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