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Original Article

Efficiency of the V3 region of 16S rDNA and the *rpo*B gene for bacterial community detection in Thai traditional fermented shrimp (*Kung-Som*) using PCR-DGGE techniques

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Abstract

Kung-Som is one of several Thai traditional fermented shrimp products, that is especially popular in the southern part of Thailand. This is the first report to reveal the bacterial communities in the finished product of *Kung-Som*. Ten *Kung-Som* samples were evaluated using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methodology combined with appropriate primers to study the dynamics of the bacterial population. Two primer sets (V3; 341f(GC)-518r and *rpo*B; *rpo*B1698f(GC)-*rpo*B2014r primers) were considered as a possible tool for the differentiation of bacteria and compared with respect to their efficiency of 16S rDNA and *rpo*B gene amplification. PCR-DGGE analysis of both the V3-region and *rpo*B amplicon was successfully applied to discriminate between lactic acid bacteria and Gram positive strains in the bacterial communities of *Kung-Som*. In conclusion, the application of these two primer sets using PCR-DGGE techniques is a useful tool for analyzing the bacterial diversity in *Kung-Som*. Moreover, these preliminary results provide useful information for further isolation of desired bacterial strains that could be used as a starter culture in order to improve the quality of *Kung-Som*.

Keywords: *Kung-Som*, *rpo*B gene, V3 region, PCR-DGGE, bacterial community

1. Introduction

Kung-Som is a traditional fermented shrimp product that is found widely distributed in the south of Thailand. It is made from shrimp, sugar, salt and water and is typically fermented with the natural, spontaneous microbial flora. The microbiology of *Kung-Som* is diverse and complex. The principal microorganisms found in *Kung-Som* are various lactic acid bacteria (LAB) (Tanasupawat *et al*., 1998; Hwanhlem *et al*., 2010). Species identification and population enumeration are critical in the study of bacterial communities. Due to the limitations of conventional microbiological methods, the identification of microorganisms that requires selective enrichment and subculturing is problematic or

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impossible. Moreover, classical microbial techniques used have not accurately analyzed the presence of the main bacterial species (Ben Omar and Ampe, 2000) and have not provided a completely accurate representation of these complex communities. On the other hand, culture-independent molecular techniques have provided better methods to give more information on the microbial diversity in complex food samples (Cocolin *et al*., 2001; Ercolini, 2004). In addition, culture-independent molecular techniques based on specific nucleotide sequences are widely used for monitoring, detection, identification and classification of bacterial diversity.

In the recent decade, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been successfully applied to determine the microbiology of fermented food such as fermented sausages (Fontana *et al*., 2005), fermented grains (Chao *et al*., 2008), fermented meat (Hu *et al*., 2009), and fermented dairy products (Liu *et al*., 2012), to name a few. This approach has provided new insight into the microbial diversity and allowed a more rapid, high-resolution description of microbial communities than did the traditional approaches since it allows the separation of DNA molecules that differ by single bases (Ercolini, 2004). The use of appropriate consensus primers is also a critical point in determining the resolution of DGGE analysis in mixed microbial systems, especially in LAB differentiation (Chen *et al*., 2008). From the evidence of several published papers, the 16S rDNA seems to be by far the most widely used as a molecular marker for the determination of the phylogenetic relationships of bacteria. The hypervariable V3-region on the 16S rDNA is the most frequently used to start the study of an unknown and complex bacterial community. In addition, the V3-region is considered to have a high grade of resolution and to be highly variable, and it is regarded as a good choice when it comes to length and inter-species heterogeneity (Coppola *et al*., 2001; Florez and Mayo, 2006; Hovda *et al*., 2007; Chen *et al*., 2008). Unfortunately, one problem related to the use of 16S rDNA in DGGE analysis is the complexity created by the existence of multiple heterogeneous copies within a genome (Dahllof *et al*., 2000; Crosby and Criddle, 2003; Rantsiou *et al*., 2004).

