Differential Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by Multiplex PCR-based Restriction Enzyme Analysis
Differential Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by Multiplex PCR-based Restriction Enzyme Analysis
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ABSTRACT

Two closely related lactic acid bacteria, *Lactobacillus sakei* and *Lactobacillus curvatus*, are very difficult to be clearly differentiated. Here we report multiplex PCR-based restriction enzyme analysis (multiplex PCR-REA) that is useful for rapid and reliable identification of these two species. This method employs both polymerase chain reaction (PCR) and restriction pattern analysis. First, multiplex-PCR using the *Lactobacillus sakei* subgroup-specific primers that were designed from 16S rDNA sequence produces two bands, a 414 bp and a 614 bp band. A 414 bp band represents only *Lb. sakei* and *Lb. curvatus* and less specific 614 bp band is used for further identification by restriction analysis. Second, restriction analysis of 614 bp band using *Hind III* restriction enzyme discriminates *Lb. sakei* from *Lb. curvatus*. This method could identify twenty-eight strains as *Lactobacillus sakei*
and *Lactobacillus curvatus*, which were isolated from kimchi (Korean fermented vegetable product). Therefore, these results suggest that this method is simple, rapid and reliable for identification of *Lactobacillus sakei* and *Lactobacillus curvatus* species.
요 약

*Lactobacillus sake*와 *Lactobacillus curvatus*은 근연 관계에 있는 유산균으로서 분별 동정하기 힘들다. 이에 multiplex-PCR based Restriction Enzyme Analysis를 고안하여 두 종의 빠르고 정확하게 두 종을 동정할 수 있었다. 이 방법은 PCR을 한 후 Restriction Enzyme Analysis법으로 *Lactobacillus sakei*와 *Lactobacillus curvatus*를 restriction digestion pattern의 분석으로 분별 동정 하였다. Multiplex PCR amplicon은 414bp와 614bp의 band를 가지며, 414bp의 amplicon은 *Lactobacillus sake*와 *Lactobacillus curvatus*에 특이적으로 반응하고, 614bp의 amplicon은 두 종반이 specific 하지 않는다. 두번째의 방법은 restriction enzyme analysis로서, *Hind III*와 614bp의 amplicon의 restriction pattern을 비교하여 kimchi에서 분리된 *Lactobacillus sake*와 *Lactobacillus curvatus*를 동정할 수 있었다. PCR-based restriction enzyme analysis는 간단하며 빠르고 정확하게 *Lactobacillus sake*와 *Lactobacillus curvatus*를 중단위로 분별 동정 할 수 있게 되었다.
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Introduction

It is well known that two species, *L. sakei* and *L. curvatus*, are best adapted to meat products and plant fermentation (Hitchener, B.J et al. 1982; Kandler, O et al. 1986; Vogel, G et al. 1993). From the food ecology, these facts implicates that two species are widely distributed, appeared simultaneously and colonized in ordinary foods and thereby governed the whole or the late phase of fermentation. *L. sakei* and *L. curvatus* have been important and familiar lactic acid bacteria to food industry and have been attracted by food workers.

These two species, however, are closely related phenotypic species and genetically homogeneous species at the high level of DNA homology. A variety of methods on the identification of *L. sakei* and *L. curvatus* have been reported for two decades (Hitchener, B.J et al. 1982; Kandler, O et al. 1986; Vogel et al. 1993; Berthier and Ehrlich 1999). The methods have been mainly based on the information molecules such as proteins, DNAs, and RNAs including numerical analysis of physiological and biochemical characteristics. All methods are successfully able to identify two species. However, such
identification is still difficult and laborious. Recently, the analysis of variable region of 16S or 23S rDNA sequences have performed for better differentiation and detection at the species and strain level (Berthier and Ehrlich 1998). Sometimes, the analysis of 16S rDNA is limited because of the high similarity between closely related species. An alternative method is proposed by Berthier et al that leads to the clear differentiation of two fastidious species, *L. sakei* and *L. curvatus* by species-specific primers chosen in 16S/23S spacer regions (Berthier and Ehrlich 1998). PCR by species-specific primers in 16S/23S spacer regions has been proposed as an alternative method (Berthier and Ehrlich 1998), but sequences for this spacer region are not fully available yet.

