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Original Article

Monitoring of changes in lactic acid bacteria during production of Thai traditional fermented shrimp (*Kung-Som*) by culturing method and PCR-DGGE technique

Kanokwan Thongruck[1], Sutanate Saelao[1], Punnanee Sumpavapol[2], Soottawat Benjakul[2], and Suppasil Maneerat[1*]

[1] *Biotechnology for Bioresource Utilization Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, 90112 Thailand*

[2] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, 90112 Thailand*

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Abstract

The lactic acid bacteria (LAB) ecology of *Kung-Som*, a traditional Thai fermented shrimp, was investigated using culture-dependent and culture-independent methods. *Kung-Som* was fermented at room temperature under anaerobic condition for 7 days using autochthonous fermentation. The viable counts revealed a dominance of LAB, from the 16S rRNA gene V6-V8 sequence analysis of the isolates, *Enterococcus faecalis*, *Enterococcus sanguinicola*, *Weissella cibaria, Weissella confusa*, *Enterococcus* sp., *Leuconostoc pseudomesenteroides* and *Lactobacillus plantarum* were observed. The total microbial community was profiled without cultivation by analyzing the DNA that was directly extracted from the *Kung-Som* samples. The 16S rRNA gene V6-V8 regions were used as targets in the denaturing gradient gel electrophoresis (DGGE), profiling of the LAB. *Pediococcus argentinicus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus garvieae*, *Lactobacillus curvatus*, *Lactobacillus sakei* and *Lact. plantarum* were observed. Our results provide evidence of the necessity to combine the two methods for better description of microbial communities in *Kung-Som* fermentation.

Keywords: culture-dependent method, culture-independent method, 16S rRNA gene, PCR-DGGE

1. Introduction

Kung-Som is a traditional fermented shrimp widely consumed in the southern part of Thailand. Differences exist in production methods and raw materials, which typically consist of banana shrimp (*Penaeus merguiensis*), salt and a carbohydrate source (brown sugar, palm sugar or boiling rice water). The traditional production of *Kung-Som* was based on spontaneous fermentation due to the development of the microflora naturally present in the raw materials. In spontaneously fermented foods, the lactic acid bacteria (LAB) derived from the raw materials or the environment to generate flavor is explained by their production of organic acids and volatiles through the fermentation of carbohydrates (Urso *et al*., 2006). This causes a decrease in pH. The combination of low pH and organic acids (primarily lactic acid) is the main preservation factor in fermented foods. Generally, the pH should be below 4.5-5.0 to inhibit pathogenic and spoilage bacteria (Saithong *et al*., 2010).

Most studies of microbial diversity in fermented foods use a culturing method that has limitations such as time-consuming, limited in terms of both discriminating ability and

* Corresponding author.
Email address: suppasil.m@psu.ac.th

accuracy and revealing only a small portion of the true microbial diversity in the environments (Cocolin *et al*., 2013; Ongal & Asano, 2009). Thus, traditional fermented foods in which culture methods not only underestimates biodiversity but fails to quantify precisely some dominant taxa (Fontana *et al*., 2005a). The reasons essentially are the inability to detect microbial diversity, which might not be cultivated with known existing media, and inability to recover known microorganisms which can be viable but in a non-cultivable state (Giraffa & Neviani, 2002). Recently, several molecular methods have been applied for study of microbial diversity from traditional fermented foods. This includes the Polymerase Chain Reaction (PCR)-Denaturing Gradient Gel Electrophoresis (DGGE), which has been used to monitor microbial dynamics during the production of fermented foods (Fontana *et al*., 2005a). PCR-DGGE is reliable, reproducible, rapid and it has a greater detection and identification potential than culture-based methods (Cocolin *et al*., 2013; Temmerman *et al*, 2003). The objective of this research work was to monitor of changes in LAB during production of *Kung-Som* by the culturing method and the PCR-DGGE technique.

2. Materials and Methods

2.1 Preparation of *Kung-Som*

Kung-Som was prepared with the following ratio of ingredients using autochthonous fermentation. The original formula of *Kung-Som* consisted of 1 kg shrimp (*Penaeus merguiensis*), 75 g table salt, 300 g sugar and 250 ml water. The mixtures were transferred to eight bottles, packed tightly and covered with polyethylene film. A rubber band was used to hold the film in place. The containers were incubated at room temperature (28±1°C) under anaerobic condition for 7 days (Hwanhlem *et al*., 2010). Every day of fermentation period, one of these bottles was taken and microbial analyses performed using culture-dependent and the PCR-DGGE techniques.

