MATERIALS AND METHODS

Materials

1. Nham

Nham samples were produced by Product Development Division, Department of Livestock and transported on ice to Food Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology National Science and Technology Development Agency (BIOTEC) for analysis. The products were separated into 2 portions for chemical and microbiological determination.

2. Starter culture

Lactobacillus plantarum BCC 9546 was obtained from Food Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology National Science and Technology Development Agency (BIOTEC). *Lb. plantarum* was stored at –80 °C in de Man Rogosa Sharpe (MRS) broth (Appendix A) with 20% glycerol, as stock culture.

3. Reference strains

The reference strains used in this study are listed in Table 1. The cultures were grown in MRS broth, while Nutrient broth (NB) (Appendix A) was used for *Staphylococcus. xylosus*. These cultures were incubated at their optimal growth temperature (LABs incubated at 30°C and 37°C for *S. xylosus*) for 18-24 h. For long-term maintenance, cell suspensions were stored at -80°C in broth cultures supplemented with 20% glycerol.

Table 1 Reference strains used in this study

Speicies	**Strains**	**Source**
Lactobacillus animalis	ATCC 35046 [T]	Dental plaque of baboon
Lactobacillus brevis	ATCC 114869 [T]	Faeces
Lactobacillus casei	ATCC 393 [T]	Cheese
Lactobacillus curvatus subsp. *curvatus*	DSM 20019 [T]	-
Lactobacillus farciminis	ATCC 29644 [T]	Sausages
Lactobacillus fermentum	ATCC 14931 [T]	Fermented beets
Lactobacillus graminis	DSM 20719 [T]	-
Lb. plantarum	DSM 2648	Silage
Lb. plantarum	DSM 12028	Dry fermented sausage
Lb. plantarum subp. *argentoratensis*	DSM 16365 [T]	Fermented cassava roots
Lb. plantarum subp. *plantarum*	DSM 20174 [T]	Pickled cabbage
Lactobacillus sakei subp. *sakei*	ATCC 15521 [T]	Moto starter of sake
Lb. sakei subp. *carnosus*	DSM 15831 [T]	-
Lactobacillus salivarius subp. *salivarius*	ATCC 11741 [T]	Saliva
Leuconostoc mesenteroides	ATCC 8293 [T]	Fermenting olives
Pediococcus acidilactici	DSM 20284 [T]	Barley
Pediococcus pentosaeceus	ATCC 33316 [T]	Dried American beer yeast
Staphylococcus xylosus	DSM 20266 [T]	-

Note: T: Type strains; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC: American Type Culture Collection, Rockville, MD, USA.

Methods

1. Preparation of starter culture

One loop of stock culture of *Lb. plantarum* BCC 9546 kept in MRS broth with 20% glycerol at -80°C was cross-streaked on MRS agar (Appendix A) and then incubated at 30°C for 48 h. A single colony of *Lb. plantarum* BCC 9546 on MRS agar was transferred into 5 ml of MRS broth and incubated at 30°C for 18-24 h. The bacterial cells were harvested by centrifugation at 4°C at 10,000 rpm for 3 min using a model 5403 refrigerated centrifuge (Eppendorf, Germany). The harvested cells were washed with 5 ml. of sterile 0.1% w/v peptone solution. Finally, the cell concentration was adjusted to 10^7cfu/ml with sterile peptone solution (0.1% w/v).

2. Nham preparation and sampling procedures

Two Nham fermentation batchs were studied: without starter culture inoculation (natural Nham) and inoculated with *Lb. plantarum* BCC 9546 (starter cultured Nham). Nham samples were prepared at Product Development Division, Department of Livestock. Nham was prepared as follows: 12 kg of minced pork was mixed with 440 g of nitrite salt, 80 g of sucrose, 40 g of sodium erythobate, 40 g of trisodium polyphosphate, 40 g of monosodium glutamate, 2 kg of minced garlic and cooked rice (garlic and cooked rice ratio was 1:1), 8 kg of shedded cooked pork rind and 400 g of whole bird chili. The mixture was divided into two equal portions, one was for control and another was inoculated with the starter cultures prepared as described above to give the final cell counts of approximately 10^4 CFU/g. Each portions was thoroughly mixed and stuffed into plastic casings with a diameter of 3 cm (approximately 200 g each) and sealed tightly prior to incubation at 30°C for 72 h. Sampling times were periodically carried out at 0 h (soon after mixture was stuffed into the casing), 6, 12, 24, 36, 48, 60 and 72 h. Four samples from each batch at each step of sampling were collected and used for analysis. One sample for microbiological and pH analysis were conducted immediately after sampling.

