International Journal of Food Microbiology 186 (2014) 61–67

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Lactic acid bacterial population dynamics during fermentation and storage of Thai fermented sausage according to restriction fragment length polymorphism analysis

Amornrat Wanangkarn [a], Deng-Cheng Liu [b], Adisorn Swetwiwathana [c], Aphacha Jindaprasert [c], Chirapiphat Phraephaisarn [d], Wanwisa Chumnqoen [b], Fa-Jui Tan [b,*]

[a] Department of Agricultural Science, Naresuan University, Phitsanulok 65000, Thailand
[b] Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan
[c] Faculty of Agroindustry, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand
[d] Research and Development, Betagro Science Center Company Limited, PathumThani 12120, Thailand

ARTICLE INFO

Article history:
Received 11 August 2013
Received in revised form 9 June 2014
Accepted 14 June 2014
Available online 26 June 2014

Keywords:
Fermented sausage
Lactic acid bacteria
Mum
Restriction fragment length polymorphism (RFLP)

ABSTRACT

This study applied restriction fragment length polymorphism (RFLP) analysis to identify the lactic acid bacteria (LAB) isolated from "mum" Thai fermented sausages during fermentation and storage. A total of 630 lactic acid bacteria were isolated from the sausages prepared using 2 methods. In Method 1, after stuffing, the sausages were stored at 30 °C for 14 days. In Method 2, after stuffing and storage at 30 °C for 3 days, the sausages were vacuum-packed and stored at 4 °C until Day 28. The sausages were sampled on Days 0, 3, 14, and 28 for analyses. The 16S rDNA was amplified and digested using restriction enzymes. Of the restriction enzymes evaluated, *Dde* I displayed the highest discrimination capacity. The LAB were classified and 7 species were identified For Methods 1 and 2, during fermentation, the *Lactobacillus sakei* and *Lactobacillus plantarum* species were dominant. For Method 2, the proportion of *Leuconostoc mesenteroides* markedly increased during storage, until *L. sakei* and *Ln. mesenteroides* represented the dominant species. The identification of LAB in the sausage samples could facilitate the selection of appropriate microorganisms for candidate starter cultures for future controlled mum production.

1. Introduction

"Mum," a traditional Thai fermented sausage, is widely produced and commonly consumed in the northeastern region of Thailand. During the preparation process, ingredients such as beef, liver, spleen, ground-roasted rice, salt, and garlic are mixed and stuffed into a natural casing, and subsequently stored at room temperature for 2 or 3 days for the development of the appropriate sour flavor (Thai Industrial Standards Institute, 2003). Traditionally, mum sausage is produced using naturally occurring lactic acid bacteria (LAB), often resulting in products with inconsistent qualities. These microfloras might consist of microorganisms beneficial to the development of the fermentation flavor and the product texture; however, they might also include spoilage species or pathogenic microorganisms (Talon et al., 2007). The raw materials used, the manufacturing techniques, and the agroecosystem of the area of production can all influence the specific characteristics and qualities of the final products (Albano et al., 2007). Therefore, understanding the microbial ecology of the fermented products is critical for the evaluation of the physicochemical and sensory changes during fermentation and maturation (Comi et al., 2005).

Lactobacilli, *Pediococci*, and *Micrococci* are the most commonly occurring bacteria in Thai fermented meat products; however, the specific influence of these bacteria on product quality has not yet to be fully elucidated (Thiravattanamontri et al., 1998). LAB cultures could ensure greater consistency in the quality and safety of products by inactivating spoilage and pathogenic microorganisms as a result of competitive growth and organic acid production, mainly lactic acid (Ammor and Mayo, 2007). Lactobacillus is commonly present as a dominant species in fermented food and selectively used as a starter culture to improve texture, flavor, taste, and aroma (Bernardeau et al., 2006). Additionally, LAB can also produce some bacteriocins which can be used as natural food preservatives to prevent the growth of pathogens in fermented meat product (Noonpakdee et al., 2003). Probiotic strains of lactobacilli were generally considered as beneficial probiotic organisms which provide consumers a health benefit (Bernardeau et al., 2008). Many advantages, such as being well adapted to the ecology of meat fermentation, against bacteria contamination and absence of decarboxylase activity to obtain fermented sausages free from biogenic amines have been illustrated (Leroy et al., 2006; Latorre-Moratall et al., 2012; Stadnik and Dolatowski, 2010). Talon et al. (2008) commented that the LAB

* Corresponding author. Tel.: +886 4 22870613; fax: +886 4 22860265.
E-mail address: tanfj@dragon.nchu.edu.tw (F.-J. Tan).

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.06.015
0168-1605/

originating from fermented meats could be used as starter cultures; however, before being selected as a starter culture for food fermentation, the microorganism should be assessed to determine if it exhibits some undesirable properties, such as opportunistic infections, productions of toxic metabolites and antibiotic resistance (Bourdichon et al., 2012). Improving the qualities of the mum sausage products, and extending their shelf-lives, is of considerable benefit and interest to mum manufacturers to fulfill consumer demands and potentially increase the market. Because of certain unique properties of *L. sakei* and *L. plantarum*, such as superior proteolytic abilities, influence on flavor development, and pH-reducing effects, these bacterial species are frequently used as starter cultures during the manufacturing of fermented products (Sriphochanart and Skolpap, 2010).