2.2 Chemical analysis

Direct pH measurement was taken using a standard pH meter (Mettler Toledo MP320U, Switzerland). The titratable acidity (TA) was measured by the method of AOAC (2000) and calculated as lactic acid.

Determination of total acid

Kung-Som juice (30 ml) from each day of fermentation periods was mixed with 30 ml sterile water and filtered through cheese cloth. The filtrates were transferred into 150-ml flasks, and mixed with 2-3 drops of phenolphthalein. Then, the solution was titrated with 0.1N sodium hydroxide until the end point which color was changed to pink color and then total acid as lactic acid was calculated as follow AOAC (2000).

$$\text{Total acid (lactic acid)} = \frac{\text{Sodium hydroxide (ml) x Normality (N) of NaOH x 90.09 x 100}}{\text{Weight of sample (g) x 100}}$$

2.3 Microbiological analysis

The appropriate ten-fold serial dilutions of *Kung-Som* juice samples (25 ml) at each day of fermentation period were prepared in 0.85% sodium chloride for the enumeration of microbial. The following media and incubation conditions were used: (1) Plate Count Agar (HiMedia Laboratories, India) incubated at 37°C for 24-48 hrs for total bacteria count; (2) MRS agar (RCI Labscan Limited, Thailand) containing 0.01% (w/v) cycloheximide (RCI Labscan Limited, Thailand), 0.01% (w/v) sodium azide (Thermo Fisher Scientific Inc., Australia) and 0.01% (w/v) bromocresol purple incubated in a glass desiccator with a candle at 37°C for 24-48 hrs under anaerobic condition for the LAB count; (3) MacConkey agar (HiMedia Laboratories, India) incubated at 37°C for 24-48 hrs for Enterobacteriaceae count and (4) Potato Dextrose Agar (HiMedia Laboratories, India) pH 4 incubated at 37°C for 24-48 hrs for yeast count. To determine the distribution of the LAB in *Kung-Som,* bacterial colonies that exhibited yellow zone on the plate were selected randomly (character of size, colony) from each plate with 30-300 colonies (15% of sampling per plate) and streaked on MRS agar. This procedure was repeated in order to purify the isolates. Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase-negative were Gram-stained and observed under light microscopic. Only those which were Gram-positive were transferred to MRS broth for further identification by the PCR-DGGE technique.

2.4 DNA Extraction from isolated LAB and *Kung-Som*

Two milliliter of isolated LAB and *Kung-Som* juice from each sample at each day of fermentation period were collected by centrifugation at 13,000 *g* (5 min, 4°C). The cell pellets were subjected to DNA extraction by Genomic DNA Mini Kit (Blood/Cultured Cell) (Geneaid Biotech Ltd., Taiwan).

2.5 PCR-DGGE analysis

PCR was performed in a total reaction volume of 50 µl containing EmeraldAmp®GT PCR Master Mix (TAKARA BIO INC., Japan) 25 µl, each primer 5 µl (2 µM), DNA template 5 µl and RNase-free water 10 µl. The primer set, U968f-GC (52CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401r (52GCG TGT GTA CAA GAC CC), spanning the V6-V8 regions of the 16S

rRNA was used. A GC-clamp (AAC GCG AAG AAC CTT AC) was added to the primer U968f (Lu *et al*., 2010) to improve the sensitivity in the detection by DGGE (Rantisiou *et al*., 2004). The PCR products were generated using an initial denaturation step of 5 min at 94°C. This was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 20 s and elongation at 72°C for 40 s. Then final chain elongation was done at 72°C for 7 min.

Parallel DGGE was performed by using a Bio-Rad DcodeTM universal mutation detection system apparatus (Bio-Rad Laboratories Ltd., England). Thirty microgram (µg) of PCR products were analyzed by DGGE that was performed on 8% (w/v) polyacrylamide gels containing acrylamide: bisacrylamide 37.5:1 and a gradient of 30-65% urea-formamide denaturant gradient. A 100% denaturant corresponded to 7 mol l^{-1} urea and 40% (w/v) formamide. The electrophoresis was conducted with a constant voltage of 20 V for 10 min and 85 V for 14 hrs at 60°C. Gels were stained with SYBR Gold solution (Thermo Fisher Scientific Inc., Australia) for 15 min, then rinsed three times in sterile water and viewed under UV transillumination. The gel images were photographed using the Gel Documentation system (UVitec Cambridge, England).