Another three samples for organic acid and sugar determination were kept at -80°C until used.

3. Chemical analysis

The measurements of pH were obtained with the pin electrode of a pH meter (Mettler Teledo 320, Switzerland) that was inserted directly into each sample. Three dependent measurements were obtained from each sample. Means was calculated. Organic acids and sugar determination (Appendix B) in both types of Nham fermentation were determined by Plengvidhaya, *et al*. (2005) at Food Biotechnology Laboratory, BIOTEC.

4. Microbiological analysis

Nham sample (25 g) was aseptically transferred into a sterile plastic bag and 225 ml of 0.1 % peptone water were added and homogenized for 1 min in a Stomacher machine (IUL Instrument, Barcelona, Spain). One milliliter of each sample homogenate was diluted serially tenfold in the peptone solution. The suspensions (0.1ml.) were plated on appropriate agar medium for microbiological analysis (in duplicate). LAB populations were counted on MRS agar and total aerobic plate count were determined on plate count agar (PCA) (Appendix A) and incubated at 30°C for 48-72 h. *Enterobateriaceae* counts were determined on violet red bile agar (VRBG) with added 1% glucose (Appendix A) and incubated at 37°C for 24 h. Plates were incubated under aerobic condition, and counts being expressed as Colony Forming Units (CFU) per gram of Nham.

5. Isolation of lactic acid bacteria

LAB was isolated by using the procedure of Plengvidha *et al.* (2004). Approximately 80-100 isolates were randomly picked from countable MRS plates (30-300 colony/plate) at each sampling point of each sample. After picked, the

isolates were grown in MRS broth and streaked for purification on MRS agar. The pure cultures were stored at -80°C in MRS broth with 20 % glycerol. Isolates from stocks were streaked onto MRS agar plate and subculture in MRS broth before being subjected to molecular analysis.

6. Nucleic acid extraction

Total genomic DNA was extracted by using a Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, with minor modification (Appendix C). In this modification, the cell lysis solution contains 5 µl of mutanolysin (2500 U/ml; Sigma, St. Louis, MO, USA) in addition to 10 µl of lysozyme (10 mg/ml, Sigma, USA). DNA quality was determined by electrophoresis on 1% agarose gel. After electrophoresis, the gels were stained in 0.5X TAE containing 0.5 µg/ml of ethidium bromide (Fluka) for 20-30 min. Pictures of the gels were digitally captured using the Bioimaging System GeneGenius (SynGene, Cambridge, England). The DNA concentration was measured with absorbance at $OD260_{nm}$ with spectrophotometer. For rep-PCR, the DNA was adjusted to 50 ng / µl with TE buffer (Appendix A).

7. ITS-PCR amplification and RFLP analysis

The modification of method from Breidt and Fleming (1996) was used to amplify the ITS region between 16S and 23S rRNA gene with primers G1-16S (5′-GAAGTCGTAACAAGG-3′) and L2-23S (5′-GGGTTTCCCCATTCGGA-3′). The reaction mixture of 50 µl in a 0.2 ml thin-wall microfuge tube (Axygen Scientific, Inc., USA), containing 36 µl of deionized water, 5 µl of 10X PCR buffer (500mM KCl and 100mM Tris-Cl, pH 8.0, Fermentas International Inc., Canada), 5.0 µl of 25mM $MgCl_2$ (Fermentas), 0.5 µl of *Taq* DNA polymerase (10.0 U/µl, Fermentas), 0.5 µl of dNTPs mixture (25mM each dNTP, Fermentas), 0.5 µl of each primers, 2 µl of DNA template. PCR amplification was performed in a GeneAmp PCR, model 9700 (PerkinElmer, U.S.A). The reaction was carried out with the initial heat denaturation step of 94°C, 5 min. The *Taq* polymerase was then added and followed by 94°C, 1