Because of the crucial role of LAB in meat fermentation, the identification of suitable starter LAB cultures becomes very critical. Previous studies have applied molecular techniques, based on DNA or RNA sequences, for accurate and efficient microbial identification. Polymerase chain reaction linked with restriction fragment length polymorphism analyses (PCR-RFLP) has been applied to differentiate LAB in several studies (Chenoll et al., 2003; Christensen et al., 2004; Claisse et al., 2007; Yanagida et al., 2008). In our study, we aimed to (1) identify the dominant LAB species and evaluate the variations in LAB community composition in Thai fermented (mum) sausages manufactured using the conventional processing method (stored at 30 °C until Day 14) or a modified processing method (fermented at 30 °C until Day 3, then vacuum-packed and stored at 4 °C until Day 28), and (2) evaluate the feasibilities and efficiencies of restriction enzymes used for the differentiation of the LAB in restriction fragment length fragment polymorphism (RFLP) analysis.

2. Materials and methods

2.1. Sausage preparation and sampling

Sausage preparation was conducted according to the methods of Wanangkarn et al. (2012). Freshly-manufactured mum sausages, prepared using conventional techniques by mixing minced beef (60% *w*/*w*), minced bovine liver (15% *w*/*w*), minced spleen (15% *w*/*w*), roasted rice powder (4.2% *w*/*w*), garlic (4.2% *w*/*w*), salt (1.6% *w*/*w*), and spices and seasonings (trace amounts), and then stuffing the mixture into bovine intestine casings (approximately 3.0 cm in diameter, 15 cm in length, weighing 250–300 g each), were collected from a local meat factory (Chaiyaphum, Thailand) for three times during 03/2010–05/2010 with three replicates. Samples were stored in a temperature-insulated container and transported to the laboratory within 4 h and further processed using 2 methods. In Method 1, the conventional method, after collection, the sausages were hung vertically on stainless steel hangers at 30 ± 2 °C and 65% ± 2% relative humidity until Day 14 without any packaging. In Method 2, the modified method, after collection, the sausages were hung vertically on stainless steel hangers at 30 ± 2 °C and 65% ± 2% relative humidity until Day 3. They were then vacuum-packed (laminated nylon/LLDPE, Chun I Gravure Co., Ltd, Taichung, Taiwan), and stored at 4 °C until Day 28. The sausages were sampled on Day 0 (after stuffing), Day 3 (end-fermentation), Day 14 (end-ripening), and Day 28 (storage) for analyses (Gonzalez-Fernandez et al., 2006; Wanangkarn et al., 2012).

2.2. Isolation of the LAB

Samples (25 g) were aseptically placed in sterile bags, each containing 225 mL of a 0.85% NaCl solution, and homogenized using a stomacher (Oskon Co., Ltd, Thailand) for 2 min. Serial dilutions were prepared and poured into a Man Rogosa Sharpe (MRS) agar (Merck, Dram Stadt, Germany), and incubated at 37 °C for 48 h in an anaerobic jar (BBL GasPak System, USA) (APHA, 2001). A total of 630 lactic acid bacteria [3 sampling times (03/2010–05/2010) × 3 replicates × 10 colonies × 7 stages (Days 0, 3, and 14 for Method 1 and Days 0, 3, 14 and 28 for Method 2)] were sampled. At each stage, 90 colonies with a clear-zone surrounding were randomly selected using the Harrison disc method (Harrigan, 1998), and purified by successive streaking on MRS agar plates. The selected colonies were transferred to a MRS broth and stored as liquid cultures with 30% (*v*/*v*) glycerol as a cryoprotectant at −80 °C prior to molecular analysis (Papamanoli et al., 2003).

2.3. Extraction and preparation of genomic DNA from LAB isolates

The LAB isolates were cultured in the MRS broth at 30 °C for 12 h. Genomic DNA of the isolates was then extracted using a GF-1 bacterial DNA extraction kit (Vivantis Technologies, Selangor DarulEhsan, Malaysia) according to the manufacturer's instructions. The extracted genomic DNA from each LAB isolate was analyzed using horizontal gel electrophoresis (Bio-RAD, CA, USA) with a 1.5% (*w*/*v*) agarose containing 0.5 μg/mL ethidium bromide in a 1 × Tris-borate-EDTA (TBE) buffer at 100 V for 20 min. The gel was visualized and photographed using an ultraviolet transilluminator (ChemiDoc™ MP System, Bio-RAD, CA, USA). The DNA concentration and purity were also determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). The DNA preparations were stored at −20 °C until use.

2.4. Amplification of the 16S rDNA of LAB isolates

The DNA from the −20 °C stock was diluted to 100 ng/μL using nuclease-free double-distilled water. The 16S rDNA fragment was amplified using the PCR, with a universal primer set containing BSF8/20 (5′-AGAGTTTGATCCTGGCTCAG-3′) as a forward primer and REVB (5′-GGTTACCTTGTTACGACTT-3′) as a reverse primer (Weisburg et al., 1991), in a PTC-200 Thermo Cycler (MJ Research Inc, Watertown, MA, USA). All reagents used during PCR amplification were purchased from Fermentas International Inc. (Ontario, Canada). The amplifications were performed in 50 μL reaction volumes, and each PCR reaction mixture consisted of 250 μM of each dNTP, 10 μM of each primer (BioDesign Co., Ltd, Pathumthani, Thailand), 50 mM of $MgCl_2$, 5 μL of a 10× PCR buffer, 5 U Taq polymerase, and 5 μL of the template DNA. The thermal cycling included an initial denaturation step at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, an annealing step at 50 °C for 1 min, an extension step at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were analyzed using 1.5% (*w*/*v*) agarose gel electrophoresis. The gel was visualized and photographed using the ultraviolet transilluminator.

