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Original Article

Safety evaluation and bacterial community of *kung-som* using PCR-DGGE technique

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Abstract

This study evaluates the safety of *kung-som* which was distributed in local markets and using PCR-DGGE technique to identify microflora in *kung-som.* Lactic acid bacteria (LAB) were found at counts of more than 7 log CFU g^{-1} in all samples and the total viable counts were about 5-8 log CFU g^{-1}. *Bacillus cereus* and yeasts were detected at around 2 log CFU g^{-1} and 5-6 log CFU g^{-1}, respectively. For DGGE analysis, LAB and coagulase negative staphylococci (CNS) bacteria dominated over other microorganisms. The sequencing of the DNA bands from DGGE gels corresponding to *kung-som* samples showed the presence of LAB as the major microflora in the products, namely: *Lactobacillus farciminis*, *Lactobacillus plantarum*, *Lactococcus garvieae*, *Tetragenococcus halophilus* and *Weissella thailandensis*. In addition, *Staphylococcus carnosus* was detected in *kung-som* as minor microflora. These dominant strains would allow the development of defined starter cultures for improving the quality of *kung-som*.

Keywords: *Kung-som*, food safety, microflora, DGGE

1. Introduction

Kung-som is a traditionally Thai fermented shrimp, which is widely consumed in southern Thailand. It is generally made by mixing 7% (w/w) salt and 30% (w/w) sugar to shrimp (Hwanhlem *et al*., 2010), and fermenting until a final acceptable sour-tasting product is obtained. As a traditional fermented food product, its recipes vary by region, depending upon the ingredients available and local consumer preferences. This results in processes that vary in microbiological terms and a production process that traditionally relies on a spontaneous fermentation. Lactic acid bacteria (LAB) play an important role in the *kung-som* fermentation, and are found in the ingredients, on the processing utensils and in the local atmosphere. The bacterial communities associated with fermented shrimp have been studied, there is no compelling evidence regarding bacterial fingerprinting during fermentation process. Previous studies have characterized bacterial communities by classical taxonomic methods using an analysis of the cellular fatty acids and carbon source utilization patterns and thus been restricted to the isolation of a small number of strains. However, the results obtained with these different methods of analysis are not directly comparable.

Currently, cultivation methods are not available for detection and identification of microbial populations involved in fermented food. This method is not providing

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reliable information on the composition of the entire microbial community (Randazzo *et al*., 2012). Many recent studies have used the culture-independent 16S rDNA-based PCR technique to determine the diversity and dynamics of the microflora (Ercolini, 2004) in foods such as fermented sausages (Fontana *et al*., 2005), fermented fish (Fujii *et al*., 2011) and kimchi (Lee *et al*., 2005). Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons has also been shown to be a suitable tool for the analysis of microbial communities. This process allows the rapid detection of the composition of microflora species in fermented products. However, there are no previous reports of bacterial communities in *kung-som*. Therefore, the aims of the present study were to assess the safety of *kung-som* products sold in local markets, and to identify the dominant bacterial species in the finished product of *kung-som*. This was done using a molecular approach combining PCR-amplification of the V3 region of the 16S rDNA and DGGE.

2. Materials and Methods

2.1 *Kung-som* samples

Eighteen samples of *kung-som*, sold as a commercial product, were bought from randomly selected vendors scattered over different markets in Songkhla Province. The samples were transported to the laboratory for analysis.

2.2 Microbiological analysis

The microbiological analysis of *kung-som* was based on the methods described in the Biological Analysis Manual (BAM, 1998) and the Thai Community Product Standard (TCPS, 2014). Representative samples (25 g) were transferred into 225 ml sterile peptone water (0.1% (w/v) peptone; Himedia, Mumbai, India) and homogenized using a stomacher machine at 230 rpm for 1.5 min. The samples were further diluted in a 10-fold serial dilution and 100 µl of the appropriate dilutions were spread-plated on plate count agar (PCA, Hi-media, Mumbai, India) for the total viable count. They were spread on MRS medium (Lab M, Lancashire, UK) for LAB, and on potato dextrose agar (PDA; Hi-media, Mumbai, India) for yeast and mould. The cultures on the PCA and MRS medium were incubated at 35°C for 24-48 h, and those on PDA medium were incubated at 30°C for 48-72 h.