2.6 Sequencing of DGGE bands and sequence analysis

The DGGE bands were excised with a sterile scalpel and the DNA of each band eluted in 20 µl of milli-Q water, overnight at 4°C. Five microlitres of the eluted DNA from each DGGE band was re-amplified using the conditions as described above and an annealing temperature at 50°C was used. Primer U968f without incorporation of a GC-clamp was used. For sequencing analysis, PCR products were purified with the PCR purification kit (QIAGEN Inc., USA) and used as templates in the sequencing reactions. The samples were analyzed with an automated DNA sequencer. Searches in GenBank with BLAST (http://www.ncbi.nlm.gov/) were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

3. Results and Discussion

3.1 Microbiological analysis of *Kung-Som*

The initial pH of *Kung-Som* was 7.46. A rapid decrease in pH was observed from 7.46 to 4.98 at the first day of fermentation. At the end of fermentation, the pH of the *Kung-Som* was 3.50. The decrease in pH could be mainly due to the production of acids such as lactic acid and acetic acid of the LAB present in the *Kung-Som*. The total acidity, that is lactic acid, followed the pH value, and at the first day of fermentation had very low lactic acidity (0.018%). However, after day 2 of fermentation, a rapid increase in acidity was observed in the *Kung-Som*. The total acidity as lactic acid was 2.76% at the end of fermentation (Figure 1).

The number of total bacteria increased from an initial value of 4.9 log cfu/ml to 9.0 log cfu/ml within the first 2 days of fermentation and remained of these levels until day 5. Thereafter, the total bacteria count gradually decreased. The number of LAB of *Kung-Som* increased drastically from 2.7 log cfu/ml to 8.3 log cfu/ml within 2 days of fermentation and this continued up to 4 days (9.2 log cfu/ml). Then the amount of LAB remained unchanged during 3-5 days of fermentation and subsequently decreased after 5 days (Table1). A rapid growth of LAB causing the pH to decrease to below 4.55 in 2 days is essential to prevent food spoilage. Cocci shape were the dominant LAB isolated from the first 2 days of fermentation when observed under light microscopic. However, after day 2 the LAB were dominated by those of rod and short rod shapes. This is because rod and short rod-shaped LAB are more tolerant to acid than cocci-shaped LAB (Adnan & Tan, 2007). Enterobacteriaceae decreased from 3 log cfu/ml to 2.6 log cfu/ml within the first day of fermentation. It could not be detected after 3 days of fermentation when the pH and lactic acid of *Kung-Som* was 4.01 and 1.44%, respectively. Yeast was not detected throughout the fermentation process (Table 1).

3.2 DGGE analysis of PCR products from isolated LAB

The PCR products of the LAB isolated from *Kung-Som* at each day of the fermentation period were analyzed by the DGGE technique as shown in Figure 2. Single pieces of the DGGE different band positions were excised and re-amplification of DNA was done for identification. The DGGE bands of different bacterial species were separated at different positions in the polyacrylamide gel because different bacterial species have differences in basepair composition (Ercolini *et al*., 2004). One hundred and thirty-three isolates of LAB isolated from *Kung-Som* at each day of fermentation period were identified as *Enterococcus* sp. (day 1, 99% similarity, 12.5% occurrences), *Enterococcus faecalis* (day 0 and 2, 99% similarity, 25% occurrences), *Enterococcus sanguinicola* (day 0, 99% similarity, 12.5% occurrences),

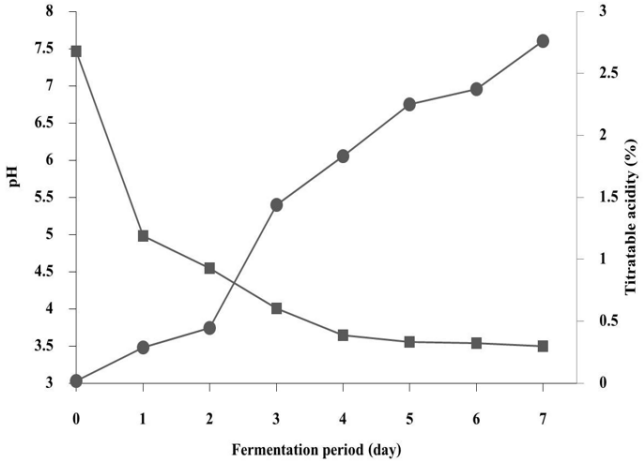

Figure 1. Changes in pH (■) and titratable acidity (●) of *Kung-Som* during fermentation.