2.5. Restriction analyses of the 16S rDNA PCR products

The 16S rDNA PCR product from each LAB isolate was cleaved using an individual restriction endonuclease enzyme: *Dde* I (C/TNAG), *Alu* I (AG/CT), *Mse* I (TT/AA), or *Aci* I (AA/CGTT), according to the methods by Bonomo et al. (2008), Claisse et al. (2007), and Rodas et al. (2003). Following the procedures specified by Fermentas International Inc. (Ontario, Canada), the restriction endonuclease reaction mixture was mixed gently and spun down for a few seconds. The reaction mixture was then incubated at 37 °C for 3 h and the restriction patterns were analyzed using 2% (*w*/*v*) agarose gels in 1× TBE buffer at 100 V for 45 min [horizontal gel electrophoresis (Bio-RAD, California, USA)] with a DNA ladder (GeneRuler™ 100 bp DNA ladder). The gel was visualized and photographed using the ultraviolet transilluminator.

2.6. 16S rDNA sequencing

A representative of each RFLP pattern group was randomly selected for sequence analysis. Prior to sequencing, the 16S rDNA was purified using a QIAquick PCR Purification Kit/250 (QIAGEN GmbH, Hilden, Germany). The same primers used in the amplification steps were

used for the sequencing of both strands of the PCR products. The sequencing reactions were analyzed using a MegaBACE 1000 sequencer (BioDesign Co., Ltd, Pathumthani, Thailand). The isolates were identified through a search of the GenBank DNA database (http://www.ncbi.nlm.nih.gov/BLAST) using the basic local alignment search tool (BLAST). The phylogenetic and molecular evolutionary analyses were conducted with the MEGA software, using the neighbor-joining method and the Kimura 2-parameter model with 1000 bootstrap replicates for the construction of distance-based trees (Antara et al., 2002).

3. Results and discussion

3.1. Extraction of genomic DNA and amplification of the 16S rDNA of LAB isolates

In this study, we randomly selected the LAB isolates from the agar plates and subjected them to molecular analysis, with each colony providing DNA templates to obtain 16S rDNA amplification fragments (Fig. 1). We extracted the genomic DNA from the LAB isolates using commercially available DNA extraction kits (Atshan and Shamsudin, 2011), and subjected all DNA samples to PCR amplification, using universal BSF8/20 and REVB primers to amplify the 16S rDNA. As shown in Fig. 1(b), we positively identified a PCR amplification product of approximately 1500 bp, which supported the results of previous studies (Rattanachomsri et al., 2011; Shukla and Goyal, 2011; Singh and Ramesh, 2009).

3.2. Restriction analyses of the 16S rDNA PCR products

We classified the 16S rDNA amplification fragments of the LAB isolates using RFLP analysis with an individual restriction enzyme (*Aci* I, *Alu* I, *Mse* I, or *Dde* I). As shown in Fig. 2(a), we observed that the *Aci* I restriction enzyme did not cleave the 16s rDNA into small fragments and was unable to distinguish the LAB isolated from the mum sausages. Similarly, among several digestion enzymes evaluated, Hsieh et al. (2010) observed that *Aci* I did not cleave the PCR products or differentiate the bacteria isolated from dry-dressed fish fillets. Claisse et al. (2007) also reported that a single *Aci* I enzyme was unable to distinguish *Lactobacillus* species from other LAB rods. The ineffectiveness of *Aci* I for DNA cleavage could be related to its recognition sequence (AA/CGTT), which has 6 nucleotides, whereas the other restriction enzymes (i.e., *Alu* I, *Mse* I, and *Dde* I) contain 4 or 5 nucleotides (Bonomo et al., 2008; Claisse et al., 2007).

In a study by Rodas et al. (2003), *Alu* I was able to discriminate *L. plantarum* and *Pediococcus pentosaceus*. In our study, as shown in Fig. 2(b), we observed 5 restriction patterns when using *Alu* I for digestion. Among them, we distinguished 4 profiles (in lanes A, B, D, and F, identified as *L. plantarum*, *L. sakei*, *Lactobacillus brevis*, and *Ln. mesenteroides*, respectively, using sequence analysis). However, we were unable to distinguish the restriction profiles of lanes C, E, and G (i.e., *Lactobacillus fermentum*, *Pediococcus pentosaceus*, and *Lactococcus lactis*, respectively). Christensen et al. (2004) used *Alu* I to identify *Lactobacillus* spp. when developing a rapid assay for the diagnosis of *Lactobacillus bacteremia*. Yanagida et al. (2008) isolated and characterized LAB from koshu vineyards in Japan, reporting the successful discrimination of *L. plantarum*, *Lc. lactis*, and *P. pentosaceus*, using 16S rDNA RFLP and *Alu* I for digestion. In our study, when using *Alu* I for digestion, *L. plantarum* (lane A) could be differentiated from *P. pentosaceus* (lane E) and *Lc. lactis* (lane G); however, *P. pentosaceus* (lane E) and *Lc. lactis* (lane G) could not be distinguished. Variations in the microbial strains evaluated could have caused the differing results among our and previous studies. Mainville et al. (2006) also suggested that RFLP analysis might be unable to detect minute differences in genetic loci, thus leading to limited discrimination among the LAB strains evaluated.