Consequently, a solution to the problem of 16S rDNA heterogeneity is provided by the analysis of a gene that exists in only a single copy (Fogel *et al*., 1999). Certain protein-coding genes, such as the gene encoding the beta-subunit of DNA-directed RNA polymerase, *rpo*B, have been proposed to fulfill this criterion. *rpo*B is used as a potential biomarker to overcome identification problems because it is considered a housekeeping gene. Targeting the *rpo*B gene allowed the reliable discrimination of species. The use of this gene as a marker was able to avoid the intraspecies heterogeneity problem caused by the use of the 16S rDNA, which appears to exist in one copy only in bacteria (Dahllof *et al*., 2000; Ko *et al*., 2002). In addition, in some strains of bacteria, an internal region of *rpo*B is a more suitable sequence than 16S RNA because of its higher nucleotide polymorphism (Khamis *et al*., 2005). However, the use of *rpo*B presents a taxonomic disavadantage: the database of the sequence is less well documented than that of the 16S rDNA (Rantsiou *et al*., 2004; Renouf *et al*., 2006).

There are no data using the PCR-DGGE technique to characterize the dominant bacteria in *Kung-Som* product. Consequently, the aim of this present study was to focus on the use of the hypervariable V3-region on the 16S rDNA and *rpo*B gene by using PCR-DGGE techniques as a tool to reveal the bacteria that commonly develop in the *Kung-Som* product and to compare the efficiency of the 16S rDNA and *rpo*B gene sequences for species discrimination in such a complex food sample. The obtained results provide preliminary information for study to apply in the microbial starter cultures in *Kung-Som* fermentation.

2. Materials and Methods

2.1 Lactic acid determination in *Kung-Som*

Kung-Som samples were purchased from different local markets in Songkhla Province, Thailand. The pH value was measured by a pH meter (420A ORION, USA). Total acidity as lactic acid was determined according to the AOAC (AOAC, 1995). Three independent measurements were made for each sample. Data presented are the calculated means and standard deviations.

2.2 DNA extraction from *Kung-Som*

DNA was extracted from the juice sample of *Kung-Som* by the method Cocolin *et al*. (2004), with slight modification. One millilitre of juice sample of each sample was centrifuged at 14,000×g for 10 min at 4°C to pellet the cells. The pellet was washed twice with 1 ml of sterile 0.85% (w/v) NaCl. The pellet was resuspended in 50 µl of 20 $mgml^{-1}$ lysozyme (Fluka, USA). After 30 min incubation at 37°C, 30 µl of 25 $mgml^{-1}$ proteinase K (AMRESCO®, USA) and 150 µl proteinase K buffer were added. The tubes were incubated at 65°C for 90 min before the addition of 400 µl breaking buffer and incubated futher at 65°C for 15 min. Then, 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7) was added for extracting DNA, RNA and protein. The tubes were centrifuged at 12,000×g at 4°C for 10 min, the aqueous phase was collected and the nucleic acid was precipitated with 1 ml of ice-cold absolute isopropanol. The DNA was obtained by centrifugation at 14,000×g at 4°C for 10 min, washed briefly with 70% (v/v) ice-cold ethanol and centrifuged again. The DNA was dried at room temperature, resuspended in 20 µl of RNase-DNase-free sterile water, and treated with 5 µl of 10 $mgml^{-1}$ DNase-free Rnase (Vivantis, USA). After 5 min incubation at 37°C, genomic DNA was stored at -20°C.

2.3 The V3 region of 16S rDNA and *rpo*B gene amplification

Primers 341f (5′-CCTACGGGAGGCAGCAG-3´) and 518r (5′ATTACCGCGGCTGCTGG-3′) were used to amplify a region of approximately 200 bp of the V3 region of 16S rDNA (Muyzer *et al*., 1993). Primers *rpo*B1698f (5′-AACAT CGGTTTGATCAAC-3′) and *rpo*B2014r (5′-CGTTGCATGTT GGTACCCAT -3′) were used to amplify a region of approximately 350 bp of the *rpo*B gene (Dahllof *et al*., 2000). Amplification reactions were carried out in volumes of 50 µl. In addition, a GC clamp was added to the forward primer to improve the sensitivity in the detection of mutations by DGGE (Sheffield *et al*., 1989). PCR products were examined by 2% (w/v) agarose gel electrophoresis. These were used to check the quality and size of PCR products before being subjected to DGGE analysis.