Table 1. Microbial counts of *Kung-Som* at day 0, 1, 2, 3, 4, 5, 6 and 7 of fermentation.

Microorganism	Fermentation period (day)							
	0	1	2	3	4	5	6	7
	log CFU ml^{-1}							
Total bacteria	4.9±0.05	6.8±0.01	9.0±0.04	9.3±0.04	9.4±0.02	9.2±0.07	8.6±0.06	8.0±0.06
Lactic acid bacteria	2.7±0.11	6.5±0.01	8.3±0.04	9.1±0.01	9.2±0.01	9.0±0.01	8.5±0.06	7.8±0.18
Enterobacteriaceae	3.0±0.01	2.6±0.06	2.4±0.07	< 1	< 1	< 1	< 1	< 1
Yeast	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1

[a] Mean ± SD from triplicate determinations.

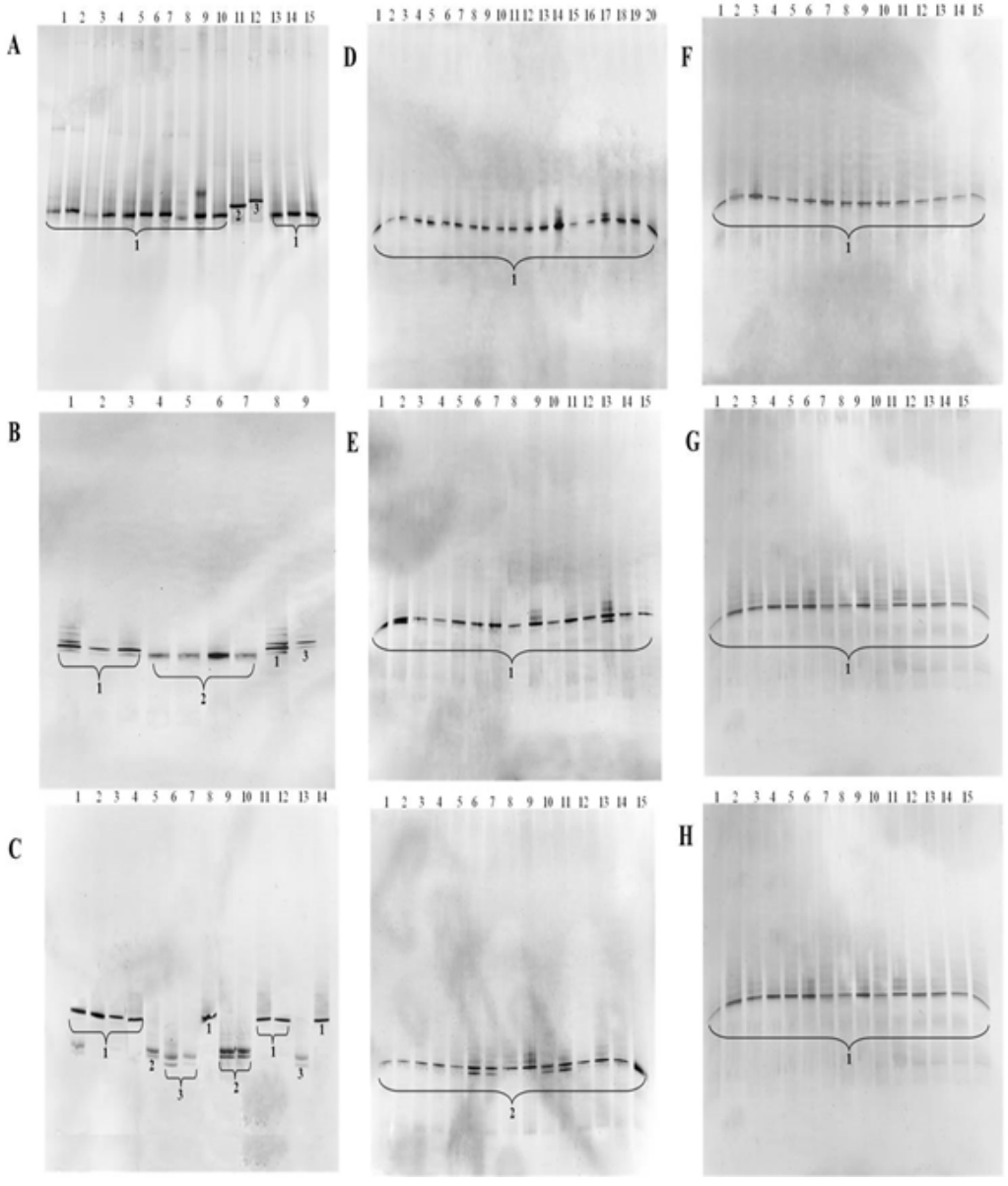

Figure 2. PCR-DGGE profiles (30-65% denaturant gradient) of amplified 16S rRNA gene (V6-V8 regions) fragments of LAB isolated from *Kung-Som* at day 0 (A), 1 (B), 2 (C), 3 (D), 4 (E), 5 (F), 6 (G) and 7 (H) of fermentation. Day 0 (A): *Ent. faecalis* (Band No. 1), *W. cibaria* (Band No. 2) and *Ent. sanguinicola* (Band No. 3). Day 1 (B): *W. confusa* (Band No. 1), *Enterococcus* sp. (Band No. 2) and *W. cibaria* (Band No. 3). Day 2 (C): *Lact. plantarum* (Band No. 1), *Leuc. pseudomesenteroides* (Band No. 2) and *Ent. faecalis* (Band No. 3). Day 3 (D): *Lact. plantarum* (Band No. 1). Day 4 (E): *Lact. plantarum* (Band No. 1 and 2). Day 5-7 (F-H): *Lact. plantarum* (Band No. 1).

Weissella cibaria (day 0 and 1, 100% similarity, 25% occurrences), *Weissella confusa* (day 1, 100% similarity, 12.5% occurrences), *Leuconostoc pseudomesenteroides* (day 2, 99% similarity, 12.5% occurrences) and *Lactobacillus plantarum* (day 2, 3, 4, 5, 6 and 7, 100%, 75% occurrences).