In our study, as shown in Fig. 2(c), the 16s rDNA amplified products of LAB digested by *Mse* I generated 7 characteristic profiles. However, this digestion enzyme was associated with disadvantages because of the presence of certain ambiguous bands on gels. Rodas et al. (2003) reported that the *Mse* I restriction enzyme provided a high discrimination capacity for the differentiation of the *Lactobacillus* and *Pediococcus* genera. Using 16S-ARDRA analyses and *Mse* I for digestion, they were able to distinguish *L. plantarum* and *L. sakei*. In our study, as shown in Fig. 2(d), the discrimination capacity of the *Dde* I restriction enzyme was superior to those of the other evaluated enzymes, including *Alu* I, which was unable to distinguish *L. fermentum*, *P. pentosaceus*, and *Lc. lactis*. *Dde* I was able to differentiate the 7 LAB isolated from the mum sausages, later identified as *L. sakei*, *L. plantarum*, *Ln. mesenteroides*, *L. brevis*, *L. fermentum*, *P. pentosaceus*, and *Lc. lactis* (Table 1). Similarly, Bonomo et al. (2008) reported that *Dde* I was able to distinguish 6 LAB isolated from traditional Italian fermented sausages, including *L. sakei*, *L. plantarum*, *L. brevis*, and *P. pentosaceus*. In a study conducted by Chenoll et al. (2003), ARDRA-*Dde* I provided the highest discrimination ability for species of the *Lactobacillus* genus. They showed that the maximum level of species differentiation among the *Pediococcus* genus could be achieved when using the *Dde* I for the restriction of intergenic spacer region (ISR).

3.3. Sequencing of the 16S rDNA gene for LAB identification

In this study, we selected 7 representative RFLP band patterns, digested using *Dde* I, for further sequencing. We aligned the sequences of the 16S rDNA of the LAB according to their identities and compared them with the DNA sequences deposited in the GenBank database

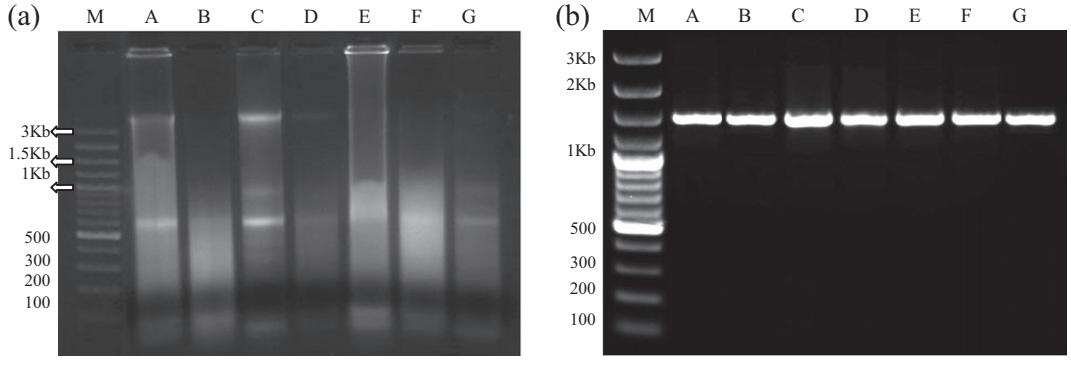

Fig. 1. Electrophoretic analysis of (a) DNA extracts on 1.5% agarose gel from lactic acid bacteria (b) PCR products on 1.5% agarose gel from lactic acid bacteria. Lane M: MW marker Bio 100 bp ladder. Lane M: MW ladder (100 bp); lane A: *L. plantarum*; lane B: *L. sakei*; lane C: *L. fermentum*; lane D: *L. brevis*; lane E: *P. pentosaceus*; lane F: *Ln. mesenteroides*; lane G: *Lc. lactis*.

