ISOLATION AND IDENTIFICATION OF SOME *LACTOBACILLUS* SP. STRAIN FROM TRADITIONAL FERMENTED FOODS

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ABSTRACT

Recent publications showed that Lactic acid bacteria (LAB) are used extensively to inhibit growth of spoilage and pathogenic bacterial strains. That is being applied in food processing such as tradition fermented food or dairy, beverage and meat products. Lactic acid bacteria can produce a variety of antibacterial agents including bacteriocin, diacetyl, etc. Thus, the isolation, identification and taxonomical characterization of each new Lactobacillus sp. strain is being more and more required. However, the large number of species in the genus Lactobacillus almost have their high phenotypic and physiological similarity which easily leads to misidentification.

The present study was aimed for isolation and reliable identification of Lactobacillus sp. strains from some traditional fermented foods by on the basis of phenotypic analysis and combination of PCR and sequencing of target sequences base on 16S-23S rRNA gene. Eight strains of LAB were isolated and characterized through morpholigical, physiological, biochemical and carbohydrate fermentation tests. All of them were determined as Lactobacillus sp. Moreover, the nucleotides sequences of 16S-23S rDNA of them was compared and phylogenetic analysis to those of Lactosbacillus species in GenBank and the results confirm that four strains: L1, L3, L4 and L7 belong to Lactobacillus platarum and four strains: L2, L5, L6 and L8 belong to species L. rhamnosus

***Keywords**: Lactic acid bacteria (LAB), Lactobacillus platarum, 16S-23S rRNA inter spacer region*

1. Introduction

Lactic Acid Bacteria (LAB) that belong to *Lactobacillaceae* family, are widely distributed in nature such as soil, water, intestinal tract of animals and human,... etc and are usually isolated in some kinds of traditional fermented foods such as kim chi, pickles, yogurt[11.13]. Interest in the lactobacilli has been stimulated in recent years by the use of these bacteria in products that are claimed to confer health benefic on the consumer (probiotic). Actually, LAB have been widely applied in food processing, food preservation, probiotic production and aquaculture as well [7,13,14,21].

In recent years, a number of new species of lactobacilli have been described. Among lactobacilli, we focus on *L. plantarum* as its widely isolated from fermented foods. This bacterium still can grow at 15°C but not at 45°C and is commonly found in many fermented food products. Also, the high levels of this organism in food makes it an ideal candidate for the development of probiotics. Especially, *L. plantarum* has significant antioxidant activities and also helps to maintain the intestinal permeability [0] as well as suppresses the growth of gas-producing bacteria in the intestines [4].

General, the identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for

example, cell wall analysis and electrophoretic mobility of lactate dehydrogenases) [12]. In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level [10]. Furthermore, the phenotypic analysis represents the most tedious task during the process of microorganism identification, as it requires time, skills, and, in order to avoid subjective observation, technical standardization [20]. Moreover, especially for LAB, similar nutritional requirements of different species due to adaptation to a particular environment hamper identification by traditional methods [2,9].

The derivation of simple yet rapid identification methods is therefore required in order to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of ecosystems such as the intestinal tract, silage and food products.

Genotype-based methods are useful to identify bacteria as a complement or alternative to phenotypic methods. Identification based on PCR amplification of targeted genes is a reliable technique. Several bacterial species, including LAB, identification methodologies using primers that target different sequences, such as the 16S rRNA, 16-23S rRNA intergenic spacer regions (ISR), as well as 23S rRNA genes have been reported so far.

16S rRNA genes are common used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene [5,25]. In addition to highly conserved regions, 16S rRNA gene sequences also contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification [15,19]. As a result, 16S rRNA gene sequencing has become prevalent in bacterial identification as a rapid and cheap alternative to phenotypic methods [6]. Considering to LAB, nucleotide base sequences of *Lactobacillus* 16S DNA provide an accurate basis for phylogenetic analysis and identification [1,8,24]. Although the species-specific sequences are contained in the first half of the 16S rRNA gene (V1-V3 region), identification is more accurate if the whole gene is sequenced. This means that about 1500 bp of DNA would have to be sequenced. Furthermore, using 16S rRNA gene is sometime insufficient to distinguish closely related species in lactobacilli genus [18,23].

The DNA encoding the 23S rRNA subunit represents a large (ca. 3300 nucleotides) source of genomic information than the 16S one (ca. 1650 nucleotides), but due to its length, not very abundant sequences are available in databases.

In case of 16-23S rRNA ISR, this sequence is much more variable than that of the 16S rRNA structural gene, both in size and sequence, even within closely related taxonomic groups which makes it a suitable target for typing microbial populations using species-species primers [16,17,22].