Enterococci strains have a beneficial role in the development of the aroma and flavor of fermented food during the fermentation process (Sarantinopoulos *et al*., 2001). *Leuc. pseudomesenteroides* was present in *Kung-Som* at the first phase of the fermentation process (day 2) and had an important role in the production of lactic acid. As a result, *Kung-Som* had a pH and the right environment for the growth of *Lact. plantarum* in the later phase of the fermentation process (day 3-7). From day 3 up to the last day of *Kung-Som* fermentation, only *Lact. plantarum* was detected due to the rapid decreased in pH of *Kung-Som*. *Lact. plantarum* is present in fermented foods at the later phase of the fermentation process because of its acid tolerance and superior ability to utilize the substrates than other bacteria (Mugula *et al*., 2003).

3.3 Monitoring of LAB in *Kung-Som* by PCR-DGGE technique

The results from the direct analysis by PCR-DGGE of the LAB community which had developed in *Kung-Som* were obtained by amplifying the V6-V8 regions of the 16S rRNA gene using primer U968f (GC) and L1401r. There was high diversity of LAB in *Kung-Som* (Figure 3). On the basis of the DGGE, different bands in the community structure of LAB were found in different samples from each day of the fermentation period. The DGGE profile indicated that within 7 days of fermented *Kung-Som* consist of at least 10 species of LAB. This is because 10 different bands were shown and

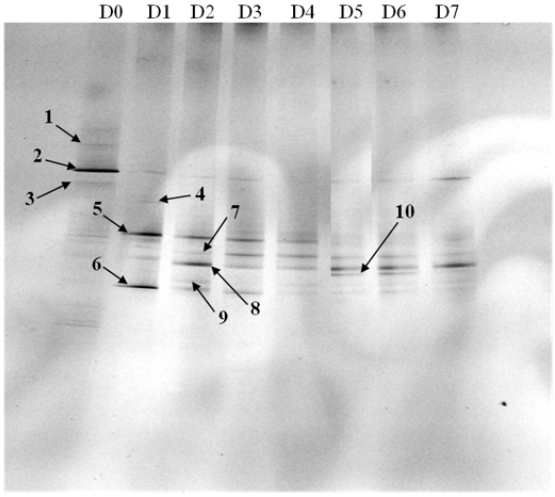

Figure 3. PCR-DGGE profiles (30-65% denaturant gradient) of amplified 16S rRNA gene (V6-V8 regions) fragments of bacterial community in *Kung-Som* during fermentation. Lane D0-D7: *Kung-Som* at day 0, 1, 2, 3, 4, 5, 6 and 7 of fermentation, respectively.

the different migrations by DGGE indicated that they have differences in basepair composition within the variable regions of the 16S rRNA (Ercolini, 2004).