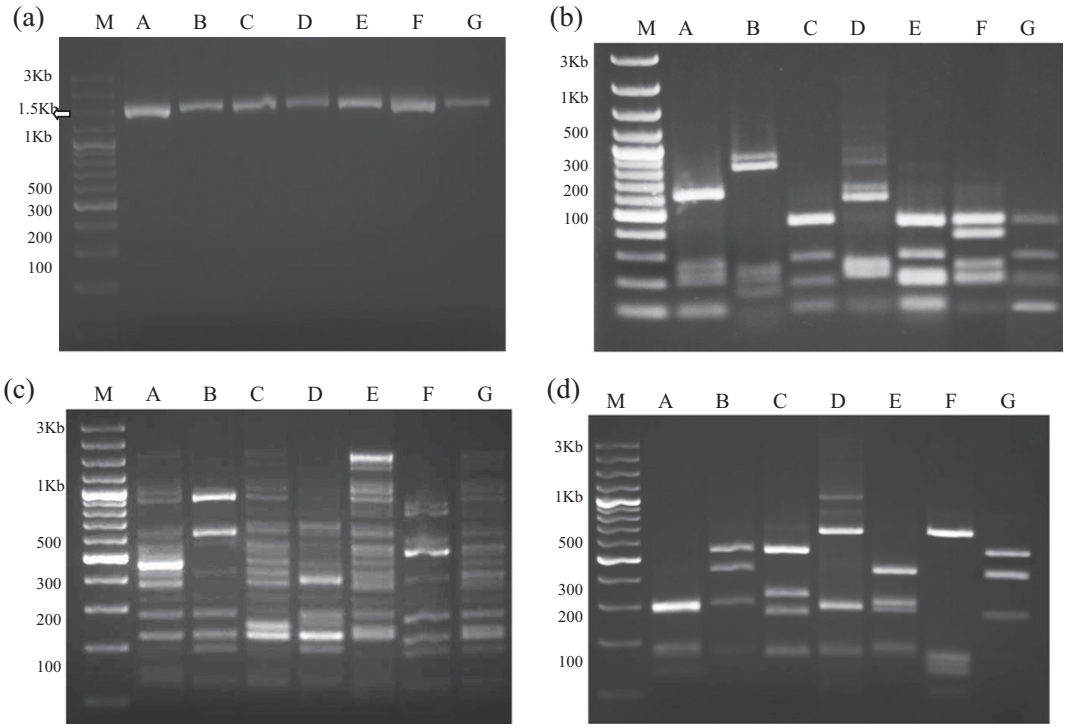

Fig. 2. RFLP patterns obtained from digestion with different restriction enzymes (a) *Aci* I, (b) *Alu* I, (c) *Mse* I and (d) *Dde* I. Lane M: MW ladder (100 bp); lane A: *L. plantarum*; lane B: *L. sakei*; lane C: *L. fermentum*; lane D: *L. brevis*; lane E: *P. pentosaceus*; lane F: *Ln. mesenteroides*; lane G: *Lc. lactis*.

(http://www.ncbi.nlm.nih.gov) by using the BLAST algorithm. Table 1 shows the identity percentages of the LAB species compared with those in the database and the provided GenBank accession numbers. We identified the 7 LAB species as *L. plantarum*, *L. sakei*, *L. fermentum*, *L. brevis*, *P. pentosaceus*, *Ln. mesenteroides*, and *Lc. lactis* (Table 1),which are the LAB that commonly occur in a variety of Thai fermented food products (Tanasupawat, 2009). The results from 16S rDNA sequencing also indicated that lanes A, B, C, D, E, and G showed close alignment with *L. plantarum* (98% identity), *L. sakei* (98% identity), *L. fermentum* (98% identity), *L. brevis* (98% identity), *P. pentosaceus* (97% identity), *Ln. mesenteroides* (99% identity), and *Lc. lactis* (99% identity), respectively. In this study, a total of 630 lactic acid bacteria were isolated including *L. sakei* (189 isolates, 30%), *L. plantarum* (156 isolates, 24.76%), *Ln. mesenteroides* (84 isolates, 13.33%), *L. brevis* (75 isolates, 11.90%), *P. pentosaceus* (48 isolates, 7.62%), *Lc. lactis* (48 isolates, 7.62%), and *L. fermentum* (30 isolates, 4.76%). In this study, we used the neighbor-joining method and the kimura2-parameter model to determine the phylogenetic relationships among the aligned 16S rDNA sequences of representative and reference strains (Fig. 3). Ennahar et al. (2003) mentioned that the phylogenetic trees based on the 16S rDNA sequences from various LAB isolates showed high consistency. In our study, the 16s rDNA sequence tree showed 2 distinct LAB groups: *Ln. mesenteroides* and all other species. In the sequence phylogenetic tree, the A, B, C, D, E, F, and G lanes were clearly assigned to the *L. plantarum*, *L. sakei*, *L. fermentum*, *L. brevis*, *P. pentosaceus*, *Ln. mesenteroides*, and *Lc. lactis* species, respectively, with a 100% bootstrap cluster.

3.4. LAB population dynamics during the fermentation and storage of mum

Previous studies have identified the *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*genera as the major LAB occurring in dry-fermented sausages (Adiguzel and Atasever, 2009; Danilović et al., 2011; Fontana et al., 2005). Thiravattanamontri et al. (1998) also described the detection of *Lactobacilli* and *Pediococci* in Thai fermented food products. In our analyses, the most commonly detected LAB genus was *Lactobacillus*, which was the dominant flora identified in dry-fermented sausages in the study by Adiguzel and Atasever (2009).

Fig. 4(a) and (b) shows the LAB dynamics for the Method 1 (the conventional method) and 2 (the modified method) samples, respectively, during various stages of treatment. The majority of the LAB isolated from the samples evaluated at Day 0 and Day 3 were *L. plantarum* and *L. sakei* species in both sample types. In Method 1 samples, after

Table 1
RFLP patterns and identification of lactic acid bacteria isolated from samples during processing of mum sausages.

RFLP pattern group [a]	Closest relative	% identity [b]	Genbank accession no. [c]	Frequency of isolation (%)
A	*Lactobacillus plantarum*	98	NR042394.1	24.76 (156/630)
B	*Lactobacillus sakei*	98	NR025719.1	30.00 (189/630)
C	*Lactobacillus fermentum*	98	JQ446568.1	4.76 (30/630)
D	*Lactobacillus brevis*	98	NR044704.1	11.90 (75/630)
E	*Pediococcus pentosaceus*	97	NR042058.1	7.62 (48/630)
F	*Leuconostoc mesenteroides*	99	NR040817.1	13.33 (84/630)
G	*Lactococcus lactis*	99	NR040955.1	7.62 (48/630)

[a] Letters from A to G correspond to RFLP patterns in Fig. 2.
[b] Identical nucleotides percentage in the sequence obtained from PCR products and the sequence obtained found in NCBI.
[c] GenBank accession numbers of the sequences obtained from the representative isolates.