We therefore carried out this study in order to isolate and identify *Lactobacillus* spp. commonly isolated from fermented foods. Then the work was to identifiy *L. plantarum*, as a first stage in order to develop the application of lactic acid bacteria isolated from local products.

2. Materials & methods

Samples

From January/2012 to March/2013, we collected the conventional fermented products such as kimchi, some types of pickles which were sold in the markets and supermarkets on HCM city and Binhduong province.

Lactic Acid Bacteria (LAB) isolation

The samples were ground and dissolved in physiological saline solution (NaCl 0.85%) at ratio 1:10. Then, the LAB isolation was carried out by streaking on MRS agar medium plates before on inoculating at 37°C for 48-96 h. The determination of pure strains of *Lactobacillus* sp. were based on colony morphology as small, round and smooth, raised colonies. The typical colonies were obtained and pure cultured on MRS agar medium.

Reference strain of *L. plantarum* was acquired from the Microbiotechnology

laboratory, HoChiMinh city Open University.

Phenotypic identification of LAB

Pure bacterial strains were performed in phenotypic identification following the bacteria's morphological observation, physiological and biochemical characterization according to Bergey's manual. Those were Gram stain appearance, lytic $CaCO_3$ test, catalase test, lactic acid test and determination of carbohydrate fermentation products. The growth ability of lactobacilli was trained at 15°C and 45°C on MRS medium. Consequently, the selected strains were used for the milk-precipitation test and the ability to resistance of *Escherichia coli*.

DNA extraction

Growth from pure cultures on Lactobacilli MRS agar plates was used to prepare a heavy suspension of cells in 1 ml of sterile deionized water. The suspensions were centrifuged at 13,000 × *g* (5 min) and washed with 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.5) buffer. The pellets were resuspended in 500 µl of TE buffer and added proteinase K (20 mg/mL), vortexing vigorously for lysis. Five hundred microliters of this crude DNA solution was extracted sequentially with 500 µl of TE-saturated phenol: chloroform (1:1). The DNA was precipitated overnight by the addition of 2 volumes of cold ethanol 99% and a 0.1 volume of 3 M sodium acetate at −20°C. The preparations were centrifuged at 13,000 × *g* (20 min, 0°C) then washed in 500 µl of cold ethanol 70%. The pellets were dried at room temperature before dissolving in 20 µl of TE buffer (pH 7.5). The amount and purity of DNA were determined by absorbance at 260 nm and 280 nm using UV-spectrophotometer.

PCR amplification of 16S -23S intergenic spacer region (ISR) sequence

Two 16-23S ISRs from each isolate were amplified by using primers [17,22] that annealed to two conserved regions of the 16S and 23S genes: with the L16RNAF primer (5'-GAATCTTCCACAATGGACG-3') and L16RNAR primer (5'-CGC TTTACGCCCAATAAATCCGG-3') corresponding to nucleotides 387 to 603 and other PL3F primer (5'-TAGGAACCAGCCGCCTAAG-3') and PL3R primer (5'-CGGTGTTCTCGGTTTCATTA-3'), corresponding to nucleotides 1497 to 1702 of the 16-23S intergenic spacer regions according to *Lactobacillus plantarum* sequence numbering NC_017482 (GenBank, NCBI).

A reaction mixture (25 µl) consisted of reaction buffer (10 mM Tris-HCl at final concentration, 3mM $MgCl_2$ concentration, and 50 mM KCl, pH 8.3), a 200 µM concentration of each deoxynucleoside triphosphate, 10 pmol of each primer, 50 ng of bacterial DNA (extracted from pure cultures as described above), and 1.75 U of *Taq* DNA polymerase (Fermentas). The amplification program was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 30 s at the appropriate annealing temperature (L16RNAF-L16RNAR primer pair at 55°C and PL3F-PL3R primer pair at 59°C), and 72°C for 40 s. A cycle of 72°C for 10 min concluded the program. Amplification products were detected by agarose gel electrophoresis (5 µl of PCR mixture, 2% agarose gel), ethidium bromide staining, and UV transillumination.