When the 16S rRNA partial sequences from the DGGE bands were compared with the database, band No. 1, 2 and 3 were not characteristic of the LAB. Band No. 1 and 3 were characteristic of *Macrococcus caseolyticus* (100% similarity, 12.5% occurrences). Band No. 2 was found to correspond to the sequence of *Staphylococcus pasteuri* (100% similarity, 100% occurrences). However, the other bands were characteristic of LAB. Band No. 4 showed 94% similarity to *Pediococcus argentinicus* (12.5% occurrences). Band No. 5 and 6 showed 98% and 99% similarity to *Lactococcus lactis* subsp. *lactis* (87.5% occurrences) and *Lactococcus garvieae* (87.5% occurrences), respectively. Band No. 7 was closest to *Lact. plantarum* (94% similarity, 75% occurrences). Band No. 8 and 9 were characteristic of *Lactobacillus curvatus* (97% similarity, 75% occurrences) and *L. garvieae* (95%), respectively. The last band (No. 10) was characteristic of *Lactobacillus sakei* (93% similarity, 37.5% occurrences). Both *Ma. caseolyticus* as well as *L. garvieae* have obviously multiple ribosomal operons with different sequences that result in more than one band in the corresponding DGGE fingerprint (Fuka *et al*., 2010).

LAB were not detected by direct analysis by PCR-DGGE of *Kung-Som* at day 0 of fermentation. However, *Ma. caseolyticus* and *Staph. pasteuri* were detected due to LAB being usually present in fresh meat at low numbers about 10^2-10^3 cfu/g (Rantsiou and Cocolin, 2006). These concentrations could not be detected by the PCR-DGGE technique because this technique can only detect individual microbial populations when the concentrations are higher than 10^4 cfu/g (Fontana *et al*., 2005b). In this study, with the conditions applied in the DGGE protocol, the detection limit for individual members was determined to be 10^4 cfu/ml (data not shown).

The results from the direct analysis by PCR-DGGE of *Kung-Som* at day 1-7 of fermentation revealed that LAB were the dominant microorganism. The anaerobic fermentation and salt (7.5%) used as an ingredient for fermented *Kung-Som* were appropriate for the growth of LAB. *Ped. argentinicus* is able to grow at pH values of 4-8 (Bruyne *et al*., 2008). Accordingly, *Ped. argentinicus* was detected in *Kung-Som* at day 1 due to the pH of *Kung-Som* being appropriate for the growth of this strain (pH 4.98). However, after that, lactic acid in the *Kung-Som* rapidly increased from 0.28% at day 1 to 2.76% at day 7 of fermentation (Figure 1) and that was not appropriate for the growth of this strain. Then, the *Ped. argentinicus* was not detected. *Pediococcus* are not acid tolerant but produce lactic acid at a faster rate and amount higher than *Lactobacillus* and *Lactococus* (Müller *et al*., 1999). *Lactobacillus* and *Lactococus* are the major producers of lactic acid responsible for the decrease in pH and the increase in acidity during fermentation (Visessanguan *et al*., 2004). During *Kung-Som* fermentation, *Lact. plantarum* and *Lact. curvatus* were detected at day 2-7. In addition, *Lact.*

sakei was also detected in *Kung-Som* at day 5-7. The results obtained in this study indicate that *Lact. plantarum*, *Lact. curvatus* and *Lact. sakei* had important roles during the fermentation of *Kung-Som*. These strains have high acid tolerance and produce lactic acid for the tangy flavor of fermented foods (Aymerich *et al*., 2003). Moreover, *Lact. curvatus* resulted in a rapid pH decrease, which affects the firmness, color, aroma, and flavor development of fermented foods (Visessanguan *et al*., 2006).