The enumeration of *Bacillus cereus* and *Staphylococcus aureus* was done by spread-plating on mannitol egg yolk polymixin agar (Hi-media, Mumbai, India), and Baird-Parker agar (Hi-media, Mumbai, India) and incubating at 35°C for 24-48 h, respectively. The *Clostridium perfringens* was pour-plated using tryptose sulfite cycloserine agar (Merck, Darmstadt, Germany), and incubated at 35°C in an anaerobic condition for 24-48 h. Total coliform bacteria and *Escherichia coli* were enumerated using the 3-tubes Most Probable Number (MPN) method. *Salmonella* sp. from each sample was applied at certain intervals according to the method described by BAM (1998).

2.3 pH and acidity measurement

The pH of undiluted juice samples was measured using a pH meter (420A ORION; Thermo Scientific, MA, USA). The determination of lactic acid (%) in *kung-som* was determined as described by AOAC (2000).

2.4 Direct extraction of bacterial DNA from *kung-som*

Ten grams of the sample was homogenized in a stomacher bag with 90 ml of peptone water for 1.5 min. The debris was allowed to precipitate for 1 min. Genomic DNA was extracted as previously described by Cocolin *et al*. (2004) with some modifications. For the first step, the supernatant was centrifuged at 6,700×*g* at 4°C for 10 min. The cell pellets were resuspended in 50 µl of lysozyme (20 mg ml^{-1}; Sigma-Aldrich, MO, USA) and incubated at 37°C for 30 min. Then 30 µl of proteinase K (25 mg ml^{-1}; Amresco, OH, USA) in 150 µl of proteinase K buffer was added and incubated at 65°C for 60 min. Then, 400 µl of breaking buffer was added and mixed by tube inversion. Four hundred microliters of phenol: chloroform:isoamyl alcohol (25:24:1 v/v/v; Amresco, OH, USA) was added for protein elimination and mixed by tube inversion. The tubes were centrifuged at 11,300×*g* at 4°C for 15 min and the aqueous phase was precipitated by adding 1 ml ice-cold isopropanol and centrifuged at 11,300×*g* at 4°C for 15 min. Then the pellet was washed with 200 µl of 70% (w/v) alcohol and centrifuged at 11,300×*g* at 4°C for 15 min. The genomic DNA was dried under room temperature for 15 min. Finally, the genomic DNA was suspended in 30 µl of sterile MiliQ water and kept at -20°C.

2.5 PCR amplification of V3 region of 16S rDNA and DGGE analysis

Genomic DNA was used as template material to amplify the V3 region of the 16S rDNA by PCR using the primers 341f and 518r (Muyzer *et al*., 1993). PCR reaction was performed in 50 µl containing 0.2 µM of each primer, 25 µl of Genei Red Dye PCR Master Mix (Merck, Darmstadt, Germany), DNase free water 14 µl and 1 µl of template DNA. The PCR was performed by using a thermal cycler TC-5000 (Techne™, Bibby Scientific, Staffordshire, UK) with an initial denaturing of 3 min at 95°C, followed by 25 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C with a final extension of 10 min at 72°C. Amplicon was analyzed by electrophoresis on 2% (w/v) agarose gel containing 1×SYBR Safe (Invitrogen, CA, USA) and then visualized by UV transillumination with the GelDoc (UVItech, Cambridge, UK).

For DGGE analysis, the PCR products of V3 region were reamplified with primer 341f-GC. The PCR program used an initial denaturing for 3 min at 95°C; followed by 25 cycles

of 30 s at 94°C, 1 min at 65°C, 30 sec at 72°C; and finally, 10 min at 72°C. Sequence specific separations of PCR amplification products were performed by using an OmniPAGE Maxi Electrophoresis Systems VS20-DGGE (Cleaver Scientific, Warwickshire, UK) PCR samples (~40 µg) were loaded on the DGGE gels that were formed with 8% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide, ratio: 37.5:1; Amresco, OH, USA) using a denaturant gradient 28-55% (w/v). The electrophoresis operation was performed at 20 V for 10 min and then 85 V for 15 h in 1×TAE buffer maintained at 60°C. After staining the gel with 1×SYBR Gold (Invitrogen, CA, USA) for 30 min, the gel was rinsed for 5 min in MiliQ water and photographed with the GelDoc.