PCR sequencing and phylogenetic analysis

PCR sequencing was performed by Nam Khoa Biotek. Consequently, proof-reading wqas carefully carried by using some professional solfwares (Chromas Pro 2.0.4; Seaview; Blast tool that integrated in NCBI) before phylogenetic analysis was carried out by using Mega 5.2. The reference sequences that were collected from Genbank including *L. plantarum* (NC_017482, NC_012984.1, NC_004567.2, CP004082.1, CP002222.1); *L.acidophilus* (CP000033.3, CP002559.1), *L. bulgaricus* (CR954253.1, CP000412.1, CP000156.1, CP002341.1), *L. rhamnosus* (AP011548, NC_017482), *L. casei* (NC_018641, P000423) , *L. crispatus* (NC_014106), *L. johsonii* (NC_005362), *L. gasseri* (CP000413), *L. helveicus* (CP003799), *L. sakei* (CR936503) and the outgroups were *E. coli* (NC_011415.1), *B. cereus (NC_004722.1), Salmonella* sp. (AE014613.1).

3. Results & discussion

Lactic Acid Bacteria (LAB) isolation and phenotypic identification

As a result, somehow, MRS is suitable selective to quantify lactobacilli (Figure 1A). Amining to select few promosing isolates, only 20 randomly picked colonies and purified isolates were selected from many strains of lactic acid bacteria homofermentative, Gram-positive and catalase-negative. Among 20 colonies, 8 colonies were randomly selected and the phenotypic characterization and PCR were performed.

All eight random selected isolates were able to grow on MRS agar at 37°C as well as 15°C, but at 45°C, only four isolates (L2, L5, L6, L8) were able to grow (Table 1). All of them were found catalase-negative, lytic $CaCO_3$ positive (Figure 1B), Gram-positive rods (Figure 1C), producing no gas and acid from ribose. The results of phenotypic characterization suggest the presumptive *Lactobacillus* species as *L. plantarum* (L1, L3, L7 isolates), *L. paraplantarum* (L4 isolate) and *L. rhamnosus* (L2, L5, L6, L8 isolates). Those of carbohydrate fermentation results are given in Table 1.

Figure 1. Lactic Acid Bacteria (LAB) isolation and phenotypic identification

Note: (a) Morphological Lactobacillus sp. on MRS plate; (b) lytic $CaCO_3$ test; (c) Gram stain

Table 1. The results of carbohydrate fermentation tests and resistant teperature test at 15°C, 45°C

Isolate	15°C/45°C	Amygdalin	Arabiose	Cellobiose	Maltose	Mannitol	Manose	Melibiose	Melizitose	Raffinose	Sorbotol	Sucrose	Xylose	Presumptive identification
L1	+/-	+	+	+	+	+	+	+	+	+	+	+	?	*L. plantarum*
L2	+/+	+	?	+	+	+	+	+	+	-	-	+	+	*L. rhamnosus*
L3	+/-	+	+	+	+	+	+	+	+	+	+	+	?	*L. plantarum*
L4	+/-	+	+	+	+	+	+	+	+	+	+	+	?	*L. plantarum/L. paraplantarum*
L5	+/+	+	?	+	+	+	+	+	-	-	+	+	-	*L. rhamnosus/*
L6	+/+	+	?	+	+	+	+	+	-	-	+	+	-	*L. rhamnosus*
L7	+/-	+	+	+	+	+	+	+	+	+	+	+	?	*L. plantarum*
L8	+/+	+	?	+	+	+	+	+	-	-	+	+	-	*L. rhamnosus*

Note: - means negative result, + means positive result,? means doubtful result

According to the phenotypic characterization, for all 8 strains, a clear species assignment was not possible because of the doubtful results due to the limitation of the used carbonhydrate tests. Although phenotypic techniques such as the API 50 CHL (BioMerieux) system are still being taken as poweful tools capable of discrimination among the species of *Lactobacillus*, but it is costly and it requires time. So, the use of molecular techniques for *Lactobacillus* taxonomy has become the backbone for a

reliable identification.

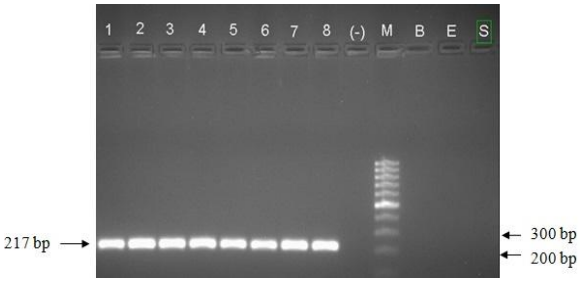
PCR for lactobacilli grouping and discrimination of *L. plantarum*

The phenotypic characterizaton corroborates with lactobacilli genus PCR (using L16RNAF - L16RNAR primers) and species-species PCR (using PL3F - PL3R) for the *L. plantarum* (Table 2).