2.4 DGGE analysis

DGGE analysis was performed using the Dcode universal mutation detection system apparatus (Cleaver Scientific, UK) according to Fontana *et al*. (2005) with slight modification. Thirty millilitres of PCR product was mixed with loading dye and applied to 8% (w/v) polyacrylamide gels. A 28% to 55% denaturing gradient (100% of denaturant corresponding to 7 mol l^{-1} urea and 40% formamide) were used for both the 341f(GC)-518r and *rpo*B1698f(GC)-*rpo* B2014r primer sets. Electrophoresis was run in 1X TAE buffer at constant temperature (60°C) for 10 min at 20 V and subsequently for 16 h at 85 V. After electrophoresis, the gel was stained for 30 min with 1X (final concentration) SYBR Gold (Invitrogen, USA) in 1X TAE buffer, rinsed in water, and then visualized and photographed under UV illumination with the Gel Documentation (UVI-TECH, England).

After running the DGGE analysis, relevant bands were punched from the gel with sterile pipette tips. Each piece was transferred into 20 µl of RNase-DNase-free sterile water and incubated overnight at 4°C to allow the diffusion of the DNA. Then, the eluted DNA was used as a template and re-amplification took place with primers without the GC clamp. The PCR products were purified by using the HiYieldGel/PCR DNA Fragments Extraction Kit (RBC, Taiwan), and sequenced by a DNA sequencer (Ward Medic Ltd., Malaysia).

2.5 Construction of phylogenetic tree

Searches in GenBank with the BLAST program on the NCBI website were performed to determine the closest known relatives of the determined 16S V3 region and *rpo*B gene sequences. Multiple sequence alignments were created by using the BioEdit version 3.3.19.0. The phylogenetic tree was obtained to compare similarities among the sequences by the neighbor-joining method (Saitou and Nei, 1987) using the MEGA software version 5. Kimura's method was followed and 1,000 repetitions were made for bootstrap (Tamura *et al*., 2011).

3. Results and Discussion

Kung-Som is a one of the traditional fermented food products from southern Thailand. The production process traditionally relies on a spontaneous fermentation initiated by natural and fortuitous microorganisms, mainly various LAB and coagulase-negative cocci (CNC). *Kung-Som* is made from the main raw materials shrimp, sugar, salt and water. These raw materials and personal hygiene can also be the possible sources of pathogenic microorganisms or spoilage bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus* and *Salmonella* sp. In all *Kung-Som* samples of this study, it was found that the pH and lactic acid concentration ranged from 3.58 to 4.04 and 1.78% to 3.12%, respectively (Figure 1). The pH of all samples was below 4.5 because LAB utilizes the carbohydrate substrates available to produce organic acids, and especially lactic acid, as part of their metabolites. These acids not only contribute to the taste, aroma and texture of the product but also lower the pH of the product which is one of the important key factors to ensure quality and safety (Visessanguan *et al*., 2006; Kopermsub and Yunchalard, 2010). Generally, a pH lower than 4.4 can inhibit growth of *E. coli* (Alvarado *et al*., 2006) and *Salmonella* sp. (Sorrells and Speck, 1970); a pH lower than 3.7 can inhibit *S. aureus* (Alvarado *et al*., 2006); a pH lower than 4.0 inhibits *B. cereus* (Yang *et al*., 2008) and a pH of 4.5-5.0 has been demonstrated to inhibit *V. parahaemolyticus* (Adams and Moss, 2008). Consequently, the pathogenic or spoilage bacteria were inhibited by organic acids that affected the bacterial growth and extracted DNA concentration in our samples. This finding is probably related to the PCR-DGGE detection limit (10^4 $cfuml^{-1}$). In accordance with these results, no DNA bands corresponding to pathogenic and spoilage bacteria were detected in *Kung-Som* samples of our study.

Renouf *et al*. (2006) reported that the first step for finding suitable primers is to assume that the primers must be present in all the species and delimit variable sequences to

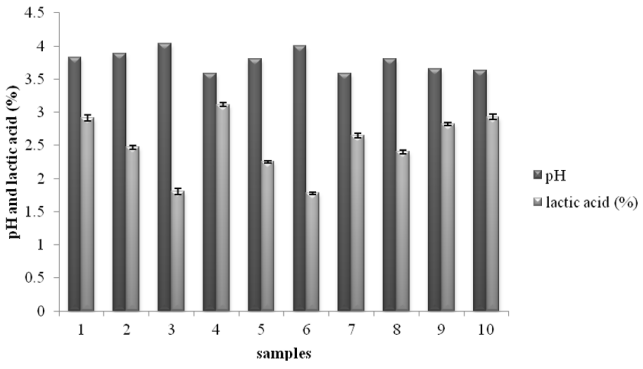

Figure 1. The pH value and titratable acidity of *Kung-Som* samples.