2.6 Excision and sequencing of the DGGE fragments and phylogenetic analysis

The bands were cut from the DGGE gels by using sterile scalpels and punched with pipette tips. The DGGE bands were resuspended in 20 µl of sterile MiliQ water and stored overnight at 4°C as previously described by Fontana *et al*. (2005). An aliquot of eluted DNA was used for reamplification with the forward primer without the GC-clamp. DNA fragments were purified using a HiYield Gel/PCR Fragments Extraction Kit (RBC, Taipei, Taiwan). The purified PCR fragments were sent to the 1st BASE company (Kuala Lumpur, Malaysia) for sequencing. DNA sequences were analyzed using Blastn of NCBI nucleotide to determine the closet known relatives of the partial 16S rDNA sequence deposited in the GenBank database (Tamura *et al*., 2011).

3. Results and Discussion

3.1 Microbiological safety

Kung-som, an indigenous salted shrimp, is characterized by acidity with a final pH of lower than 4.6 (TCPS, 2014). The microbiological safety of *kung-som* is of major importance to consumers and food industry. Generally, *kung-som* is not heated or boiled before consumption so that there is risk of foodborne illnesses if organic acids in the fermentation process do not inhibit all the pathogenic bacteria, which are found in raw materials. This study therefore offers the first information about the microbial status of traditionally fermented shrimp in southern of Thailand. A total of 18 samples of *kung-som* were bought from different local markets in Songkhla Province, Thailand. They were analyzed for microbiological safety based on the TCPS (2014) as shown in Table 1. The microbial population was predominated by LAB (>7 log CFU g^{-1}). Number of total viable counts (TVC) ranged from 5-8 log CFU g^{-1}, that was similar to results (4-8 log CFU g^{-1}) in fermented shrimp (*kung-chom*) (Prachasitthisak *et al*., 2009). Lee *et al*. (2002) reported that the number of TVC of salted (15% and 20%) fermented shrimp (*jeotkal*) was 5

Table 1. Microbiological characteristic of *kung-som* collected from local markets in Songkhla Province.

Sample	Microbiological analyses (log CFU/g)								
	TVC	LAB	*S. aureus*	*C. perfringens*	*B. cereus*	Total coliform (MPN/g)	*E. coli* (MPN/g)	*Salmonell*a sp.	Yeasts and Moulds
Sample A1	6.9	8.5	ND[a]	ND	ND	<3	ND	ND	5.7
Sample A2	6.6	7.9	ND	ND	ND	<3	ND	ND	4.7
Sample A3	5.4	7.0	ND	ND	1.5	<3	ND	ND	<1.0
Sample A4	5.5	8.6	ND	ND	1.5	<3	ND	ND	3.8
Sample A5	8.2	7.9	ND	ND	1.3	<3	ND	ND	6.0
Sample A6	5.7	8.0	ND	ND	1.3	<3	ND	ND	3.1
Sample A7	5.6	7.5	ND	ND	1.3	<3	ND	ND	5.6
Sample A8	5.8	8.3	ND	ND	1.5	<3	ND	ND	5.3
Sample A9	5.2	7.7	ND	ND	1.3	<3	ND	ND	6.0
Sample A10	6.7	8.1	ND	ND	ND	<3	ND	ND	5.1
Sample A11	5.5	8.2	ND	ND	1.3	<3	ND	ND	5.4
Sample A12	5.8	8.1	ND	ND	1.5	<3	ND	ND	5.6
Sample A13	8.6	8.8	ND	ND	ND	<3	ND	ND	6.8
Sample A14	5.8	8.5	ND	ND	ND	<3	ND	ND	3.5
Sample A15	5.6	8.2	ND	ND	1.5	<3	ND	ND	5.3
Sample A16	6.7	8.1	ND	ND	1.8	<3	ND	ND	5.2
Sample A17	6.4	7.8	ND	ND	1.5	<3	ND	ND	6.5
Sample A18	7.5	8.5	ND	ND	2.0	7.2	ND	ND	5.9
TCPS[b]			<2.0	ND			<10	ND	<2.7 (moulds)

[a]ND: Not detected
[b]TCPS: Thai Community Product Standard.

log CFU ml^{-1}. The microbial population was predominated by LAB (>7 log CFU g^{-1}). Various authors have reported LAB as microflora in Thai fermented products (*pla-ra*, *plaa-som, kung-chom*) (Kopermsub and Yunchalard, 2010; Tanasupawat, 2009). Count of total coliforms on samples showed low bacterial population. Pathogenic bacteria, such as *C. perfringens*, *E. coli*, *S. aureus* and *Salmonella* sp., were not detected in any of the samples. The presence of *B. cereus* at a low level of 2 log CFU g^{-1} was detected in some of the samples (Table 1). However, it did not exceed the threshold risk level of 4 log CFU g^{-1} (Dong, 2013). EFSA (2005) reported that in most instances, foodborne diseases caused by *B. cereus* were associated with 5-8 log CFU g^{-1}. Yeasts were detected at a level of 5-6 log CFU g^{-1} and moulds were not recovered in any samples.

