Full Paper

Rapid identification of lactic acid bacteria isolated from home-made fermented milk in Tibet

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Fermented milk is a very delicious and nutritional food distributed in the international markets. Rapid preliminary identification of lactic acid bacteria to the species level is an important issue for the fermentation industry. One hundred seventy-one strains of lactic acid bacteria (LAB) were isolated from twenty-nine kurut samples and fifteen traditional fermented mongolian cattle milk (FMCM) samples in Tibet, China. All of the strains were isolated and divided into bacilli or cocci by phenotype, and then differentiated by restriction fragment length polymorphism (RFLP) analysis using a set of restriction enzymes, *AluI*, *HaeIII*, *BsmI*, *TspRI* and *HinfI*. Restriction pattern analyses indicated that the lactobacilli could be clearly identified at the species level and the LAB cocci at the genera level by the five restriction enzymes. Further studies on 16S rDNA sequences of representative and undefined strains showed that the combination of RFLP analysis of polymerase chain reaction (PCR) for amplifying 16S rDNA and 16S rDNA sequence analysis is rapid, easy to perform, and effective for large-scale preliminary identification of LAB.

Key Words—identification; lactic acid bacteria; restriction fragment length polymorphism (RFLP); 16S rDNA sequence analysis

Introduction

Kurut is one of the important fermented products from yak milk by a traditional method using a special-made tung. After at least 3–6 days' fermentation around 10–20°C, kurut can be simply prepared with the proper proportion of lactic acid and alcohol content, like kefir and koumiss. Fermented Mongolian cattle milk (FMCM) is mainly produced in agricultural areas of Tibet. The fermentation technology of FMCM is different from that of kurut in the fermentation time (10–48 h)

and the fermentation temperature (20–30°C).

The Tibetan plateau lies in northwest China and some other countries with an average altitude of over 4,000 m (Wu, 2001). It is a distinct ecological region with a wide biodiversity. Such diversity of species has an impact on the formation of the specific microflora found in yak's milk (Luo et al., 2005). LABs and yeasts are the major microbial communities in kurut and Tibetan FMCM.

LABs are a group of gram-positive rod- or coccus-shaped organisms that are non-spore-forming and non-motile and produce lactic acid as their major end product during fermentation of carbohydrates (Holzapfel et al., 2001; Stiles and Holzapfel, 1997). Lactic acid bacteria have played a long and important role in food technology (Yanagida et al., 2005). They constitute a significant portion of human intestinal microflora, and their association with the general state of human health

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is under intensive investigation.

Traditional bacterial classification methods based on morphology, physiology, and biochemical tests can be time-consuming and misleading (Yanagida et al., 2007). Commercial kits based on biochemical, enzymatic and fatty acid analyses were found to be unstable, perhaps due to their susceptibility to different bacterial physiological conditions (Teapaisan and Dahlen, 2006). So, efforts have been made to develop simple, fast and discriminating methods for identification of LAB. Because the 16S ribosomal DNA (rDNA) is ubiquitous and composed of alternating conserved and hypervariable (V) regions, it represents the prototype genetic marker in molecular microbial ecology (Huys et al., 2007). Several studies have rapidly classified LAB based on 16S rDNA restriction fragment length polymorphism (RFLP) analysis (Gurtler et al., 1991; Johansson et al., 1995; Randazzo et al., 2004; Yanagida et al., 2007). Therefore, in this study, 16S rDNA RFLP analysis and 16S rDNA sequence analysis were used to identify the LAB isolated from the homemade fermented milk in Tibet, China.

The aim of this study was to design an accurate and rapid method to identify LAB species from twenty-nine kurut samples and fifteen FMCM samples, and convenient methods were devised to amplify 16S rDNA of all isolates, analyze genes with RFLP, and obtain 16S rDNA sequences of partial isolates complementarily. The combination of 16S rDNA PCR-RFLP and phylogenetic analysis of 16S rDNA proved to be useful for identification of lactobacilli at the species level and cocci at the genera level.

Materials and Methods

Fermented milk samples. All samples were collected from scattered households in three regions in Tibet of China (Table 1), Rikaze, Nakqu and Lasa. Samples were picked in sterilized bags, kept in ice boxes and transported to the microbiology laboratory for analysis.

