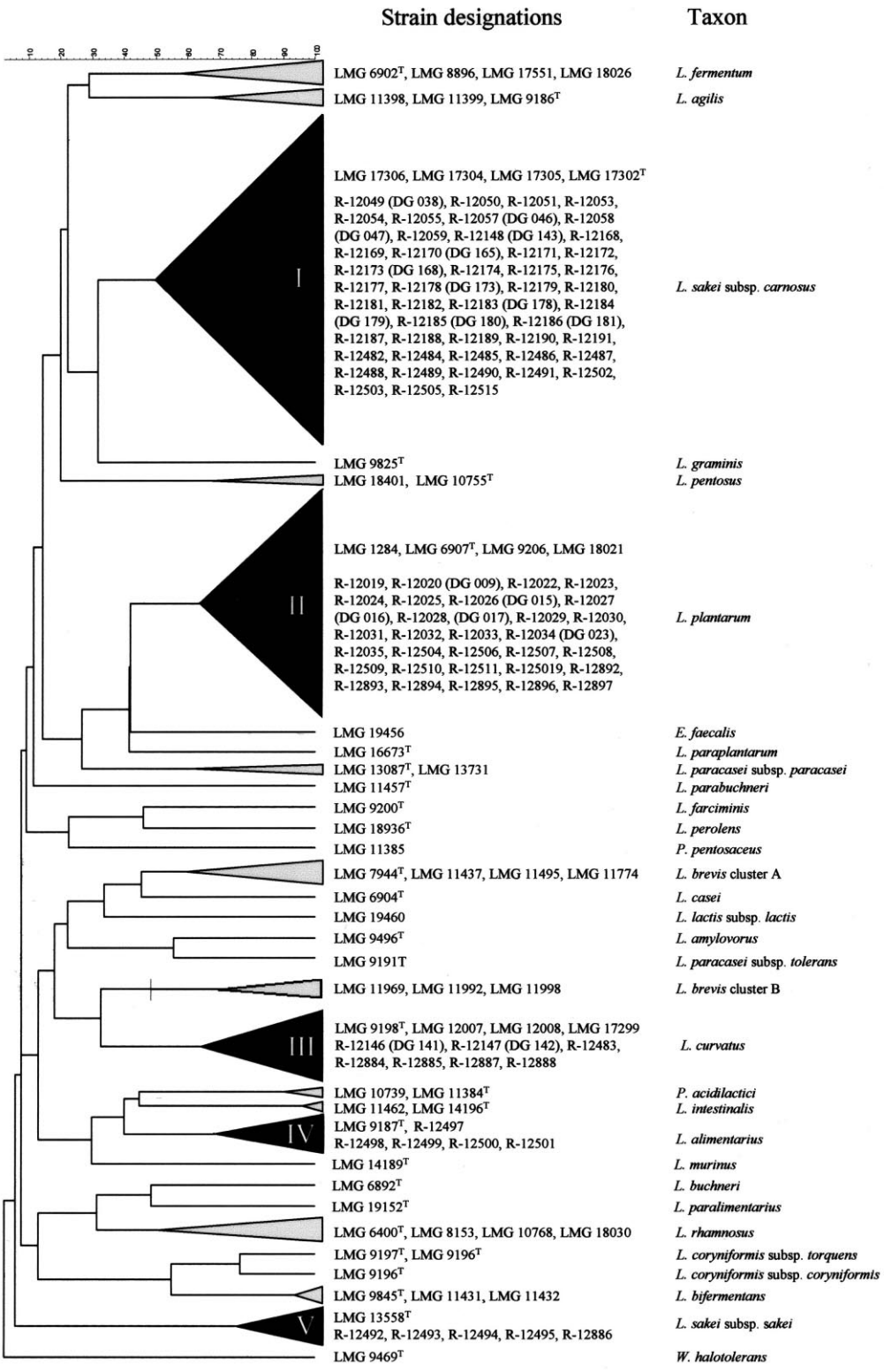

Fig. 2. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of the 61 reference strains and 94 isolates. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient. Block clusters I–IV contain reference strains and isolates. DG numbers refer to numbers used in Gevers et al. [21].

REP, ERIC and BOX primers for typing of rhizobial strains. A combined REP+ERIC+BOX+$(GTG)_5$ dendrogram was generated because a maximized specificity of the patterns was preferred.

3.2. Reproducibility of rep-PCR fingerprinting

A selection of 16 isolates and five reference strains was used to assess the reproducibility of the banding pattern

with rep-PCR fingerprinting using the $(GTG)_5$ primer ($(GTG)_5$-PCR). PCR amplification and electrophoresis were performed in three separate trials starting from the same DNA preparation and using the same PCR reagents. None of the strains tested showed qualitative differences in banding patterns, i.e. presence versus absence of a band. On the other hand, minor quantitative variations in band intensity were occasionally found, but with no pronounced effect on the stability of cluster analysis. The similarity index between three separately obtained banding patterns of the same strain ranged between 91 and 97%. In a second reproducibility test, we investigated the influence of the DNA preparation, and again, no qualitative differences in banding patterns could be detected. Moreover, each PCR reaction is controlled for reproducibility by inclusion of the reference strains LMG 6907^T and LMG 17302^T. In a series of 10 PCR reactions, an average of 93% similarity between the corresponding banding patterns of each reference strain was found, and no qualitative variations were noticed. In order to maintain a high reproducibility in $(GTG)_5$-PCR fingerprinting, we strongly recommend the use of filter tips, small aliquots of PCR reagents, and the same thermal cycler for all PCR reactions. From our experience, and in contrast to the original protocol of Versalovic and co-workers [17], working on ice to prepare the PCR reaction mixture was preferred above room temperature performance.