separate each species. The last step is to find the most suitable and accurate gradient and the best DGGE conditions (temperature, time). Two sets of primers (V3-region of 16S rDNA and *rpo*B gene) were considered suitable in this study since it is a housekeeping gene. Consequently, the bacterial diversity of the *Kung-Som* product was revealed by DGGE analysis. Figure 2 and 3 show the DGGE profiles obtained by the DNA directly extracted from *Kung-Som* in different regions in Songkhla Province, Thailand. Both the V3 region of 16S rDNA and the *rpo*B gene were amplified from these DNA templates. The V3 region of 16S rDNA and *rpo*B gene profiles displayed different patterns. The DGGE profile of the *rpo*B gene amplification showed that the numbers of the bands were lower than those of the V3 region of 16S rDNA amplification. For the DGGE profile and the phylogenetic relationship of the V3 region of 16S rDNA amplification (Figure 2A and 2B), bands corresponding to *Tetragenococcus halophilus* (band *b*), *Lactobacillus farciminis* (band *d*) and *L. plantarum* (band *l* and *k*) were prominent in all *Kung-Som* samples, which is in agreement with data published by Hwanhlem *et al*. (2010). Although band *k* was found in all samples, it was faint. *L. acetotolerans* (band *g*) and *L. rapi* (bands *j, o*, *p*) were present in all samples except samples 3 and 6. *Salinivibrio sharmensis* (band *a*) and *Macrococcus* sp. (band *e*) appeared only in sample 3 and *L. crustorum* (bands *h* and *i*) was only found in sample 6. *Staphylococcus piscifermentans* (band *c*), *Weissella thailandensis* (band *m*) and *W. cibaria* (band *n*) were exhibited in some samples but with very weak intensity. *Salinivibrio sharmensis* (band *a*) was found only in sample 3. Several bands originating from a single species were observed on the DGGE gels, and these were from *L. crustorum*, *L. rapi* and *L. plantarum*. The reason for this is the sequence heterogeneity as described by Crosby and Criddle (2003).

For the DGGE profile and the phylogenetic relationship of *rpo*B gene amplification (Figure 3A and 3B), bands 4 and 5, corresponding to *W. thailandensis* and *S. piscifermentans*, were predominating in every sample. Furthermore, *L. fermentum* (band 2) and *L. reuteri* (band 3) were present in all samples except sample 3 and 6. Band 1 was only detected