Bacterial strains. Reference strains: *Lactobacillus acidophilus* ATCC 4356^T, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T, *Lactobacillus helveticus* ATCC 15009^T, *Lactobacillus casei* ATCC 334, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus plantarum* ATCC 14917^T, *Lactobacillus rhamnosus* ATCC 7469^T, *Lactobacillus animalis* ATCC 35046^T, *Lactobacillus coryniformis* subsp. *coryniformis* ATCC 25602^T,

Enterococcus faecalis ATCC 19433^T, *Enterococcus faecium* ATCC 19434^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293^T, *Leuconostoc lactis* ATCC 19256^T, *Pediococcus pentosaceus* ATCC 33316^T, *Pediococcus acidilactici* DSM 20284^T, *Lactococcus lactis* subsp. *Lactis* ATCC 19435^T, and *Lactococcus lactis* subsp. *cremoris* ATCC 19257^T, were obtained from China General Microbiological Culture Collection Center (CGMCC) and were kept at -80°C in 20% glycerol.

Bacterial strains isolated: One hundred seventy-one strains of LAB to be identified in this study were isolated from traditional fermented milk products (Kurut and FMCM) in Tibet, China (Table 1).

Microbial classification by phenotypic characterization. All LAB strains were classified as bacilli or as cocci based on phenotypic characteristics such as properties of Gram stain, cell shape and catalase test (bioMe'rieux, France). These are essential for the whole identification procedure.

Genomic DNA extraction. Two methods (Ausubel et al., 1994; Zhou et al., 1996) were combined to extract the total DNA. Briefly, 1 ml liquid culture of each strain incubated overnight in MRS broth was pelleted by centrifugation at 8,000×g for 5 min. The pellets were washed with 500 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) in a clean 1.5-ml microcentrifuge tube and re-pelleted by centrifugation. Washed cell pellets were resuspended in 500 µl TE buffer; tubes were frozen for 5 min by liquid nitrogen, then incubated at 65°C for 5 min. This freezing-thawing step was repeated 4 times. Ten microliter proteinase K solution (20 mg proteinase K/ml in TE; Amresco, Inc.) and 60 µl SDS solution (10%) were added. After incubation at 37°C for 1 h, 100 µl NaCl (5 M) and 80 µl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7 M NaCl) were added and tubes were incubated at 65°C for 30 min. The mixture was extracted with 1 vol. phenol/chloroform/isoamylalcohol. After centrifugation, DNA was obtained by the addition of isopropanol (one equal volume), and then washed in 500 µl ethanol (70%). DNA was pelleted, dried, and dissolved in 100 µl RNase solution (100 µg/ml RNase in TE; Sigma-Aldrich). After incubation for 1 h at 37°C, the sample volumes were adjusted to 400 µl with TE. Then phenol/chloroform extraction was performed and DNA precipitated with 0.5 M NaAc (final concentration) and two volumes of ethanol (99%). Pellets were washed in 500 µl ethanol (70%). Finally DNA was solubilized as

Table 1. Collection of fermented milk and strains used in this study.