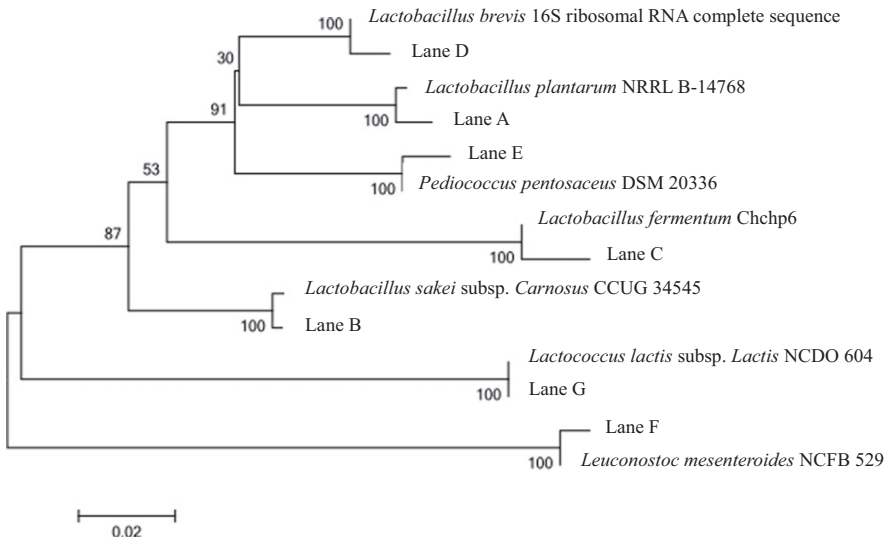

Fig. 3. Phylogenetic relationship of LAB present in mum sausage based on maximum-likehood analysis of 16S rDNA sequences. The numbers indicate the confidence level (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.

Day 3, the percentages of the *L. sakei*, *L. fermentum*, and *Lc. lactis* increased, whereas the percentages of *L. brevis*, *P. pentosaceus*, and *Ln. mesenteroides* decreased. At Day 14, the prevalence of the microorganisms was in the ascending order of *L. sakei*, *L. plantarum*, and *L. fermentum*. We observed small amounts of *Lc. lactis* but did not detect *Ln. mesenteroides*, *P. pentosaceus*, or *L. brevis*. These results supported those of Lee et al. (2006), in which the percentages of *Lactococcus* and *Pediococcus* increased initially, and then decreased during the ripening process. In Method 2 samples, *Ln. mesenteroides* showed the greatest increase in proportion, becoming the dominant species, followed by *L. sakei*, *L. brevis*, and *Lc. Lactis*. On Days 14 and 28, *L. plantarum* and *L. fermentum* were undetectable. According to the studies by Lee et al. (2006) and Wu et al. (2009), *Lactobacilli* can grow continuously throughout the fermentation process because they show greater acid tolerance than do other bacterial species.

Lee et al. (2006) stated that temperature and fermentation have considerable influence on the growth of LAB. *L. plantarum* and *L. sakei* are able to grow in various conditions. According to the study by Papamanoli et al. (2003), the optimal temperature for *L. plantarum* growth is 15–45 °C, and growth might not occur at temperature slower than 4 °C, whereas *L. sakei* is capable of growing in a wider temperature range of 4–45 °C, and is reportedly predominant in vacuum-packed or

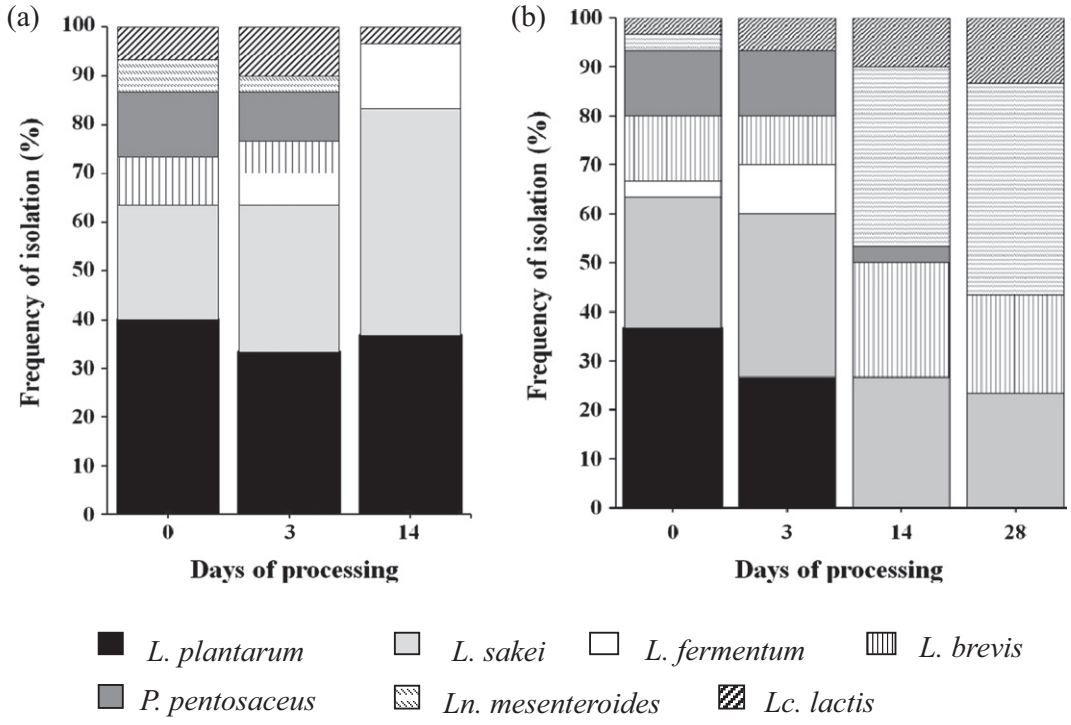

Fig. 4. Lactic acid bacteria dynamics of (a) method I mum sausage (stored at ~30 °C for 14 days) and (b) method II mum sausages (stored at ~30 °C for 3 days, vacuum-packaged, and stored at 4 °C until day 28) during processing and storage.

unpacked sausages (Danilović et al., 2011). These characteristics could explain our detection of *L. sakei*, but not *L. plantarum*, in Method 1 (30 °C and aerobic) and 2 (4 °C and anaerobic) samples after Day 14. Suutari and Laakso (1992) further reported that *L. fermentum* does not grow at temperatures lower than 10 °C and requires oxygen to maintain a fermentative metabolism, which could explain its absence in Method 2 samples after Day 14.