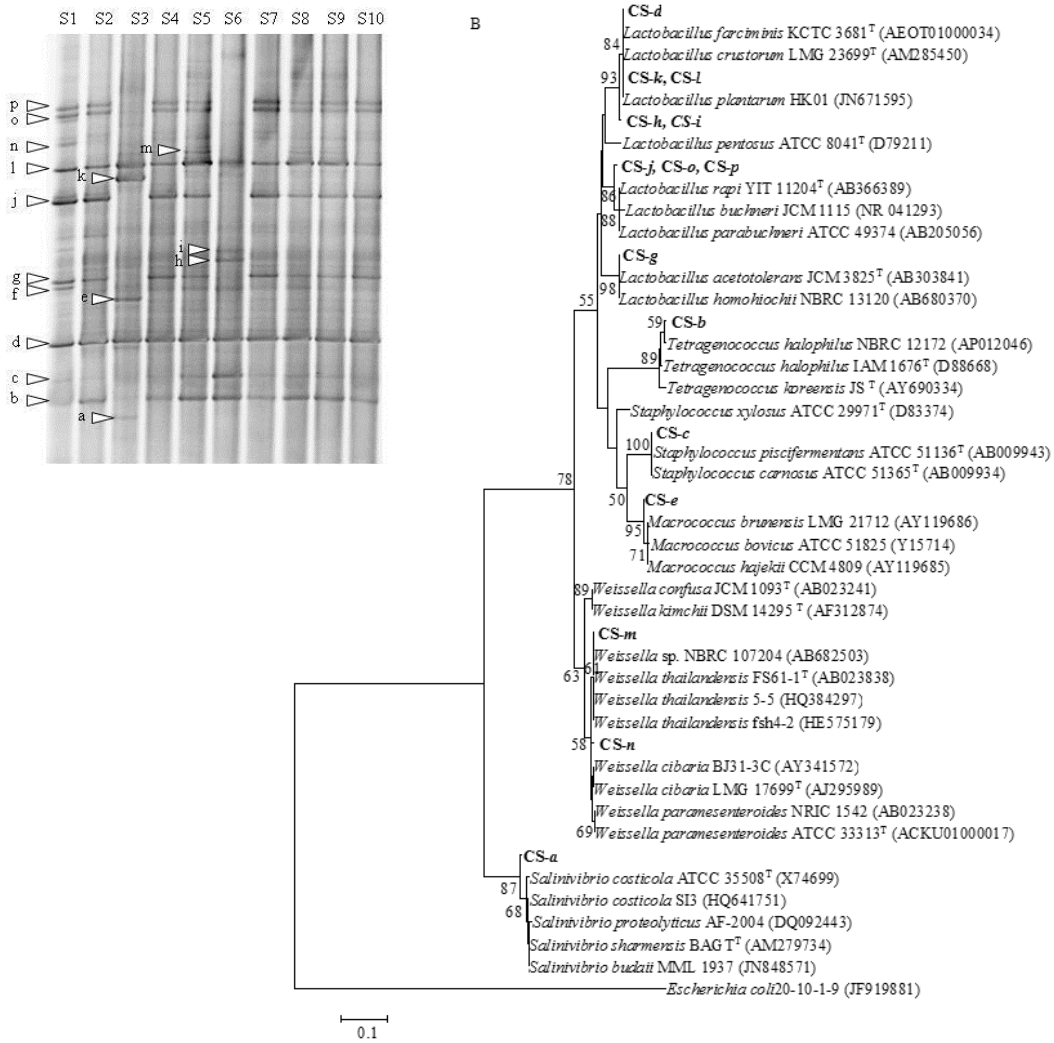

Figure 2. DGGE profiles (A) and the phylogenetic tree based on V3-region on 16S rDNA (B) of the bacteria community obtained from DNA directly extracted from *Kung-Som* samples. The scale bar represents the number of inferred substitutions per site.

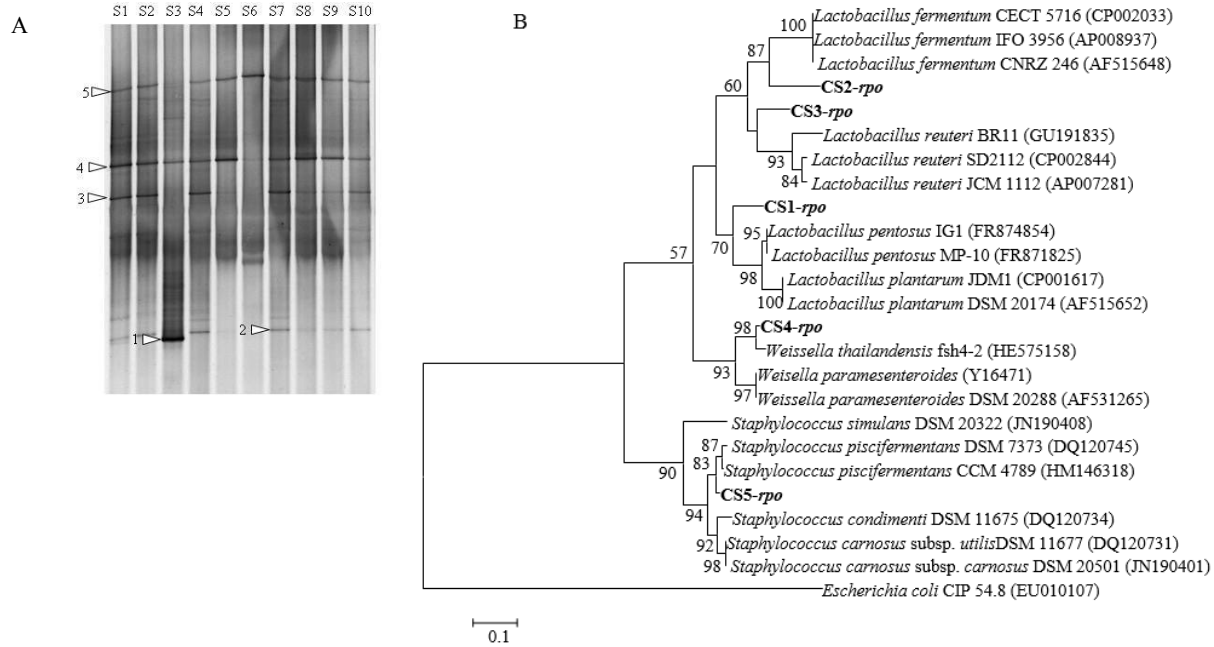

Figure 3. DGGE profiles (A) and the phylogenetic tree based on *rpo*B gene (B) of the bacteria community obtained from DNA directly extracted from *Kung-Som* samples. The scale bar represents the number of inferred substitutions per site.

in sample 3.