Samples	Collection place	Isolated strains
FMCM	Shigatse	IMAU60001, IMAU60002, IMAU60003, IMAU60004, IMAU60005
FMCM	Shigatse	IMAU60006, IMAU60007, IMAU60008
FMCM	Shigatse	IMAU60009, IMAU60010
FMCM	Shigatse	IMAU60011, IMAU60012, IMAU60013, IMAU60014, IMAU60015, IMAU60016
FMCM	Shigatse	IMAU60017, IMAU60018, IMAU60019, IMAU60020
FMCM	Shigatse	IMAU60021, IMAU60022, IMAU60023, IMAU60024
FMCM	Shigatse	IMAU60025, IMAU60026, IMAU60027, IMAU60028
FMCM	Shigatse	IMAU60029, IMAU60030
FMCM	Shigatse	IMAU60031, IMAU60032, IMAU60033
FMCM	Shigatse	IMAU60034, IMAU60035, IMAU60036, IMAU60037
FMCM	Shigatse	IMAU60038, IMAU60039, IMAU60040
FMCM	Shigatse	IMAU60041, IMAU60042, IMAU60043, IMAU60044, IMAU60045
FMCM	Shigatse	IMAU60046, IMAU60047, IMAU60048, IMAU60049, IMAU60050, IMAU60051, IMAU60052
FMCM	Shigatse	IMAU60053, IMAU60054, IMAU60055, IMAU60056, IMAU60057
FMCM	Shigatse	IMAU60058, IMAU60059, IMAU60060, IMAU60061, IMAU60062, IMAU60063
Kurut	Nakqu	IMAU60064, IMAU60065, IMAU60066
Kurut	Nakqu	IMAU60067, IMAU60068
Kurut	Nakqu	IMAU60069, IMAU60070, IMAU60071
Kurut	Nakqu	IMAU60072, IMAU60073, IMAU60074, IMAU60075, IMAU60076, IMAU60077, IMAU60078
Kurut	Nakqu	IMAU60079, IMAU60080
Kurut	Nakqu	IMAU60081, IMAU60082, IMAU60083
Kurut	Nakqu	IMAU60084, IMAU60085, IMAU60086, IMAU60087
Kurut	Nakqu	IMAU60088, IMAU60089, IMAU60090, IMAU60091
Kurut	Nakqu	IMAU60092, IMAU60093, IMAU60094, IMAU60095
Kurut	Nakqu	IMAU60096, IMAU60093, IMAU60098, IMAU60099
Kurut	Nakqu	IMAU60100
Kurut	Nakqu	IMAU60101, IMAU60102
Kurut	Nakqu	IMAU60103, IMAU60104, IMAU60105, IMAU60106, IMAU60107, IMAU60108, IMAU60109, IMAU60110, IMAU60111
Kurut	Nakqu	IMAU60112, IMAU60113, IMAU60114, IMAU60115
Kurut	Nakqu	IMAU60116, IMAU60117
Kurut	Nakqu	IMAU60118, IMAU60119, IMAU60120, IMAU60121
Kurut	Nakqu	IMAU60122, IMAU60123, IMAU60124
Kurut	Nakqu	IMAU60125, IMAU60126, IMAU60127, IMAU60128
Kurut	Lasa	IMAU60129, IMAU60130, IMAU60131, IMAU60132
Kurut	Lasa	IMAU60133
Kurut	Lasa	IMAU60134, IMAU60135, IMAU60136, IMAU60137
Kurut	Lasa	IMAU60138, IMAU60139
Kurut	Lasa	IMAU60140, IMAU60141, IMAU60142, IMAU60143
Kurut	Lasa	IMAU60144, IMAU60145, IMAU60146, IMAU60147, IMAU60148, IMAU60149, IMAU60150
Kurut	Lasa	IMAU60151, IMAU60152, IMAU60153, IMAU60154, IMAU60155
Kurut	Lasa	IMAU60156, IMAU60157, IMAU60158, IMAU60159
Kurut	Lasa	IMAU60160, IMAU60161, IMAU60162, IMAU60163, IMAU60164, IMAU60165, IMAU60166
Kurut	Lasa	IMAU60167, IMAU60168
Kurut	Lasa	IMAU60169, IMAU60170, IMAU60171

above in 50–250 μ l TE, and stock solutions were stored at -20°C . The quality of the extracts was routinely checked in 1% (wt/vol) agarose-TBE 0.5 $\times$ gels.

PCR amplification. The 16S rDNA sequences were

amplified by PCR using the universal primers 27f and 1495R. The primer sequences were: 27f, 5'-AGAGTTT-GATCCTGGCTCAG-3' (Mora et al., 1998); and 1495R, 5'-CTACGGCTACCTTGTTACGA-3' (Jensen et al., 1993).

The 25 µl reaction mixture contained 100 ng template DNA, 2.5 µl 10× buffer with 1.5 mM MgCl₂, 3.0 unit *Taq* DNA polymerase, 0.2 mM of dNTP, 10 pmol of each primer. Amplification was done using a PCR System PTC2200 (MJ Research, USA) programmed as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min; and finally, 72°C for 10 min. PCR products were electrophoresed in a 1.0% agarose gel and visualized by UV transillumination after ethidium bromide staining.