The study showed that different LAB strains were detected in *Kung-Som* at each day of the fermentation periods in accord with the culturing method. *Ent. faecalis*, *Ent. sanguinicola*, *W. cibaria*, *W. confusa*, *Enterococcus* sp., *Leuc. pseudomesenteroides*, and *Lact. plantarum* were isolated. However, DGGE analysis highlighted the presence of *Ped. argentinicus*, *L. lactis* subsp. *lactis*, *L. garvieae*, *Lact. curvatus* as well as *Lact. sakei* and *Lact. plantarum*. Monitoring changes in the composition of LAB in *Kung-Som* showed that only *Lact. plantarum* was detected by both techniques. This could be due to *Lact. plantarum* being the most important microorganism in fermented foods and its frequently being detected throughout the fermentation process. The different amounts of the species present in the sample can also affect the concentration of the DNA extracted and its detectability. Moreover, the presence of natural constituents in *Kung-Som* such as lipids, proteins, carbohydrates and salt may render the DNA extraction very hard and some of these molecules can persist until the end of the extraction and are found in the extract. When the extracted DNA is used as template for the PCR, the matrix residues might act as inhibitors (Ercolini *et al*., 2004). In addition, in mixed populations, individual members can be identified by PCR-DGGE when the concentrations are higher than 10^4 CFU/g. The detection limit depends on the species and perhaps even the strain considered. Moreover, the number and the concentration of the other members of the microbial community, the nature of food, all represent variables influencing the detection limit of DGGE by affecting both the efficiency of DNA extraction and the PCR amplification due to the possible competition among templates (Ercolini *et al*., 2004; Fontana *et al*,. 2005b).

Ent. faecalis, *Ent. sanguinicola*, *W. cibaria*, *W. confusa*, *Enterococcus* sp. and *Leuc. pseudomesenteroides* were detected in *Kung-Som* at day 0-2 only by the culturing method. As the MRS agar used in the study was a complex nutrient for the growth of these strains. Furthermore, these strains are the dominant microflora in raw materials for fermented *Kung-Som*. However, DGGE analysis did not detect these strains. This was probably because the cell numbers of these strains were lower than the detection limit (10^4 cfu/ml, data not shown) of PCR-DGGE. In addition, biases at the level of DNA extraction and PCR specificity and efficiency could also have been reasons (Madoroba *et al*., 2011). The concentration of the DNA extracted was insufficient to hybridize with primers in the PCR reaction. Thus, the DNA of these strains was not amplified due to the competition in DNA hybridization between the DNA templates of LAB strains with primers. The high concentration of the DNA templates were hybridized with primers and amplified in the PCR reaction, whereas the low concentration of the DNA templates were not hybridized with primers and not amplified.

Ped. argentinicus, *L. lactis* subsp. *lactis*, *L. garvieae*, *Lact. curvatus* and *Lact. sakei* were detected in *Kung-Som* by DGGE analysis but not detected by the culturing method. This may be due to these strains being present in numbers higher than the detection limit of PCR-DGGE and MRS agar has insufficient substrates for the growth of these strains. *Pediococcus* grow in the presence of NaCl (Chen *et al*., 2006). *Lactococcus* such as *L. lactis* subsp. *lactis* grow in M17 agar and may be explained by the fact that M17 agar is an elective medium (Beukes *et al*., 2001). In addition these strains enter a viable but non-cultivable state, characterized by metabolically active cells that do not produce colonies on MRS agar (Madoroba *et al*., 2011). Furthermore, in this culturing method, the LAB colonies were randomly selected from each plate with 30-300 colonies. Thus, the predominant LAB that plays an important role in the fermented *Kung-Som* was able to grow on MRS agar in the range of 30-300 colonies, which could usually be counted at dilutions of 10^{-4} -10^{5}. The minor LAB group present could not be detected on MRS agar at the high dilutions (10^{-4}-10^{-5}) but could be detected at the lower dilutions (10^{-1}-10^{-3}). With the high dilutions, the LAB was able to grow on MRS agar in numbers greater than 300 colonies. Therefore, some LAB strains on MRS agar at the high dilutions were not analyzed.

4. Conclusions

LAB play important roles during *Kung-Som* fermentation. This is the first report showing the differences and similarities in the populations of LAB in *Kung-Som* using culture-dependent and culture-independent (PCR-DGGE) techniques. This study indicated that *Kung-Som* contained a diverse spectrum of LAB including *Enterococcus*, *Weissella*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Lactobacillus*. Furthermore, both methods detected different LAB strains in *Kung-Som* on each day of the fermentation periods. Thus, the study of the bacterial community in *Kung-Som* should combine culture-dependent and culture-independent methods. Our findings also provide important information for the designing of autochthonous starter cultures.

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References

Aymerich, T., Martin, B., Garriga, M., & Hugas, M. (2003). Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausage. *Applied & Environmental Microbiology, 63*, 4583-4594.

Beukes, E. M., Bester, B. H., & Moster, J. F. (2001). The microbiology of South African traditional fermented milks. *International Journal of Food Microbiology, 63*, 189-197.