Species-specific DGGE bands from two sets of primers for the main members of LAB and CNC were exhibited. LAB (*Lactobacillus, Tetragenococcus* and *Weissella*), including CNC species (*Macrococcus* and *Staphylococcus*), are the most commonly isolated bacteria from fermented foods, especially meat and fish (Hu *et al*., 2008; Kopermsub and Yunchalard, 2010; Hwanhlem *et al*., 2011). In addition, the genus *Salinivibrio* was found to be the dominant species in sample 3 of the V3 region amplification. These species are generally isolated from fermented fish samples (Chamroensaksri *et al*., 2009). The differences in the results obtained, such as the DGGE pattern from the *rpo*B gene amplification, indicated the occurrence of very low bacterial diversity when compared with the V3-region amplification. These results depended on the specificity of the primers (Endo and Okada, 2005; Renouf *et al*., 2006) and the PCR conditions (Hongoh *et al*., 2003).

Rantsiou *et al*. (2004) and Renouf *et al*. (2006) reported that the use of *rpo*B gene amplification combined with PCR-DGGE can only reveal the predominant species in a sample. Moreover, Chen *et al*. (2008) suggested that the use of appropriate consensus primers is a critical point in influencing the resolution of DGGE analysis in mixed microbial systems, especially in LAB differentiation. Endo and Okada (2005) indicated that LAB could not be detected by universal bacterial PCR primer but were detected when groups of LAB-specific primer were used. This is because the DGGE profile can demonstrate only the diversity of bacteria present at more than 1% of the target bacteria. Therefore, the detection of numerous different species present at low concentrations appeared to be difficult using PCR-*rpo*B/DGGE. Thus, the *rpo*B gene pattern exhibited the different species which did not appear in the V3-region pattern.

In both the *rpo*B gene and V3-region patterns, samples 3 and 6 showed different DGGE patterns from those of compared to other samples. This is because these samples originated from different recipes or processes of preparation which could vary the initial food matrix, fermentation process, personal hygiene, local tradition or local geographic preferences. All of these are crucial factors in determining the growth of specific microbial communities (Cocolin *et al*., 2004; Ercolini, 2004; Chen *et al*., 2008). This outcome was related to the higher pH and lower lactic acid content of samples 3 and 6 (Figure 1). In accordance with this lower lactic acid concentration, a difference in the LAB groups of these samples was detected (Figures 2 and 3).

A number of faint bands could not be identified because of their low content which might be related to the heterogeneous distribution of microorganism in the food matrix (Florez and Mayo, 2006). In a detection limit analysis, an individual species (*Pediococcus pentosaceus* DMST 18752) was identified by PCR-DGGE when its number was higher than 10^4 cfuml^{-1} (data not shown). The detection limit of PCR-DGGE depends on the species or perhaps even the strain considered. Furthermore, the number and the concentration of the other members of the microbial community, along with the nature of the food matrix, all represent variables influencing the detection limit of DGGE. These factors affect both the efficiency of DNA extraction and the PCR amplification due to possible competition among templates (Ercolini, 2004; Temmerman *et al*., 2004; De Vero *et al*., 2006).

4. Conclusions

The suitability of the primers used was based on the discriminatory efficiency of the hypervariable V3-region and the *rpo*B gene that allowed species differentiation from the dominant groups of bacteria in *Kung-Som*. Although the applications of PCR-DGGE techniques combined with appropriate consensus primers to study complex microbial communities originating from food samples have been shown to be an efficient tool for detection of complex bacteria populations, we believe that our findings represent a preliminary analysis. The data cannot be considered sufficient to achieve a confident identification at species level, but should suggest that the relevant genes together with other target sequences such as other region of 16S rDNA, *gyr*A, *gyr*B, *rec*A or *rpo*C, should be used for unequivocal identification of individual species. Moreover, these preliminary results provide useful information for improving product quality. An improved understanding of the changing microflora in *Kung-Som* fermentations could be used to develop a starter culture in the future.

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