On-line restriction analysis and choice of restriction endonucleases. For digest experiments, a cost-effective set of eleven restriction endonucleases (*Bsm*I, *Mse*I, *Hae*III, *Alu*I, *Taq*I, *Tsp*RI, *Hind*III, *Hha*I, *Fok*I, *Rsa*I and *Hinf* I; New England Biolabs) was chosen on the basis of restriction sites giving DNA fragments clearly resolvable by theoretical agarose gel electrophoresis with NEBcutter V2.0, an on-line tool for restriction mapping nucleotide sequences (Internet address: <http://tools.neb.com/NEBcutter2/index.php>) (Giraffa et al., 2003).

Restriction fragment length polymorphism (RFLP). Restriction was separately carried out over 2.5 h at 37°C (for *Mse*I, *Hae*III, *Alu*I, *Fok*I, *Rsa*I, *Hha*I, *Hind*III and *Hinf* I), 65°C (for *Taq*I, *Tsp*RI) or 30°C (for *Bsm*I) in 20 µl volumes of reaction mixtures containing 2 µl of buffer, 2 U of restriction enzyme, and 2 µl of PCR amplified product. Restriction digests were analyzed through 5.0% polyacrylamide gel at 150 V for 1.5 h in 0.1× TBE (40 mM Tris-acetate and 1 mM EDTA pH 8.0). A DL2000 Ruler (TaKaRa) was used as a size marker. Restriction fragments were compared with those of reference strains to identify the lactic acid bacteria at the species level. Fragments shorter than 100 bp were not considered in the analysis.

RFLP pattern analysis. RFLP patterns were stored and analyzed by Cross Checker software. Strain clustering was performed by the unweighted pair group method with arithmetic averages (UPMGA) of NT-SYS2.1.

16S rDNA sequence determination and phylogenetic analysis. Sequencing of the 16S rDNA was performed in Shanghai Sangni Biosciences Corporation. Sequences were submitted to the National Center for Biotechnology Information (Bethesda, Md.) for similarity searches through BLAST (<http://www.ncbi.nlm.nih.gov/blast>) and were compared with type strains by multiple alignments by DNAMAN. The obtained sequences (continuous stretches of more or less 1,450 bp)

were deposited and clustered in the NCBI database under the accession numbers FJ211390(IMAU60018), FJ211391(IMAU60042), FJ211392(IMAU60026), FJ211393(IMAU60027), FJ211394(IMAU60109), FJ211395(IMAU60125), FJ211396(IMAU60078), FJ215667(IMAU60081), FJ215668(IMAU60069), FJ215669(IMAU60100), FJ215670(IMAU60148), FJ215671(IMAU60022), FJ215672(IMAU60037), FJ215673(IMAU60040), FJ215674(IMAU60052), and FJ215675(IMAU60116). A phylogenetic dendrogram based on sequence data was constructed by the unweighted pair-group method with arithmetic averages (UPGMA) of MEGA4.0 (Tamura et al., 2007).

Results

All isolates were Gram-positive and catalase-negative bacteria. They were classified as bacilli or as cocci according to morphological properties. The 16S rDNA sequences of 171 strains were amplified by PCR with the universal primer in this study. The expected fragment length of about 1,450 bp was observed for the PCR products of all the strains. This confirmed the effectiveness of PCR conditions performed. The 16S rDNA of bacilli were digested with six restriction enzymes: *Alu*I, *Hae*III, *Hinf*I, *Hind*III, *Mse*I and *Taq*I. The results showed that *Alu*I, *Hae*III, *Hinf*I were discriminative among the six restriction enzymes. In the study on digestion of 16S rDNA fragments of cocci with five restriction enzymes (*Tsp*RI, *Hha*I, *Fok*I, *Rsa*I, and *Bsm*I), considerably differential patterns of bands were produced by *Tsp*RI and *Bsm*I, and few differences were observed by using the other enzymes (data not shown). Reference strains gave profiles as expected by the NEB cutter V2.0 analysis.

Restriction fragment analysis of lactobacillus

The RFLP profiles of bacilli generated by *Hae*III, *Alu*I and *Hinf*I digestion are shown in Fig. 1. *Hae*III patterns of IMAU60027 and IMAU60109 (Fig. 1A, lanes1 and 4) appeared to be the closest to those of *L. casei* and *L. rhamnosus* (Fig. 1A, lanes12 and 14), while *Hae*III patterns of IMAU60018, IMAU60116, *L. acidophilus* and *L. helveticus* were similar (Fig. 1A, lanes 2, 3, 9 and 13). It was noticed that the *Hae*III pattern of *L. plantarum* looked quite similar to those of *L. casei* and *L. rhamnosus* (Fig. 1A, lanes 15, 12 and 14), except that the band of *L. plantarum* at about 700 bp were clearly lower than the bands of *L. casei* and *L. rhamnosus*. Nevertheless,

A

B

C

Fig. 1. 16S rDNA RFLP patterns of nine reference strains and eight isolates digested with *Hae*III (panel A), *Hin*fI (panel B) and *Alu*I (panel C) and the dendrogram based on the 16S rDNA profile analysis.