Kung-som had a pH value lower than 4.2 and the range of lactic acid values ranged from 1.4-3.5% (Figure 1). The reduction of pH was mainly due to organic acids produced by LAB, and it has been proposed that the organic acid greatly inhibits many pathogenic and food spoilage bacteria. According to the results, *kung-som* samples which were collected from Songkhla Province are safe for consumer consumption (TCPS, 2014).

3.2 Bacterial community of *kung-som* using DGGE technique

Kung-som fermentation continues to be performed in a traditional manner, resulting in a great bacterial diversity of the final products. In the present paper, a PCR-DGGE technique was used for the first time to systematically study the bacterial communities of Thai traditional fermented shrimp. The fingerprints obtained from the bacterial community in finished products of *kung-som* were analyzed using the DGGE technique, which demonstrated 16 differently visible bands as shown in Figure 2. No difference was detected in

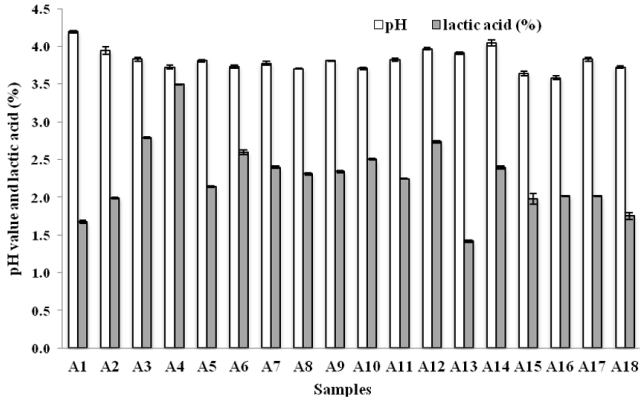

Figure 1. pH and lactic acid (%) of *kung-som* collected from local markets in Songkhla Province.

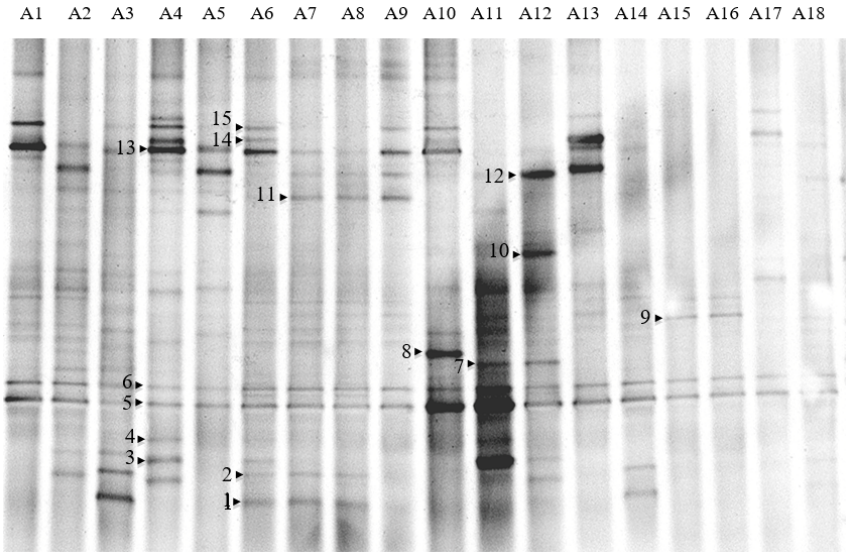

Figure 2. Denaturing gradient gel electrophoresis (DGGE) profiles of DNA amplicons obtained directly from *kung-som*. Sequence of bands (1-15) was searched in the GenBank with the BLAST program to determine the closest known relatives of the partial 16S rDNA sequences obtained (Table 2).

the DGGE profiles when replicates obtained from the same samples were analyzed (data not shown). To identify the main DGGE bands, each band was recovered from the DGGE gel and sequenced. Figure 3 shows the phylogenetic relationships based on the sequence results. These sequences exhibited a greater than 96% identity to the sequences deposited in the databases (Table 2) which were identified as belonging to 7 genera: *Alkalibacterium*; *Enterococcus; Lactobacillus; Lactococcus; Staphylococcus; Tetragenococcus* and *Weissella*.