The aim of this study, we report the Multiplex PCR-based restriction enzyme analysis (Multiplex PCR-REA) is simple and reliable, and has proven useful for both detection and identification of *Lb. sakei* and *Lb. curvatus*. 
Materials and methods

I. Materials

1. Bacterial strains and culture conditions

Bacterial strains used in this study were *Lactobacillus sakei* subsp. *sake* DSM 20017\(^T\), *Lb. curvatus* subsp. *curvatus* DSM 20019\(^T\), *Lb. brevis* KCTC 3498\(^T\), *Lb. plantarum* KFCC 11322\(^T\), *Leuconostoc gelidum* DSM 5578\(^T\), *Leuconostoc kimchii* KCTC 2368\(^T\), and *Weissella confusa* KCTC 3499\(^T\), *Weissella hanii* KCTC 3755\(^T\) as reference type strains. Twenty-eight strains isolated from retail kimchi (Korean fermented vegetables products) were previously characterized as *Lb. sakei* /*Lb. curvatus* group and were named HU 1 to 28. All strains were grown in MRS broth at 25 °C and kept in 20% glycerol solution (w/v) at -70 °C. Cultures were activated in the same broth, if necessary.
II. Methods

1. Primer design

To search for the forward and reverse primers specific for a subgroup *Lb. sakei*, the 16S rDNA sequences of most lactic acid bacteria including *Lb. sakei* and *Lb. curvatus* were aligned by using Clustal X multi-alignment program (version 1.81). A few tentative signature sequences were compared with other DNA sequences in the GenBank and RDP II (http://rdp.cme.msu.edu/) database by using BLAST to confirm their specificity as the subgroup-specific primers. Among them two primers showing no matches with other (bacterial) DNA sequences were selected. One was the forward primer that the sequence was 5’-GAGCTTGCTCCTCATTGATAA-3’ (CS-f) and corresponded to positions 60-80 of *Lb. curvatus* (GenBank accession no. AJ270951). Another one was the reverse primer that the sequence was 5’-TTGGATACCGTCACTACCTGG-3’ (CS-r) and corresponded to positions 472-492 of the *Lb. curvatus*. PCR reaction using these two primers (set A) produces a 414 bp product only from *Lb. curvatus*. Another primer pairs (set B) substitute CS-f of set A with the primer
(5’-GATAAACAAATGTGTAGGG-3’, Uni-f) that is located at the conserved region in rRNA operon and produces a 614 bp band (Table 1). These two primer sets rapidly detect *Lb. sakei, Lb. curvatus*, and very closely related species.

2. Isolation of template DNA and PCR condition

The isolation of template DNA for PCR was performed as described previously by Kim *et al.* (2000). Amplifications for set A or set B were performed in 50 µl total volume containing 0.2 mM each dNTP, 50 ng genomic DNA, 1.5 mM MgCl₂, 0.5 µM of each primer (Bioneer, Korea) and *Taq* DNA polymerase of 1.25 U (Applied Biosystems). In case of multiplex PCR, the concentration of each primer was adjusted to achieve thick bands of amplified products. The primers, CS-f, CS-r and Uni-f, were mixed to be 0.75 µM, 0.5 µM, and 0.25 µM, respectively. Reactions were carried out in a Perkin-Elmer thermal cycler as follows: after one cycle of denaturation for 5 min at 94 °C, 35 cycles consisting of denaturation at 94 °C for 30 sec, primer annealing at 58 °C for 30 sec, extension at 72 °C for 1 min and final extension was carried out at 72 °C for 7 min. PCR products were run on 3% (w/v) agarose gel in 0.5
X borate-EDTA buffer at 100 V for 50 min. Gels were stained with ethidium bromide, and photographed.