Lane 1, IMAU60027. Lane 2, IMAU60018. Lane 3, IMAU60116. Lane 4, IMAU60109. Lane 5, IMAU60042. Lane 6, IMAU60026. Lane 7, IMAU60125. Lane 8, IMAU60078. Lane 9, *Lactobacillus acidophilus* ATCC 4356^T. Lane 10, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T. Lane 11, *Lactobacillus coryniformis* subsp. *coryniformis* ATCC 25602^T. Lane 12, *Lactobacillus rhamnosus* ATCC 7469^T. Lane 13, *Lactobacillus helveticus* ATCC 15009^T. Lane 14, *Lactobacillus casei* ATCC 334. Lane 15, *Lactobacillus plantarum* ATCC 14917^T. Lane 16, *Lactobacillus fermentum* ATCC 9338. Lane 17, *Lactobacillus animalis* ATCC 35046^T. Lane M, DL2000 marker.

L. plantarum could be easily distinguished from *L. casei* and *L. rhamnosus* by using *Hin*fI (Fig. 1C, lanes 15, 12 and 14). So it is easy to identify IMAU60126 and

IMAU60042 under *L. plantarum* by *Hin*fI and *Hae*III profiles.

In contrast, PCR-RFLP using *Hin*fI could clearly dis-

tinguish most of the strains (Fig. 1B), except for *L. rhamnosus* and *L. animalis* (Fig. 1B, lanes 12 and 17), and *L. plantarum* and *L. coryniformis* subsp. *coryniformis* (Fig. 1B, lanes 11 and 15); RFLP with *AluI* could differentiate the *L. rhamnosus* and *L. animalis* (Fig. 1C, lanes 12 and 17), and *L. plantarum* and *L. coryniformis* subsp. *coryniformis* (Fig. 1C, lanes 11 and 15), but produced identical restriction patterns of *Lactobacillus casei*, *Lactobacillus plantarum* and *L. rhamnosus*. *L. acidophilus* appeared to be the closest to those of the *L. helveticus*. The others yielded quite dissimilar digestion profiles within themselves. In addition, *AluI* and *HinfI* patterns of IMAU60027 are unique. Therefore, *HaeIII*, *AluI* and *HinfI* were complementary for species identification of the strains.

One hundred twenty-four isolates classified as *Lactobacillus* were examined by 16S rDNA PCR-RFLP analysis using *HaeIII*, *HinfI* and *AluI* digestion for spe-

cies identification. It was found that the most common species of *Lactobacillus* in homemade fermented milk in Tibet were *L. fermentum* (55/124), followed by *L. casei* (51/124), *L. plantarum* (10/124) and *Lactobacillus helveticus* (6/124). Two unique isolates (IMAU60027 and IMAU60028) were further identified as *Lactobacillus crustorum* by 16S rDNA sequence.

Restriction fragment analysis of cocci

The restriction patterns of *BsmAI* and *TspRI* are shown in Fig. 2. Small differences in band sizes were insufficient to differentiate reliably *Enterococcus faecalis* from *Enterococcus faecium*, *Leuconostoc mesenteroides* subsp. *mesenteroides* from *Leuconostoc lactis*, and *Lactococcus lactis* subsp. *cremoris* from *Lactococcus lactis* subsp. *Lactis*. Therefore, it is difficult to identify *Leuconostoc*, *Lactococcus* and *Enterococcus* to the species level by *BsmAI* (Fig. 2A). How-

Fig. 2. 16S rDNA RFLP patterns of eight reference strains and five isolates digested with *BsmAI* (panel A), *TspRI* (panel B) and the dendrogram based on the 16S rDNA profile analysis.