As the result, PCR products generated the following expected for lactobacilli representative reported in Fig. 2: ca. 217 bp, whereas no PCR products obtained from any reference strains *Bacillus cereus*, *Escherichia coli* and *Salmonella* sp. Those results were completely compatible with Song *et al*. (2000)[22] due to the primer selecting. Lactobacilli genus grouping primers [2] were chosen on the regions showing sequence homogeneity among lactobacilli strains grouped together but characterized by a high level of heterogeneity with other genus.

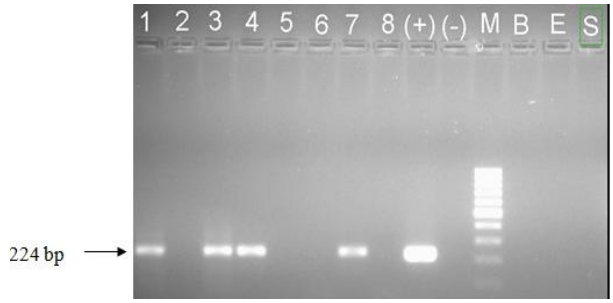
Individual *L. plantarum* strains were successful detected and discriminated with other lactobacilli strains by the second PCR using species-species primers [17]. As the result, PCR products generated the following expected for only four presumptive *L. plantarum* (L1, L3, L7) or *L. paraplantarum* (L4) reported in Fig. 3: ca. 227 bp, whereas no PCR products obtained from any other lactobacilli strain or reference strains *Bacillus cereus*, *Escherichia coli* and *Salmonella* sp. This also means that the PL3F - PL3R primers can not discriminate *L. plantarum* and *L. paraplantarum* by PCR product observation.

Figure 2. PCR products obtained from lactic acid bacteria isolates using L16RNAF - L16RNAR primers



Note: Lane 1 – 8: eight presumptative Lactobacillus spp L1 - L8; M: 100 bp ladder; (-): negative control, B: Bacillus cereus, E: Escherichia coli, S: Salmonella sp

Figure 3. PCR products obtained from lactic acid bacteria isolates using PL3F - PL3R



Note: Lane 1 – 8: eight presumptative Lactobacillus spp L1 - L8; M: 100 bp ladder; (+) positive control, reference L. plantarum; (-): negative control, B: Bacillus cereus, E: Escherichia coli, S: Salmonella sp

Sequence analysis of the 16-23S rRNA ISR and phylogenetic grouping

In order to confirm the specificity of PCR products and aim to discriminate between *L. plantarum* and *L. paraplantarum*, both polynucleotide strands of the purified DNA were sequenced, using both primer pairs: L16RNAF - L16RNAR and PL3F - PL3R for two groups of PCR products L2, L5, L6, L8 and L1, L3, L4, L7, respectively.

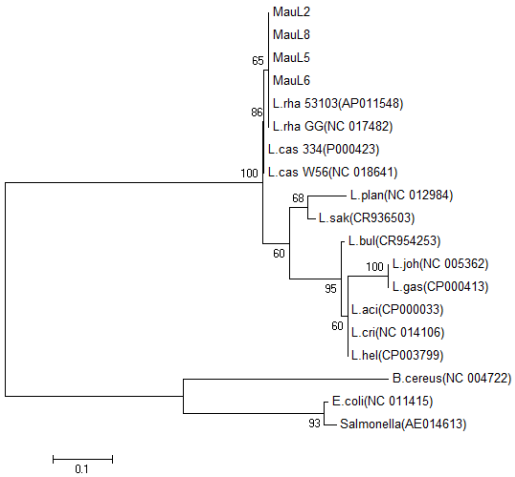
The sequencing results showed that the quality scores were so good, clearly chromatography of each sequence, unique peak at each nucleotide and completely match between two strands of each PCR product sequences by proof-reading analysis (data not shown). Using these sequences after proof-reading for analysis, the comparison of 16-23S rRNA ISR sequences held in Genbank showed that all were greater than 98% similarity with other lactobacilli references (data not shown). We therefore constructed the phylogenetic analysis afater manual adjustment of the nucleotide sequence alignment.

The phylogram constructed from 16-23S rRNA ISR sequences amplified by L16RNAF - L16RNAR primers clearly distinguished four L2, L5, L6, L8 sequences grouping with two *L. rhamnosus* references (AP011548 and NC017482) with other lactobacilli and eubacteria representatives (Fig. 4). Especially, values for bootstraping were convinced as 86% between L2, L5, L6, L8, *L. rhamnosus* with two *L. casei* references (P000423 and

NC018641); and 100% between those L2, L5, L6, L8, *L.* rhamnosus, *L. casei* with other remained representatives (Fig. 4). This also means that the amplified sequence between L16RNAF - L16RNAR primers can be used to discriminate each lactobacilli strain and deduce that L2, L5, L6, L8 as *L. rhamnosus* species.