3.3. Identification and typing of lactobacilli with $(GTG)_5$-PCR

A total of 61 reference strains were subjected to $(GTG)_5$-PCR fingerprinting. The results of numerical analysis of the generated $(GTG)_5$-PCR banding patterns are shown in a dendrogram (Fig. 1). All reference strains clearly grouped in separate clusters according to their respective taxonomic designations, except for representatives of *L. brevis*, which were dispersed in two clusters. *L. brevis* is known to be a phenotypically and genotypically heterogeneous species, as observed from protein profiling (D. Gevers, unpublished data) and DNA–DNA hybridization data [5]. The newly obtained $(GTG)_5$-PCR fingerprinting results confirm this previous finding, hereby indicating the need for a revision of the species status of *L. brevis*. In a number of cases, it was also noted that the taxonomic resolution of the $(GTG)_5$-PCR method was higher than that of protein profiling. The closely related species *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Lactobacillus paraplantarum*, and the species *Lactobacillus alimentarius* and *Lactobacillus paralimentarius* cannot be differentiated using protein profiling (data not shown). As shown in Fig. 1, this was not a problem in numerical analysis of $(GTG)_5$-PCR fingerprints. For each of the investigated taxa, group-specific bands could be observed that allowed visual verification of the clusters obtained with numerical analysis. It was found that the complexity of the $(GTG)_5$-PCR band pattern was not the same for all species. Generally, the number of bands ranged between 10 and 20, with an average of 16.5. For *L. alimentarius* and *Lactobacillus intestinalis*, however, the number of bands was lower, i.e. six and five respectively. The banding patterns of seven strains belonging to other Gram-positive genera (i.e. *Enterococcus*, *Lactococcus*, *Pediococcus* and *Weissella*) displayed a comparable complexity as seen among the majority of *Lactobacillus* fingerprints. On the other hand, the grouping obtained in Fig. 1 clearly did not reflect classification in different genera as lactobacilli were not clearly separated from strains of other Gram-positive genera. A fairly high discriminatory power up to the strain level was found for the set of 61 reference strains as all of these strains could be differentiated from each other on the basis of at least one band difference in their respective $(GTG)_5$-PCR fingerprints (Fig. 1).

In order to evaluate the applicability of $(GTG)_5$-PCR for identification of unknown isolates, two different sets of isolates were subjected to $(GTG)_5$-PCR fingerprinting (Fig. 2). The first set contained 52 isolates that were previously identified at the (sub)species level by means of protein profiling [21]. The results of numerical analysis of the generated $(GTG)_5$-PCR banding patterns confirms identification and the clustering based on the protein profiles for all 52 isolates. For the second set of 42 previously unidentified isolates, the $(GTG)_5$-PCR banding patterns were clustered together with the reference strains and isolates of the first set. The previously unidentified LAB isolates were assigned to the clusters representing *L. alimentarius*, *Lactobacillus curvatus*, *L. plantarum*, *L. sakei* subsp. *carnosus* and *sakei*. Clearly, the addition of these isolates had no pronounced effect on the stability of cluster analysis based on the $(GTG)_5$-PCR banding patterns of the reference strains. Overall, $(GTG)_5$-PCR banding patterns displayed a much higher heterogeneity among isolates, compared to the corresponding protein profiles (data not shown). In this way, $(GTG)_5$-PCR analysis revealed that a given species was represented by different strains within the same sample of fermented dry sausage. On the other hand, isolates with highly similar or even identical $(GTG)_5$-PCR fingerprints were frequently found within the set of isolates recovered from the same sample of fermented dry sausage. From our experience, $(GTG)_5$-PCR fingerprinting can be used for identification and possibly for intraspecies differentiation and is especially useful for screening a large number of strains. In specific cases, however, it may be necessary to further subtype a cluster of similar $(GTG)_5$-PCR fingerprints using PFGE. Further research will include more strains of a broader group of LAB and investigate the usefulness of $(GTG)_5$-PCR for high-resolution typing.

In conclusion, the rep-PCR fingerprinting technique using $(GTG)_5$-PCR is a rapid, easy-to-perform, and reproducible tool for differentiation of a wide range of food-associated lactobacilli at the species, subspecies and poten-

tially up to the strain level, with a single-performance protocol. In our hands, this technique is a promising genotypic tool for rapid and reliable speciation and typing of lactobacilli and other LAB in food-fermentation industries.

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