Lane 1, IMAU60034. Lane 2, IMAU60069. Lane 3, IMAU60037. Lane 4, IMAU600100. Lane 5, IMAU60148. Lane 6, *Lactococcus lactis* subsp. *cremoris* ATCC 19435^T. Lane 7, *Lactococcus lactis* subsp. *Lactis* ATCC 19257^T. Lane 8, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293^T. Lane 9, *Leuconostoc lactis* ATCC 19256^T. Lane 10, *Pediococcus pentosaceus* ATCC 33316^T. Lane 11, *Pediococcus acidilactici* DSM 20284^T. Lane 12, *Enterococcus faecalis* ATCC 19433^T. Lane 13, *Enterococcus faecium* ATCC 19434^T. Lane M, DL2000 marker.

ever, visible differences were observed among the genera, which indicated that it may be feasible to conduct genus identification of LAB cocci by this means. Moreover, *Pediococcus acidilactici* yielded unique banding patterns that distinguished it clearly from *Pediococcus pentosaceus*. *TspRI* (Fig. 2B) produced identical restriction patterns with *BsmAI* for LAB cocci.

The dominant genera isolated from home-made fermented milk were *Enterococcus* (19/47), *Lactococcus* (19/47) and *Leuconostoc* (8/47). Only one strain

(IMAU60022) showed a profile unique to all the strains.

16S rDNA sequence analysis

The constructed phylogenetic tree, based on 16S rDNA sequences, is shown in Fig. 3. Sixteen isolates, bacilli and cocci, were subjected to 16S rDNA sequence analysis. The 16S rDNA sequences determined in this study were compared with 16S rDNA sequences of reference strains previously published,

Fig. 3. Phylogenetic tree of the 16S rRNA gene of 19 reference strains and 16 isolates based on the UPGMA method of software MEGA4.0.

which were obtained from the database of the National Center for Biotechnology Information. 16S rDNA sequence analysis indicated that the 16 isolates clearly belong to *Lactobacillus helveticus* (IMAU60018, IMAU60116), *Lactobacillus plantarum* (IMAU60126, IMAU60042), *Lactobacillus casei* (IMAU60109) and *Lactobacillus fermentum* (IMAU60125, IMAU60078), *Lactococcus lactis* subsp. *cremoris* (IMAU60040), *Lactococcus lactis* subsp. *lactis* (IMAU60037), *Leuconostoc lactis* (IMAU60081), *Leuconostoc mesenteroides* (IMAU60052, IMAU60148), *Enterococcus durans* (IMAU60069, IMAU60110), *Lactococcus garvieae* (IMAU60022), and *Lactobacillus crustorum* (IMAU60027), showing 16S rDNA sequence similarities of over 98% together (Fig. 3). Of the 16 isolates, results of 16S rDNA sequence analysis for the 16 isolates were in agreement with results of RFLP analysis. 16S rDNA sequence analysis of the cocci isolates will be subject to further study.

Discussion

LAB have similar nutrition and growth requirements. Therefore, it is considerably difficult to identify them by classical methods (José et al., 2007). Simple and reliable methods for identification of such bacteria are required. Recently, several methods based on molecular techniques have been developed to identify LAB quickly and conveniently. These include randomly amplified polymorphic DNA analysis (Torriani et al., 1999), DNA-DNA hybridization analysis (Duan et al., 2008), restriction enzyme analysis (REA), pulsed field gel electrophoresis (PFGE), ribotyping, and phage typing (Barrangou et al., 2002; Jensen et al., 1993; Kullen et al., 2000; Ortua et al., 2007; Schleifer et al., 1995).

In this study, with the aim to develop a rapid tool for distinguishing largely unknown strains of LAB, we used two molecular techniques, PCR-RFLP and 16S rDNA sequencing, to characterize several strains of them isolated from dairy products. Initially 171 isolates of LAB, including bacilli and cocci, were analyzed by 16S rDNA RFLP, and representative strains of them were further studied by 16S rDNA sequencing. It was showed that each method has its own advantages and disadvantages. The identification results of RFLP analysis for lactobacillus were in excellent agreement with those of 16S rDNA sequencing. 16S rDNA RFLP offers an effective and rapid method for genus identification of LAB cocci, such as distinguishing *Enterococcus*,

Lactococcus and *Leuconostoc*. High sequence homologies of 16S rDNA sequences were observed among *Enterococcus* species (99.3% similarity), *Lactococcus* species (99.2% similarity) and *Leuconostoc* species (98% similarity), and the same restriction sites in those strains. Therefore, RFLP analysis failed to identify *Leuconostoc*, *Lactococcus* and *Enterococcus* to the species level. However, it is feasible to conduct species identification of *Pediococcus* (98% similarity) by RFLP analysis.