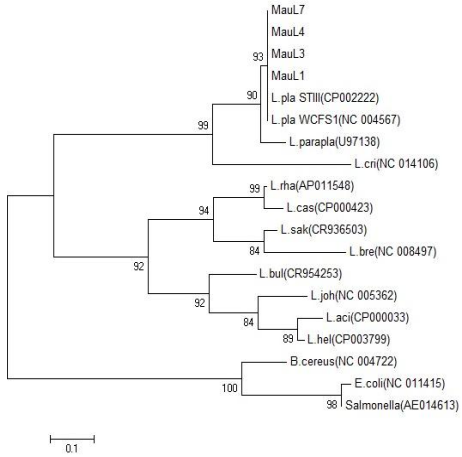
Considering to discriminate between *L. plantarum* and *L. paralantarum*, the phylogram constructed from 16-23S rRNA ISR sequences amplified by PL3F - PL3R primers clearly grouped four L1, L3, L4, L7 sequences with two *L. plantarum* references (CP002222 and NC004567) in monophyletic clade and distinguished this group with *L. paraplantarum* reference (U97138) as well as other lactobacilli and eubacteria representatives (Fig. 5). Especially, values for bootstraping were very convinced as 90% between L1, L3, L4, L7, *L. plantarum* with *L. paraplantarum*; and greater than 90% between those L1, L3, L4, L7, *L. plantarum*, *L. paraplantarum* with other remained representatives (Fig. 5). This also means that the amplified sequence using PL3F - PL3R primers can be used to discriminate *L. plantarum* and *L. paraplantarum* and deduce that L1, L3, L4, L7 as *L. plantarum* species.

Figure 4. Phylogenetic tree showing the relative positions of 12 *Lactobacillus* and 3 other referecen strains representing L2, L5, L6, L8 species based on 16-23S rRNA ISR sequence.



Note: Bar, 0.1 nucleotide substitution per site; Maximum likelihood tree was shown.

Figure 5. Phylogenetic tree showing the relative positions of 12 *Lactobacillus* and 3 other referecen strains representing L1, L3, L4, L7 species based on 16-23S rRNA ISR sequence.



Note: Bar, 0.1 nucleotide substitution per site; Maximum likelihood tree was shown.

Finally, the combination of phenotypic, PCR, phylogenetic results was shown on table 2.

Table 2. Phenotypic, PCR and phylogenetic identification of lactic acic bacteria (LAB) isolated from fermented foods and reference *L. plantarum*

Isolates	Phenotypic characterization	PCR		Phylogenetic analysis
		LAB genus	*L. plantarum*	
L1	*Presumptive L. plantarum*	*Lactobacillus* spp	*L. plantarum*	*L. plantarum*
L2	*Presumptive L. rhamnosus*	*Lactobacillus* spp	***ND***	*L. rhamnosus*

Isolates	Phenotypic characterization	PCR		Phylogenetic analysis
		LAB genus	*L. plantarum*	
L3	*Presumptive L. plantarum*	*Lactobacillus* spp	*L. plantarum*	*L. plantarum*
L4	*Presumptive L. plantarum/L. paraplantarum*	*Lactobacillus* spp	*L. plantarum/L. paraplantarum*	*L. plantarum*
L5	*Presumptive L. rhamnosus/*	*Lactobacillus* spp	***ND***	*L. rhamnosus*
L6	*Presumptive L. rhamnosus*	*Lactobacillus* spp	***ND***	*L. rhamnosus*
L7	*Presumptive L. plantarum*	*Lactobacillus* spp	*L. plantarum*	*L. plantarum*
L8	*Presumptive L. rhamnosus*	*Lactobacillus* spp	***ND***	*L. rhamnosus*
L. plantarum (reference)	*L. plantarum*	*Lactobacillus* spp	*L. plantarum*	

Note: ND means not detected.

4. Conclusion

Our study have shown that we were successful in lactobacilli identification, especially, identification of *L. plantarum* in discrimination with *L. paraplantarum* by combination of some simple phenotypic characterization with genotype-based method.

Eight strains of lactic acid bacteria were isolated and characterizated through morpholigical, physiological, biochemical and carbohydrate fermentation tests. All of them were determinated as *Lactobacillus* sp. Moreover, the nucleotides sequences of 16S-23S rDNA of them was phylogenetic analysis to those of *Lactosbacillus* species in GenBank and the results confirm that four strains: L1, L3, L4 and L7 belong to *Lactobacillus platarum* and four strains: L2, L5, L6 and L8 belong to species *L. rhamnosus*.

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