These LAB were the major component in the bacterial composition of *kung-som*. The bands indicative for *Lb. farciminis* (band 4, 5 and 6) and *Lb. plantarum* (band 12) were observed with a high intensity in *kung-som*, indicating their important role in *kung-som* fermentation. *Lb. farciminis* showed multiple bands in DGGE gels. Ercolini (2004) has reported that a single species with multiple rDNA copies could demonstrate maniple bands in a DGGE gel, which overestimates the community diversity detected by PCR-DGGE technique. *Lactobacillus* species, especially *Lb. farcinis* and *Lb. plantarum* have been frequently isolated from various aquatic products such as commercial cold smoked salmon (Tomé *et al*., 2006) and Asian fermented seafood products such as Thai fermented fish and shrimp (Tanasupawat *et al*., 2000; Tanasupawat, 2009), Japanese fermented fish (Fujii *et al*., 2010) and Taiwanese fermented clams (Chen *et al*., 2012). In the present study, *Lb. farciminis* and *Lb. plantarum* were detected in the final stage of the fermentation process using DGGE technique; these are acid-tolerant LAB and can grow in high acidity conditions (Kopermsub and Yunchalard, 2010; Miyashita *et al*., 2012). In addition, *Lc. garvieae* (band 13), *T. halophilus* (band 1 and 2) and *W. thailandensis* (band 14 and 15) were also identified as significant components of the microflora in *kung-som*.

Lc. garvieae has been previously reported as the strain isolated from fermented fish products (*plaa-som*) during an early stage of fermentation (Kopermsub and Yunchalard, 2010) and it can be isolated from brined shrimp and fermented clams (Chen *et al*., 2012; Dalgaard *et al*., 2003). Chuon *et al*. (2014) reported that *T. halophilus* is a bacterial flora in various fermented seafood products that was detected by culture dependent and culture independent techniques.

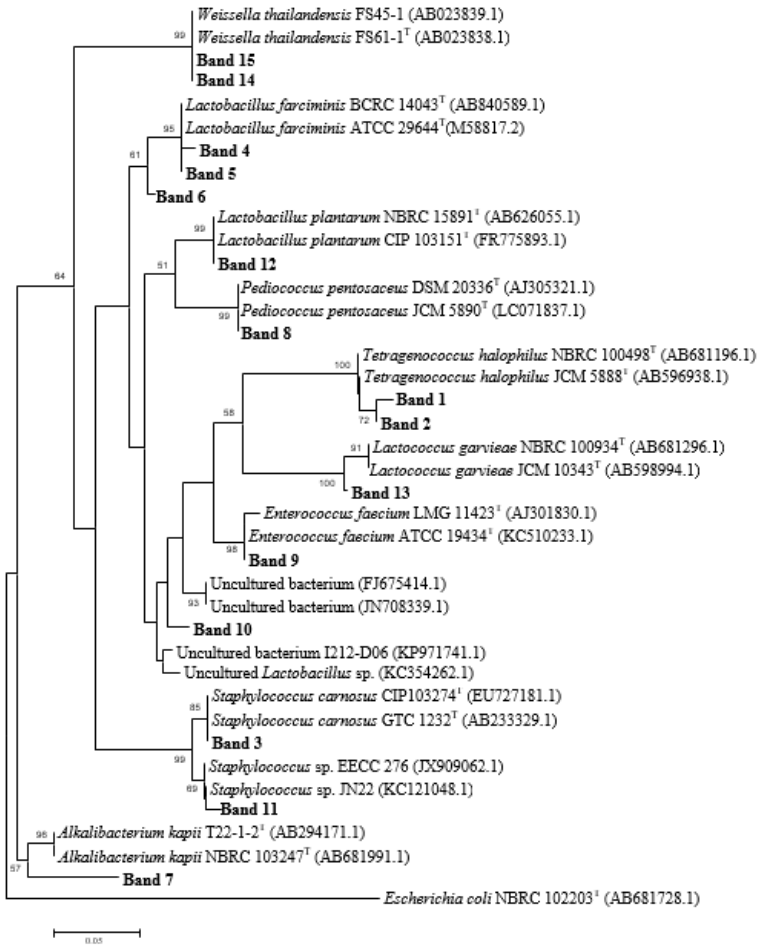

Figure 3. Phylogenetic tree analysis was based on V3 region of 16S rDNA sequence. The tree was constructed by the neighbor-joining method, and *E. coli* NBRC 102203^T was used as the out-group. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown.