3. Restriction enzyme analysis (REA)

Amplified fragments by primer set B were digested with Hind III to identify _Lb. sakei_ and _Lb. curvatus_ at the species level. Suitable restriction sites were identified though REBASE web site (http://rebase.neb.com) (Roberts, R.J. _et al_ 2001). Primer set A has no recognition site of Hind III, while primer set B has a single one. Enzyme digestion was carried out for 1 h at 37 °C in a total volume of 10 μl reaction mixture containing 1 μg PCR product from primer set A and set B, 1 μl 10X M Buffer (100 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 10 mM dithiothreitol, 500 mM NaCl), 1U Hind III. Resultant digests were separated by electrophoresis on agarose gel (3% Nusieve 3:1, BMA) at 100V for 50 min. Ethidium bromide was added to the agarose gels to be a final concentration of 0.5 μg /ml. A 100 bp ladder DNA (Gibco BRL) was used as a DNA molecular weight marker. All restriction digests (10 μl) were mixed with 2 μl 6 X Gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol
in water). The resultant gels were photographed, and the size of each fragment was determined with interpolation by using the Image Master VDS image analyzer (Pharmacia biotech).

4. 16S rDNA sequencing

For 16S rDNA sequencing, we amplified about 800 nucleotides (proximal variable region of 16S rDNA sequences among lactic acid bacteria) by using both universal forward primer and group-specific reverse primer for lactic acid bacteria (Kim, J. et al 2000). The PCR products were purified by use of an EXELUTOR PAK6 purification instrument (FINEPCR, Korea). 16S rDNA sequences were determined by using a Sequencing Kit (Thermo sequenase CY 5.5 Terminator Cycle, Amersham Pharmacia Biotech, Inc) and an automatic DNA sequencer (SEQ4X4 personal sequencing system, Amersham Pharmacia Biotech, Inc).

5. Hybridization

DNA-DNA hybridization was performed in slot blotting equipment
(Hoefer PR648 Slot blot Manifold, Amersham Pharmacia Biotech Inc.) using the protocol described in the enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (RPN 3000, Amersham Pharmacia Biotech). For the preparation of DNA probes ranging from approximately 250 bp to 1 kb, chromosomal DNAs were cleaved with sonication. These DNA fragments were purified twice by ethanol precipitation and denatured by boiling in 50% ethylene glycol solution for 5 min. For hybridization, chromosomal DNA (200 ng) was denatured using an alkaline method (0.2 M NaOH, 20 mM EDTA, 80 °C) and fixed to Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech Inc.). DNA probes of 100 ng were labeled with the ECL labeling and detection kit. The slot blots were prehybridized in a hybridization solution at 42 °C for 1 h, and then hybridization was performed in 10 ml of hybridization solution containing DNA probes (10 ng ml⁻¹) at 42 °C overnight. After hybridization, the blots were washed twice with each of the wash solutions: a primary wash solution (0.4% SDS, 0.1 X SSC) at 42 °C for 20 min and a secondary wash solution (2 X SSC) at room temperature for 5 min. Detection reagents were added to the blots and kept for 1 min at room temperature, and then discarded. The blots wrapped in Saran-Wrap were exposed to
Hyperfilm-ECL (Amersham Pharmacia Biotech Inc.) for 10-15 min. The signal produced by self-hybridization was taken as 100%, and the values of percent homology were calculated for the duplicate samples.