In our study, we detected small amounts of *Ln. mesenteroides* in Method 1 and 2 samples during fermentation, and markedly increased amounts (becoming predominant) in Method 2 samples after Day 14. We detected *Ln. mesenteroides* in Method 2 samples after Day 14 only. *Ln. mesenteroides* has the unique characteristics of being able to grow at 4 °C, but not above 30 °C, and preferring anaerobic to aerobic conditions (Cai et al., 1998; Danilović et al., 2011). Several studies have demonstrated that *Ln. mesenteroides* is able to produce bacteriocins which function against some pathogen, such as *Listeria monocytogenes* (Herchard et al., 1992; Xiraphi et al., 2008); it can also produce some metabolites, such as acetic acid, acetaldehyde, diacetyl, and ethanol, which can eventually enhance flavor development in fermented products (Sanchez et al., 2005; Lee et al., 2006).

4. Conclusion

Our results show that RFLP analysis is capable of rapidly and easily differentiating and identifying the LAB isolated from mum sausages during fermentation and storage. Of the restriction enzymes evaluated, *Dde* I provided the highest discrimination capacity for the LAB. Our results indicate the distribution of the dominant LAB species: *L. sakei* and *L. plantarum* are predominant during fermentation, whereas *Ln. mesenteroides* spp. show marked increases during storage. These finding also illustrated that chilled storage combined with vacuum packaging validly influenced the microbial ecology of the mum sausage. Our results could facilitate the development of LAB starter cultures that enable controlled processing during mum manufacturing, and provide a more consistent and higher quality product.

References

Adiguzel, G.C., Atasever, M., 2009. Phenotypic and genotypic characterization of lactic acid bacteria isolated from Turkish dry fermented sausage. Rom. Biotechnol. Lett. 14, 4130–4138.

Albano, H., Oliveira, M., Aroso, R., Cubero, N., Hogg, T., Teixeira, P., 2007. Antilisterial activity of lactic acid bacteria isolated from "Alheiras" (traditional Portuguese fermented sausages): *in situ* assays. Meat Sci. 76, 796–800.

Ammor, M.S., Mayo, B., 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. Meat Sci. 76, 138–146.

Antara, N.S., Sujaya, I.N., Yokota, A., Asano, K., Aryanta, W.R., Tomita, F., 2002. Identification and succession of lactic acid bacteria during fermentation of 'urutan', a Balinese indigenous fermented sausage. World J. Microbiol. Biotechnol. 18, 255–262.

APHA, 2001. Compendium of Methods for the Microbiological Examination of Foods, 4th ed. American Public Health Association, Washington, DC.

Atshan, S.S., Shamsudin, M.N., 2011. Evaluation of phenotypic and genotypic detection methods for biofilm-forming methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates. Ann. Microbiol. 61, 825–831.

Bernardeau, M., Guguen, M., Vernoux, J.P., 2006. Beneficial *lactobacilli* in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol. Rev. 30, 487–513.

Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Guéguen, M., 2008. Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int. J. Food Microbiol. 126, 278–285.

Bonomo, M.G., Ricciardi, A., Zotta, T., Parente, E., Salzano, G., 2008. Molecular and technological characterization of lactic acid bacteria from traditional fermented sausages of Basilicata region (Southern Italy). Meat Sci. 80, 1238–1248.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Schure, E.T., Boven, A.V., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., Hansen, E.B., 2012. Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 154, 87–97.

Cai, Y., Benno, Y., Takeda, A., Yoshida, T., Itaya, T., Nakase, T., 1998. Characterization of *Leuconostoc* species isolated from vacuum-packaged ham. J. Gen. Appl. Microbiol. 44, 153–159.

Chenoll, E., Macián, M.C., Aznar, R., 2003. Identification of *Carnobacterium*, *Lactobacillus, Leuconostoc* and *Pediococcus* by rDNA-based techniques. Syst. Appl. Microbiol. 26, 546–556.

Christensen, J.E., Reynolds, C.E., Shukla, S.K., Reed, K.D., 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.

Claisse, O., Renouf, V., Lonvaud-Funel, A., 2007. Differentiation of wine lactic acid bacteria species based on RFLP analysis of a partial sequence of *rpoB* gene. J. Microbiol. Methods 69, 387–390.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., Cocolin, L., 2005. Characterisation of naturally fermented sausages produced in the North East of Italy. Meat Sci. 69, 381–392.

Danilović, B., Joković, N., Petrović, L., Veljović, K., Tolinački, M., Savić, D., 2011. The characterisation of lactic acid bacteria during the fermentation of an artisan Serbian sausage (Petrovská Klobása). Meat Sci. 88, 668–674.

Ennahar, S., Cai, Y., Fujita, Y., 2003. Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. Appl. Environ. Microbiol. 69, 444–451.

Fontana, C., Cocconcelli, P.S., Vignolo, G., 2005. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. Int. J. Food Microbiol. 103, 131–142.

Gonzalez-Fernandez, C., Santos, E.M., Rovira, J., Jaime, I., 2006. The effect of sugar concentration and starter culture on instrumental and sensory textural properties of chorizo-Spanish dry-cured sausage. Meat Sci. 74, 467–475.