Table 2. Identification of dominant fragments in DGGE patterns in *kung-som* collected from local markets in Songkhla Province.

Band no.[a]	Highest match with accession number in parenthesis	Identity (%)	Accession numbers[b]
1	*Tetragenococcus halophilus*	99	AB681296.1
2	*Tetragenococcus halophilus*	100	AB681296.1
3	*Staphyloccous carnosus*	100	EU727181.1
4	*Lactobacillus farciminis*	98	AB840589.1
5	*Lactobacillus farciminis.*	100	AB840589.1
6	*Lactobacillus farciminis*	96	AB840589.1
7	*Alkalibacterium kapii*	97	AB681991.1
8	*Pediococcus pentosaceus*	100	AJ305321.1
9	*Enterococcus faecium*	96	AJ301830.1
10	Uncultured bacterium	98	JN708339.1
11	*Staphylococcus* sp.	99	KC121048.1
12	*Lactobacillus plantarum*	100	AB626055.1
13	*Lactococus garvieae*	100	EU121676.1
14	*Weissella thailandensis*	100	AB023838.1
15	*Weissella thailandensis*	99	AB023839.1

[a]Band number as indicated on DGGE gels as shown in Figure 2.
[b]Accession number of the sequence of the closest relative species identified using the Blast software.

It has been reported that *Weissella* species was found in certain aquatic products. Tanasupawat *et al*. (2000) reported that *W. thailandensis* was the most frequently found *Weissella* species in Thai fermented fish food. From the data of previous studies, it is known that *W. hellenica* was the most abundant LAB in Taiwanese fermented clams (Chen *et al*., 2012). This is the first report of *W. thailandensis* in fermented shrimp, *kung-som*, using DGGE technique.

A. kapii (band 7), *Ent. faecium* (band 9) and *P. pentosaceus* (band 8) were found in some of the samples. As previously described, fermented foods are made differently in different regions owing to location-specific factors such as raw materials for fermentation, ingredients available in the area, climate condition and fermentation methods and these affect the bacterial flora in the product (Lee *et al*., 2005). Literature reports also confirmed the isolation *Ent. farciminis* and *P. pentosaceus* from different kind of marine food products and Thai fermented food (Miyashita *et al*., 2012). *Alkalibacterium* spp. with alkaliphilic and halophilic properties were isolated from marine organism. Few studies have reported the presence of *A. kapii* in fermented foods. Ishikawa *et al*. (2009) reported that *A. kapii* is a halophillic bacterium isolated from Thai fermented shrimp paste (*ka-pi*), salted fish and raw fish.

Among the bacteria isolated from *kung-som*, CNS made up the minor part of the community; *S. carnosus* (band 3) and *Staphylococcus* sp. (band 11) were detected in DGGE gels. *S. carnosus* are halotolerant bacteria and, as with CNS, their pathogenicities have not yet been reported (Guan *et al*., 2011). They have nitrite and nitrate reductase activity, enhance the flavor of fermented foods, promote the desired red color development and help stabilization, which limits lipid oxidation and prevents rancidity. *S. carnosus* was the dominant species detected in fermented sausage and is used as a starter culture in fermented sausages (Fontana *et al*., 2005; Talon *et al*., 2007).

4. Conclusions

We studied the bacterial flora of the traditional Thai fermented shrimp (*kung-som*) using DGGE analysis. *Lb. farciminis*, *Lb. plantarum*, *Lc. garvieae*, *T. halophilus* and *W. thailandensis* were detected as the dominant bacteria species. The subdominant bacterial species in *kung-som* identified as *S. carnosus*. This provides useful information for the further development of bacterial starter culture in order to establish a more controllable *kung-som* process that gives a product with more consistent quality.

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