It was demonstrated that *AluI*, *HinfI* and *HaeIII* are complementary for identification of lactobacillus; thus the three enzymes could be used when one of them alone did not distinguish the strains. The results also indicated that *BsmAI*, *AluI*, *HinfI*, *TspRI* and *HaeIII* were discriminating among the restriction enzymes used in this study.

The 16S rDNA sequence results showed that it would be possible to initially discriminate species of LAB by 16S rDNA RFLP analysis. However, differentiation of species was less obvious due to the high degree of BLAST identity scores between 16S rDNA sequences of *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum* subsp. *argentoratis* and *Lactobacillus plantarum* strains, and also among some *Enterococcus* strains. The BLAST analysis of 16S rDNA for IMAU60026 and IMAU60042 indicated high species similarities of the two isolates to reference strains, *Lactobacillus pentosus* (100%), *Lactobacillus paraplantarum* (99.8%) and *Lactobacillus plantarum* (99.9%). In particular, the point mutation of C (instead of T) at position 193 of 16S rDNA distinguishes between *Lactobacillus plantarum* and *Lactobacillus pentosus*.

The BLAST analysis of 16S rDNA for IMAU60069 and IMAU60100 indicated high species similarities of them to *Enterococcus faecalis* and *Enterococcus durans* strains (99.6%). Base differences located at positions 286, 620, 787 and 835 (depending on the strain) were found between 16S rDNA sequences of the reference strains *Enterococcus faecalis* ATCC 19443^T and *Enterococcus durans* ATCC 59607^T. Other studies have shown the same results, indicating that it is difficult to identify these strains to the species level by sequencing results alone (Devriese et al., 1993). Therefore, many microbiologists suggested that it should be accompanied with phenotypic analysis, DNA-DNA hybridization and 16S rDNA sequencing to exactly identify strains to the species level (Vuyst and Vancanneyt,

2007).

It was reported that 16S rDNA sequence comparison is sometimes difficult to conduct for identification of bacteria in large-scale studies and for small laboratories because it is time-consuming and expensive (Jeffrey et al., 2004). Our study showed that the combination of PCR-RFLP analysis and 16S rDNA sequencing techniques provides a rapid, easy to perform, and effective tool for identification of LAB. Unknown strains of LAB isolated from samples can be analyzed by 16S rDNA RFLP analysis firstly, and then *Lactobacillus* species not distinguished clearly are taken into account by 16S rDNA sequencing. This method may be the direction for further identification of isolates in the study of microbiology.

However, this method only provide a preliminary identification for larger-scale strains, as for each strain's exact identification, polyphasic taxonomy method is necessary. The application of DNA-DNA hybridization, and 16S rDNA sequence analysis, in combination with physiological traits such as fructose fermentation and mannitol production, allowed us to classify the LAB isolates into the species.