6. Polyphasic taxonomic identification

In order to confirm whether the identification of twenty-eight isolates by multiplex PCR-REA is reliable, additional tests for polyphasic classification (Bjorkroth, J., et al. 1998) were carried out as follows: the strains were characterized biochemically using the API CH50 and API CHL medium systems according to the manufacturer’s instructions (API, bioMérieux). Arginine dihydrolase activity and catalase activity were tested according to Methods for General and Molecular Bacteriology (Smibert, R.M., et al 1994).
RESULTS

To develop a novel method for the identification of *Lb. sakei* and *Lb. curvatus* species (named *Lb. sakei* subgroup), we first designed *Lb. sakei* subgroup-specific forward (CS-f) and reverse (CS-r) primer using the alignment of 16S rDNA sequences of all *Lactobacillus* species and other related organisms including *Leuconostoc*, *Pediococcus*, and *Weissella* species (Table 3). We found that *Lb. sakei* subgroup primer pair was commonly specific for *Lb. sakei* and *Lb. curvatus* strains. Accordingly, *Lb. sakei* and *Lb. curvatus* strains generated a PCR product sized 414 bp (Fig. 1), while non-targeted organisms showed no PCR bands by this primer set (data not shown). These results indicate that *Lb. sakei* subgroup-specific product (414 bp) leads us to classify isolates as *Lb. sakei* and/or *Lb. curvatus*. Unfortunately, this PCR product is not suitable for the separation between *Lb. sakei* and *Lb. curvatus* species, because the distinctive restriction site from these two species is absent in subgroup-specific PCR products.