Bruyne, K. D., Franz, C. M. A. P, Vancanneyt, M., Schillinger, U., Mozzi, F., Valdez, G. F. D., Vuyst, L. D., & Vandamme, P. (2008). *Pediococcus argentinicus* sp. nov. from Argentinean identification of *Pediococcus* species by *pheS*, *rpoA* and *atpA* sequence analysis. *International Journal of Systematic and Evolutionary Microbiology, 58*, 2909-2916.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., & Rantisiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *International Journal of Food Microbiology, 167*, 29-43.

Chen, Y. S., Yanagida, F., & Hsu, J. S. (2006). Isolation and characterization of lactic acid bacteria from dochi (fermented black beans), a traditional fermented food in Taiwan. *Letters in Applied Microbiology, 43*, 229-235.

Ercolini, D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods, 56*, 297-314.

Fontana, C., Cocconcelli, P. S., & Vignolo, G. (2005a). Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *International Journal of Food Microbiology, 103*, 131-142.

Fontana, C., Vignolo, G., & Cocconcelli, P. S. (2005b). PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. *Journal of Microbiological Methods, 63*, 254-263.

Fuka, M. M., Engel, M., Skelin, A., Redzepovic, S., & Schloter, M. (2010). Bacterial communities associated with the production of artisanal Istrian cheese. *International Journal of Food Microbiology, 142*, 19-24.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiology Review, 26*, 163-171.

Hwanhlem, N., Watthanasakphuban, N., Riebroy, S., Benjakul, S., H-Kittikun, A. & Maneerat, S. (2010). Probiotic lactic acid bacteria from *Kung-Som*: isolation, screening, inhibition of pathogenic bacteria. *International Journal of Food Science and Technology, 45,* 594-601.

Lu, S., Xu, X., Zhou, G., Zhu, Z., Meng, Y., & Sun, Y. (2010). Effect of starter cultures on microbial ecosystem and biogenic amines in fermented sausage. *Food Control, 21*, 444-449.

Madoroba, E., Steenkamp, E. T., Theron, J., Scheirlinck, I., Cloete, T. E., & Huys, G. (2011). Diversity and dynamics of bacterial populations during spontaneous sorghum fermentations used to produce *ting*, a South African food. *Systematic and Applied Microbiology, 34*, 227-234.

Mugula, J. K., Nnko, S. A. M., Narvhus, J. A., & Sørhaug, T. (2003). Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology, 80*, 187-199.

Müller, C. P., Huss, H. H., & Gram, L. (1999). Characterization of lactic acid bacteria isolated from a Thai low-salt fermented fish product and the role of garlic as substrate for fermentation. *International Journal of Food Microbiology, 46*, 219-229.

Ongol, M. P., & Asano, K. (2009). Main microorganisms involved in the fermentation of Ugandan ghee. *International Journal of Food Microbiology, 133*, 286-291.

Rantsiou, K., & Cocolin, L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods. *International Journal of Food Microbiology, 108*, 255-267.

Rantsiou, K., Comi, G., & Cocolin, L. (2004). The *rpoB* gene as a target for PCR-DGGE analysis to follow lactic acid bacteria population dynamics during food fermentations. *Food Microbiology, 21*, 481-487.

Saithong, P., Panthavee, W., Boonyaratanakornkit, M., & Sikkhamondhol, C. (2010). Use of a starter culture of lactic acid bacteria in *plaa-som*, a Thai fermented fish. *Journal of Bioscience and Bioengineering, 110*, 553-557.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D., Rea, M. C., Lombardi, A., Cogan, T. M., Kalantzopoulos, G. & Tsakalidou, E. (2001). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal, 11*, 621-647.

Temmerman, R., Scheirlinck, I., Huys, G., & Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 69,* 220-226.

Urso, R., Comi, G., & Cocolin, L. (2006). Ecology of lactic acid bacteria in Italian fermented sausage: isolation, identification and molecular characterization. *Systematic and Applied Microbiology, 29,* 671-680.

Visessanguan, W., Benjakul, S., Riebroy, S., & Thepkasikul, P. (2004). Changes in composition and functional properties of proteins and their contributions to Nham characteristics. *Meat Science, 66,* 579-588.

Visessanguan, W., Benjakul, S., Smitinont, T, Kittikun, C., Thepkasikul, P., & Panya, A. (2006). Changes in microbiological, biochemical and physico-chemical properties of *Nham* inoculated with different inoculum levels of *Lactobacillus curvatus*. *LWT Food Science and Technology, 39*, 814-826.