Harrigan, W.F., 1998. Laboratory Methods in Food Microbiology, 4th ed. Academic Press, California.

Herchard, Y., Derijard, B., Letellier, F., Cenatiempo, Y., 1992. Characterization and purification of mesentericin Y 105, an anti-listeria bacteriocin from *Leuconostoc mesenteroides*. J. Gen. Microbiol. 138, 2725–2731.

Hsieh, C.H., Chang, W.H., Chang, H.C., Hsieh, H.S., Chung, Y.L., Hwang, D.F., 2010. Puffer fish-based commercial fraud identification in a segment of cytochrome *b* region by PCR–RFLP analysis. Food Chem. 121, 1305–1311.

Latorre-Moratall, M.L., Bover-Cid, S., Veciana-Nogués, M.T., Vidal-Carou, M.C., 2012. Control of biogenic amines in fermented sausages: role of starter cultures. Front. Microbiol. 3, 1–9.

Lee, J.Y., Kim, C.J., Kunz, B., 2006. Identification of lactic acid bacteria isolated from *kimchi* and studies on their suitability for application as starter culture in the production of fermented sausages. Meat Sci. 72, 437–445.

Leroy, F., Verluyten, J., Vuyst, L.D., 2006. Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106, 270–285.

Mainville, I., Robert, N., Lee, B., Farnwort, E.R., 2006. Polyphasic characterization of the lactic acid bacteria in kefir. Syst. Appl. Microbiol. 29, 59–68.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Panyim, S., 2003. Isolation of nisin-producing *Lactococcuslactis* WNC 20 strain from nham, a tradition Thai fermented sausage. Int. J. Food Microbiol. 81, 137–145.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E., Kotzekidou, P., 2003. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci. 65, 859–867.

Rattanachomsri, U., Kanokratana, P., Eurwilaichitr, L., Igarashi, Y., Champreda, V., 2011. Culture-independent phylogenetic analysis of the microbial community in industrial sugarcane bagasse feedstock piles. Biosci. Biotechnol. Biochem. 75, 232–239.

Rodas, A.M., Ferrer, S., Pardo, I., 2003. 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. Syst. Appl. Microbiol. 26, 412–422.

Sanchez, J.I., Martınez, B., Rodrıguez, A., 2005. Rational selection of Leuconostoc strains for mixed starters based on the physiological biodiversity found in raw milk fermentations. Int. J. Food Microbiol. 105, 377–387.

Shukla, S., Goyal, A., 2011. 16S rRNA-based identification of a glucan-hyperproducing *Weissella confuse*. Enzyme Res. 1–10.

Singh, A.K., Ramesh, A., 2009. Evaluation of a facile method of template DNA preparation for PCR-based detection and typing of lactic acid bacteria. Food Microbiol. 26, 504–513.

Sriphochanart, W., Skolpap, W., 2010. Characterization of proteolytic effect of lactic acid bacteria starter cultures on Thai fermented sausages. Food Biotechnol. 24, 293–311.

Stadnik, J., Dolatowski, Z.J., 2010. Biogenic amines in meat and fermented meat products. ACTA Sci. Pol. Technol. Aliment. 9 (3), 251–263.

Suutari, M., Laakso, S., 1992. Temperature adaptation in *Lactobacillus fermentum*: interconversions of oleic, vaccenic and dihydrosterulic acids. J. Gen. Microbiol. 138, 445–450.

Talon, R., Lebert, I., Lebert, A., Leroy, S., Garriga, M., Aymerich, T., Drosinos, E.H., Zanardi, E., Ianieri, A., Fraqueza, M.J., Patarata, L., Lauková, A., 2007. Traditional dry fermented sausages produced in small-scale processing units in Mediterranean countries and Slovakia. 1: Microbial ecosystems of processing environments. Meat Sci. 77, 570–579.

Talon, R., Leroy, S., Lebert, I., Giammarinaro, P., Chacornac, J.P., Latorre-Moratalla, M., Vidal-Carou, C., Zanardi, E., Conter, M., Lebecque, A., 2008. Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. Int. J. Food Microbiol. 126, 227–234.

Tanasupawat, S., 2009. Thai lactic acid bacteria: diversity and applications. SWU Sci. J. 25, 1–13.

Thai Industrial Standards Institute, 2003. Ministry of industry. http://app.tisi.go.th/otop/pdffile/tcps14646.pdf ((In Thai). Accessed 01.05.11).

Thiravattanamontri, P., Tanasupawat, S., Noonpakdee, W., Valyasevi, R., 1998. Catalases of bacteria isolated from Thai fermented foods. Food Biotechnol. 12, 221–238.

Wanangkarn, A., Liu, D.C., Swetwiwathana, A., Tan, F.J., 2012. An innovative method for the preparation of mum (Thai fermented sausages) with acceptable technological quality and extended shelf-life. Food Chem. 135, 515–521.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., Guo, M., Zhang, H., 2009. Isolation and preliminary probiotic selection of lactobacilli from koumiss in Inner Mongolia. J. Basic Microbiol. 49, 318–326.
Xiraphi, N., Georgalaki, M., Rantsiou, K., Cocolin, L., Tsakalidou, E., Drosinos, E.H., 2008. Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroides* E131. Meat Sci. 80, 194–203.
Yanagida, F., Srionnual, S., Chen, Y.S., 2008. Isolation and characteristics of lactic acid bacteria from koshu vineyards in Japan. Lett. Appl. Microbiol. 47, 134–139.