To discriminate *Lb. curvatus* from *Lb. sakei*, we next produced another PCR product containing one *Hind* III restriction site for *Lb.
*curvatus*, but not for *Lb. sakei*. This PCR product (614 bp) is obtained by using two primers; a universal primer (Uni-f) and the CS-r reverse primer (Fig. 1). However, we found that this PCR reaction was less specific for *Lb. sakei* and *Lb. curvatus*, because the CS-r reverse primer can be also targeted to *Lb. fuchuensis* and *Lb. plantarum*, resulting in a 614 bp product from these two species (data not shown). Therefore, we next developed a multiplex PCR system by using three primers; CS-f, Uni-f, and CS-r. Only *Lb. sakei* and *Lb. curvatus* produced two distinct PCR products (414 & 614 bp), while non-target species including *Lb. fuchuensis* and *Lb. plantarum* produced no bands or sometimes only 614 bp band (Fig. 2). Two non-specific background PCR products of 580 bp and >2,000 bp differ in product size from the specific products (414 & 614 bp), resulting in no confusion in identification of *Lb. sakei* and *Lb. curvatus* (Fig. 2 & Fig. 3). Finally, we digested multiplex PCR products with the restriction endonuclease *Hind* III, and thereby *Lb. curvatus* was distinguished from *Lb. sakei*. *Hind* III-digestion of a 614 bp PCR product yielded two fragments of 210 bp and 404 bp (Fig. 3, lane 2). In contrast, the same product from *Lb. sakei* did not cleaved by *Hind* III (Fig. 3, lane 1).
This method was next applied to identify twenty-eight isolates of *Lb. sakei/Lb. curvatus*-like isolates. We found that thirteen isolates were identified as *Lb. sakei* by the production of both PCR products (414 & 614 bp) and non-cleavage by *Hind* III, and other fifteen isolates were identified as *Lb. curvatus* by the production of both PCR products (414 & 614 bp) and *Hind* III-digestion of 614 bp band (data not shown). To examine whether such an identification system is valid, we further identified these twenty-eight isolates. The data obtained from polyphasic taxonomy as described in the method are not presented here, because we aimed at just verifying our multiplex PCR-REA method. Only results will be discussed instead. Partial 16S rDNA sequences (about 440 nucleotides containing 1 – 347 nt of *E. coli* 16S rRNA gene) containing three primer regions showed that the identification is reliable. 16S rDNA sequences of thirteen isolates that are classified as *Lb. sakei* were nearly identical to that of *Lb. sakei* DSM 20017\(^T\) (99.8 – 100%), but less similar to that of *Lb. curvatus* DSM 20019\(^T\) (99.4%). This identification is also supported by DNA-DNA hybridization that thirteen isolates showed high level of DNA homology (>60%) with the type strain of *Lb. sakei* DSM 20017\(^T\), and phenotypic characteristics that melibiose is fermented and arginine is hydrolysed (Table 3.) [4].
Fifteen isolates classified as *Lb. curvatus* also exhibited the same 16S rDNA sequence to the type strain of *Lb. curvatus* DSM 20019\(^T\) (100%), but lower similarity to *Lb. sakei* (99.4%). They also had high DNA homology values of >82% with the type strain of *Lb. curvatus* DSM 20019\(^T\). Phenotypic characteristics that did not produce acid from melibiose and not hydrolyze arginine also support this identification (Table 3.). In addition, we also found that primer and recognition sites of restriction enzyme were perfectly conserved among all twenty-eight *Lb. sakei/Lb. curvatus* isolates. Therefore, these results show that our method partly represents 16S rDNA sequence similarity and is in agreement with data from polyphasic taxonomy.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer (CS-f)</th>
<th>Reverse primer (CS-r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>5’- GACCTTGCTCCTSA-TTGATAA –3’</td>
<td>5’- TTGGATACCGTCACTACCTG –3’</td>
</tr>
<tr>
<td>Lb. sakei</td>
<td>5’- GACCTTGCTCCTCA-TTGATAA –3’</td>
<td>5’- TTGGATACCGTCACTACCTG –3’</td>
</tr>
<tr>
<td>Lb. curvatus</td>
<td>5’- AAGCTTGCTTCTGA-TTGATAA –3’</td>
<td>5’- TTGGATACCGTCACTACCTG –3’</td>
</tr>
<tr>
<td>Lb. fuchuensis</td>
<td>5’- AGCTTTGCTTCTGA-TTGAAAGC –3’</td>
<td>5’- TTGGATACCGTCACTACCTG –3’</td>
</tr>
<tr>
<td>Lb. plantarum</td>
<td>5’- GTGCTTGATC-ATGATT –3’</td>
<td>5’- TTAAATACCGTCAATACCTG –3’</td>
</tr>
<tr>
<td>Ped. parvulus</td>
<td>5’- GTGCTTGCAACCGAAGATGATTT –3’</td>
<td>5’- TTGGATACCGTCACTGCATG –3’</td>
</tr>
<tr>
<td>Wei. hani</td>
<td>5’- GAACCTTG=TTCAGATTTGATTT –3’</td>
<td>5’- TAAAGATACCGTCACACATTG –3’</td>
</tr>
<tr>
<td>Leu. kimchii</td>
<td>5’- GTGCTTGCAACC– -3’</td>
<td>5’- TATGGTACCGTCTCACTAAA –3’</td>
</tr>
</tbody>
</table>

Table 1

Comparison of DNA sequences targeting at forward and reverse primers. Abbreviations; Lb., Lactobacillus; Ped., Pediococcus; Wei., Weissella; Leu., Leuconostoc.

*The recognition site of Hind III is underlined.
Table 2. *Lb. sakei* and *Lb. curvatus* 16S rDNA specific/universal primer site (set A and set B) and restriction site.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Lb. sake</th>
<th></th>
<th>Lb. curvatus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSM20017&lt;sup&gt;†&lt;/sup&gt;</td>
<td>HU(13)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DSM20019&lt;sup&gt;†&lt;/sup&gt;</td>
<td>HU(15)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Cell shape</td>
<td>rod</td>
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<td>rod</td>
<td>rod</td>
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<tr>
<td>Gram stain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sporulation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 7% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acid production from</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-Methyl-D-glucoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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<td>Cellobiose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saccharose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>CO₂ from gluconate and glucose</td>
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<td></td>
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<tr>
<td>Glucose - grown cells</td>
<td>-</td>
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<tr>
<td>Gluconate-grown cells</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.