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References

- Ausube, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Suppl. 27, Vol. I, Chap. II, pp. 2.4.1–2.4.5.
- Barrangou, R., Yoon, S. S., Breidt, F., Fleming, H. P., and Klaenhammer, T. R. (2002) Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. *Appl. Environ. Microbiol.*, **68**, 2877–2884.
- Devriese, L. A., Pot, B., and Collins, M. D. (1993) A review. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species group. *J. Appl. Bacteriol.*, **75**, 399–408.
- Duan, Y. H., Tan, Z. F., Wang, Y. P., Li, Z. W., Li, Z. Y., Qin, G. Y., Huo, Y. P., and Cai, Y. M. (2008) Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese. *J. Gen. Appl. Microbiol.*, **54**, 51–60.
- Giraffa, G., Lazzi, C., Gatt, M., Rossetti, L., Mora, D., and Neviani, E. G. (2003) Molecular typing of *Lactobacillus delbrueckii* of dairy origin by PCR-RFLP of protein-coding genes. *Int. J. Food Microbiol.*, **82**, 163–172.
- Gurtler, V., Wilson, V. A., and Mayall, C. (1991) Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J. Gen. Microbiol.*, **137**, 2673–2679.
- Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J., and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am. J. Clin. Nutr.*, **73**, 365S–373S.
- Huys, G., Vanhoutte, T., Marie, J., Mahious, A. S., Brandt, E. D., Vermeire, S., and Swings, J. (2007) Coamplification of eukaryotic DNA with 16S rRNA gene-based PCR primers: Possible consequences for population fingerprinting of complex microbial communities. *Curr. Microbiol.*, **56**, 553–557.
- Jeffrey, E. C., Cory, E. R., Sanjay, K. S., and Kurt, D. R. (2004) Rapid molecular diagnosis of *Lactobacillus bacteremia* by terminal restriction fragment length polymorphism analysis of the 16S rRNA gene. *Clin. Med. Res.*, **2**, 37–45.
- Jensen, M. A., Webster, J. A., and Straus, N. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.*, **59**, 945–952.
- Johansson, M. L., Quednau, M., Ahrne, S., and Molin, G. (1995) Classification of *Lactobacillus plantarum* by restriction endonuclease analysis of total chromosomal DNA using conventional agarose gel electrophoresis. *Int. J. Syst. Bacteriol.*, **45**, 670–675.
- José, L. B., Ignacio, D. B., Imanol, R. Z., Daniel, V., Olivia, G., and José, L. M. (2007) Sequencing of variable regions of the 16S rRNA gene for identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids. *Comp. Immunol. Microbiol. Infect. Dis.*, **30**, 111–118.
- Kullen, M. J., Sanozky-Dawes, R. B., Crowell, D. C., and Klaenhammer, T. R. (2000) Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. *J. Appl. Microbiol.*, **89**, 511–516.
- Luo, Z., Shi, Y., Yu, L., and Han, B. (2005) Study on microbiological and chemical composition of Tibetan yoghurt. *China Brew.*, **10**, 40–41.
- Mora, B., Fortina, M. G., Nicastro, G., Parini, C., and Manachini, P. L. (1998) Genotypic characterisation of thermophilic bacilli: A study on new soil isolates and several reference strains. *Res. Microbiol.*, **149**, 711–722.
- Ortua, S., Felisa, G. E., Marzottob, M., Deriua, A., Moliccottia, P., Sechia, L. A., Dellagliob, F., and Zanettia, S. (2007) Identification and functional characterization of *Lactobacillus* strains isolated from milk and Gioddu, a traditional Sardinian fermented milk. *Int. Dairy J.*, **17**, 1312–1320.
- Randazzo, C. L., Restuccia, C. A., Romano, A. D., and Caggia, C. (2004) *Lactobacillus casei*, dominant species in natu-

- rally fermented Sicilian green olives. *Int. J. Food Microbiol.*, **90**, 9–14.
- Schleifer, K. H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W., and Amann, R. (1995) Application of molecular methods for the classification and identification of lactic acid bacteria. *Int. Dairy J.*, **5**, 1081–1094.
- Stiles, M. E. and Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.*, **36**, 1–29.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**, 1596–1599.
- Teanpaisan, R. and Dahlen, G. (2006) Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. *Oral Microbiol. Immunol.*, **21**, 79–83.
- Torriani, S., Zapparoli, G., and Dellaglio, F. (1999) Use of PCR-based methods for rapid differentiation of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. *Appl. Environ. Microbiol.*, **65**, 4351–4356.
- Vuyst, L. D. and Vancanneyt, M. (2007) Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiol.*, **24**, 120–127.
- Wu, T. (2001) The Qinghai-Tibetan plateau: How high do Tibetans live? *High Alt. Med. Biol.*, **2**, 489–499.
- Yanagida, F., Chen, Y., and Shinohara, T. (2005) Isolation and characterization of lactic acid bacteria from soils in vineyards. *J. Gen. Appl. Microbiol.*, **51**, 313–318.
- Yanagida, F., Chen, Y., and Yasaki, M. (2007) Isolation and characterization of lactic acid bacteria from lakes. *J. Basic Microbiol.*, **47**, 184–190.
- Zhou, J. Z., Bruns, M. A., and Tiedje, J. M. (1996) DNA recovery from soil of diverse composition. *Appl. Environ. Microbiol.*, **62**, 316–322.