min; 55°C, 2 min; 72°C, 2 min for 25 cycles. The final extension step was performed at 72°C for 5 min, followed by cooling to 15°C. After PCR amplification, 20 µl of reaction mixtures containing DNA products were treated with 1 µl of *Rsa*I enzyme solution (Fermentas International Inc., Canada) for 2 h at 37°C in a thermo-mixture (model 5355 Comfort, Eppendorf, Germany). The restriction digested samples were stored at -20°C prior to electrophoresis in 2.0% agarose gel using a horizontal submerged gel apparatus (Bio-Rad, Wide Mini Sub Cell, Bio-Rad Laboratories, Hercules, CA) in a 0.5 X Tris-borate buffer (TBE, Sambrook *et al*., 1989). Gels were run for approximately 2 h and 20 min at 120 volts. A 100 bp DNA molecular weight marker (Bioexcellence, Thailand) was used as a standard. After electrophoresis, the gels were stained in 0.5X TBE containing 0.5 µg/ml of ethidium bromide (Fluka) for 20-30 min. Pictures of the gels were digitally captured using the Bioimaging System Gene Genius (SynGene, Cambridge, England), and Gel Compar II, Version 4.5 (Applied Math, Kortrijk, Bellgium) was used for pattern analysis. The similarity of the electrophoretic profiles was determined using Dice coefficients and the unweighted pair group method with average linkage (UPGMA)

8. 16S-PCR amplification and sequencing

The procedure of Barrangou *et al.,* (2002) was used to amplify variable regions of the 16S rDNA gene from selected isolates with primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′GTCTCAGTCCCAATG TGGCC-3′. PCR amplification of 16S rDNA was carried out in a final volume of 50 µl comprising 33 µl of deionized water, 2.0 µl of each primer, 5 µl of 10X PCR buffer (500mM KCl and 100mM Tris-Cl, pH 8.0, Fermentas), 5 µl of 25mM $MgCl_2$ (Fermentas), 0.5 µl of Taq DNA polymerase (10.0 U/µl, Fermentas), 0.5 µl of dNTPs mixture (25mM each dNTP, Fermentas), 2 µl of DNA template. PCR amplification was performed in a GeneAmp PCR, model 9700 (PerkinElmer, U.S.A). The PCR conditions were the following: an initial heat denaturation step was performed at 94°C for 10 min. The Taq polymerase was then added, and the amplification cycle was as follows: 25 cycles of 94°C for 1 min, 61°C for 2 min, and 72°C for 2 min. The samples were incubated

for 5 min at 72°C for final extension and followed by cooling to 15°C. The 16S-PCR products were purified by using PCR Clean-Up System (Qiagen), according to manufacturer's instruction. The purified PCR products were sequenced commercially. Homology searches of the 16S rDNA sequences were performed using the BLAST basic local alignment search tool in the Genebank database on internet (http://www.ncbi.nlm.nih.gov/Blast/) to determine the most likely identities of the isolates.

9. rep-PCR genomic fingerprint

DNA extracted from selected *Lb. plantarum* isolates were subjected to rep-PCR analysis using primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3)′ as previous described (Gevers *et al*., 2001). Reaction mixtures were carried out in a final volume of 25 µl containing 18.38 µl of deionized water, 2.5 µl of 10X buffer (with 15 mM $MgCl_2$), 2 µl of 2.5 mM each dNTPs, 1 µl of 20 µM primer $(GTG)_5$, 0.125 µl of *Taq* polymerase (5.0 U/µl, Takara Bio Inc., Japan) and 1 µl of DNA template (50 ng/ µl) in 0.2 ml thin-wall microfuge tube (Axygen Scientific, Inc., USA). The reactions were performed in a Thermo cycler (DYAD ALD 1244, MJ research Inc., Massachussets). The PCR conditions were as followed: an initial heat denaturation step was performed at 95°C for 5 min, followed by 30 cycles of 94°C for 45 seconds, 40°C for 1 min, and 65°C for 10 min, and final extension at 65°C for 20 min. Amplicons were separated on 1% LE Seakem® agarose (BME, Rockland, ME, U.S.A) in 0.5X TBE at 120 V for 2 h and 40 min prior to stain in 0.5X TBE containing 5 µg/ml ethidium bromide (Sigma, USA) for 5 min. and destained in tap water for 20 min, with shaking. The gel image was captured by using an image scanner Typhoon 9410 (Amersham Biosciences). The DNA patterns were analyzed by using a pattern analysis software package, Gel Compar II, Version 4.5 (Applied Math, Belgium). Pearson product-moment correlation coefficient was used to calculate similarities between patterns and a dendrogram is obtained by means of unweighted pair group method with arithmetic average (UPGMA).