Differential characteristics of *L. sake*, *L. curvatus* and isolated strains

<sup>a</sup> The numbers in parentheses indicate numbers of isolates.
Fig. 1. PCR products amplified by primer set A (414 bp) and set B (614 bp). Lanes: 1,3, \textit{Lb. sakei} DSM 20017$^\text{T}$; lanes: 2,4, \textit{Lb. curvatus} DSM 20019$^\text{T}$. M, 100-bp ladder DNA as size marker (Gibco BRL). \textit{Lb. sakei} and \textit{Lb. curvatus} produce both 414-bp and 614-bp bands.
Fig. 2. Multiplex PCR products amplified by the mixture of primer set A and set B (CS-f, CS-r and Uni-f). Lanes: 1, *Lb. sakei* DSM 20017T; 2. *Lb. curvatus* DSM 20019T. M, 100-bp ladder DNA (Gibco BRL). The simultaneous appearance of two bands enables the detection of *Lb. sakei*/ *Lb. curvatus* at the subgroup level.
Fig. 3. Digestion patterns of multiplex PCR products with Hind III. Lanes: 1, *Lb. sakei* DSM 20017T; 2, *Lb. curvatus* DSM 20019T. M, 100 bp ladder DNA (Gibco BRL). Two species are differentiated with these patterns.
DISCUSSION

In this study, we demonstrated that multiplex PCR-based restriction enzyme analysis was a rapid and reliable method for differential identification of *Lb. sakei* and *Lb. curvatus* species, so called the subgroup *Lb. sakei* belonging to the genus *Lactobacillus*. This system is unique.

First, PCR with the primer set A specific for the subgroup *Lb. sakei* exhibited that this primer set A is specific for only these species without any non-specific binding to other 16S rDNA sequences from non-target organisms, such as leuconostocs, weissellas, and other lactobacilli. In particular, *Lactobacillus plantarum* and *Weissella* species are frequently isolated from kimchi samples, and these organisms are therefore the most possible non-target organisms in this study. In this context, our method can well discriminates the subgroup *Lb. sakei* from other related organisms by multiplex PCR yielding two PCR products (414 and 614 bp; Fig. 2), comparing with the production of a single (614 bp) or no PCR products from other related organisms.
Second, the digestion patterns of PCR products with *Hind* III restriction enzyme lead to clear identification of *Lb. sakei* or *Lb. curvatus* at the species level. Identification becomes very clear, because the number of the restriction fragment is not only three, but the size is also quite different (Fig. 3). Moreover, any possible variation of digestion patterns will be neglected, because *Hind* III restriction site in 614 bp band is likely to be conserved among strains of these two species. 16S rDNA sequencing of twenty-eight isolates of *Lb. sakei/Lb. curvatus* used in this study showed strict conservation in the recognition site of *Hind* III as well as the targeting site of two primers. Therefore, the digestion patterns of *Lb. sakei* subgroup-specific PCR products enables twenty-eight isolates of this group to identify at the species level.

This method is very reproducible. Unlike RAPD-PCR that annealing temperature is 37 °C (Berthier, F et al 1999), the temperature in this study is high (50 - 65 °C), since primers used are relatively long in length (20 or 21 bp). This high and broad annealing temperature gives rise to very high reproducibility. However, the subspecies of *Lb. sakei* and *Lb. curvatus* that were classified by Torriani et al. (1996) could not
be differentiated, because our method is limited to a few polymorphisms in partial 16S rRNA gene, but probably not in whole genome. Generally speaking, our multiplex PCR-REA method is considered suitable for rapid identification of only *Lb. sakei* and *Lb. curvatus* at the species level.

In conclusion, these results indicate that our method for the identification of *Lactobacillus sakei* and *Lactobacillus curvatus* is simple and rapid in comparison with the analyses of RAPD or total soluble protein patterns, and therefore may help their rapid identification in food analysis laboratories, in particular, dealing with many food samples.
References


