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Full Length Research Paper

Detection and quantification of probiotic bacteria using optimized DNA extraction, traditional and real-time PCR methods in complex microbial communities

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The aim of this study is to optimize molecular detection and quantification methods of probiotic bacteria in complex microbial communities that have long been difficult for traditional culture-based methods. Traditional and real-time PCR were optimized to detect and quantify *Lactobacillus* spp. and *Bifidobacterium* spp. in complex microbial community. Fish and shrimp sauce were used as a model for complex microbial community. Directly form samples, 4 DNA extraction methods, primers specificity, PCR, and real-time PCR procedures were optimized, tested in comparison with samples, enriched bacteria and related standard bacterial strains, *E. coli*, *Bacteroides*, *Enterococcus* and *Salmonella*. Results showed that extracted genomic DNA using Wizard® Genomic DNA Purification Kit showed the highest yield, quality and performance. Moreover, the specificity of the primer set specific for *Lactobacillus* spp. and *Bifidobacterium* spp. was checked and found highly specific. The sensitivity of real-time PCR was higher than the conventional PCR and its quantifying potential is very precise for the detection and quantification of *Lactobacillus* spp. but not *Bifidobacterium* spp. which was absent in the tested samples. In conclusion, PCR and real-time PCR assays could be used very efficiently in quantifying and detecting *Lactobacillus* spp. that are present in very PCR-suppressive and complex microbial environment.

Key words: PCR, real-time PCR, DNA extraction, *Bifidobacterium* spp., *Lactobacillus* spp., fermentation, probiotic.

INTRODUCTION

Probiotic bacteria are essential for healthy gastro-intestinal function. The action of probiotics on intestinal flora results in vital benefits, including protection against pathogens, development of the immune system (Isolauri et al., 2002) and positive effects on colonic health and host nutrition (Umesaki and Setoyama, 2000). The use of *Bifidobacterium* as probiotics in food or pharmaceutical products is of high value. The most representative application of *Bifidobacteria* in diet is *Bifidobacteria* yogurt and *Bifidobacteria* milk, used as non-fermented milk (Ishibashi et al., 1997). Alike, *Lactobacilli* bacteria are extensively used as probiotics and used technologically as food-associated micro-organism as they are generally recognized as safe (GRAS) (Salminen et al., 1998).

Probiotic bacteria are found in some diet abundantly rendering such diet as a health supply for proper function of the intestine. Several indigenous microorganisms are found in fermented fish products including *Bifidobacteria* and *Lactobacilli* (Salinas et al., 2008). The use of fermentation as a preservation method for fish has been of great value from earliest times. Among these products, fish sauce and shrimp sauce are the most popular products which are being used as a condiment in Southeast Asia (Yung et al., 2006). As fish fermentation involves minimal bacterial conversion of carbohydrates to lactic acid but entails extensive tissue degradation by

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proteolytic and lipolytic enzymes, fish and shrimp sauce have been shown to be the best specimens for the highly complex microbial communities (Muhling et al., 2008). Although fish and shrimp sauce harbor significant amounts of probiotic bacteria, the detection, isolation and enumeration of *Bifidobacteria* and *Lactobacilli* bacteria has always not been successful (Yongsawatdigul et al., 2007). This was attributed to the fact that fish and shrimp sauce are extremely complex microbial specimens in which most traditional culture methods of bacteria proved to be non-specific, non-accurate and due to the complexity of microbial mixture, too lengthy (Jaspers and Overmann, 1997). Unfortunately, after PCR advent, most detection and isolation of probiotic bacteria turned unsuccessful due to the lack of proper design and optimization of the molecular protocols to fit with such highly complex microbial specimens namely, fish and shrimp sauce (Muhling et al., 2008). PCR assays were not successfully applied on fermented products due to the presence of potent and complex diverse PCR inhibitors such as fermented fish products and due to difficult extraction of intact DNA (Podar et al., 2007).

Prior to the advent of PCR, identification of micro-organisms relied on bacteriological methods and subsequent biochemical tests (Fairchild et al., 2006). Moreover, some of the biochemical tests such as gram-staining, oxidase and catalase tests for identification of bacteria were also not precise or perfect. On the other hand, anti-16S rRNA conventional and real-time PCR proved to be superior in the detection and enumeration of many microorganisms. Nevertheless, their use has been limited to simple and uncomplicated specimens (Ray and Bhunia, 2008). Upon the rapid evolution of PCR assay, the real-time PCR provided an accurate, unambiguous identification and quantification of nucleic acid sequence (Klein, 2002) compared to conventional PCR. The real-time PCR allowed simultaneous amplification and quantification of specific nucleic acid sequences cycle-by-cycle which provided the platform for easy, rapid and accurate method of bacterial detection and enumeration (Logan and Edwards, 2004).

There is evident shortage of suitable and precise detection methods of *Lactobacillus* spp. and *Bifidobacterium* spp in complex microbial communities. Therefore, the purpose of this study was to optimize DNA extraction methods, test the most specific PCR primers and adjust the best molecular methods for precisely enumerating *Lactobacillus* spp. and *Bifidobacterium* spp. bacteria using real-time PCR. Two different genus-specific primer sets were being used in this study, (g-Bifid-F / g-Bifid-R) for *Bifidobacterium* and (Lacto-16S-F / Lacto-16S-R) for *Lactobacillus*. These primers were used to detect and quantify the 16S rRNA gene. PCR quantification offers the advantage that no prior knowledge of bacterial content is required when using genus-specific primers, since no correction for multiple gene copies has to be performed. For securing stringent conditions of the current study's objectives, a very complicated microbial community was chosen as a model in this study, namely fermented fish sauce and shrimp sauce.

MATERIALS AND METHODS

Samples and media

Three commercial samples of fish sauce, fish sauce A (Malaysia), fish sauce B (Thailand) and fish sauce C (China) and three samples of shrimp sauce D (Malacca, Malaysia), shrimp sauce E (Cheras, Malaysia) and shrimp sauce F (Muar, Malaysia) were obtained from local market. Growth media used in this study were MRS Broth (Difco, USA), TPY Broth (Scharlau, European Union), Nutrient Agar (Oxoid LTD, England), MRS Agar (Difco, USA) and Ringer Solution (Merck kGaA, Germany). They were prepared according to manufacturers' instructions. All used media and instruments were autoclaved for 15 min at 121°C before being used.

Preparation of samples and inocula

A 10 g sample was taken aseptically from sample bottles and homogenized in 90 ml of sterilized Ringer solution (Merck, Germany) to be prepared for DNA extraction (Podar et al., 2007; Yi-Ting et al., 2007). In parallel, for detecting target bacteria indirectly through an enrichment step for comparison with DNA extracted directly from samples, 1ml of the homogenized mixture was inoculated into 9 ml of MRS Broth (Oxoid, UK) for *Lactobacillus* spp. and TPY Broth (Oxoid, UK) for *Bifidobacterium* spp. before incubated anaerobically for 72 h at 37°C in an anaerobic jar, which contained Anaerocult ® A (Merck, Germany). A 0.1 ml of the inoculum from TPY and MRS broths was spread onto the surface of TPY and MRS Agar (Oxoid, UK) before incubated for 72 h at 37°C in anaerobic condition. The colonies observed on both agar surfaces were picked and streaked onto nutrient agar slope in triplicate. All the agar slopes were incubated again for 72 h at 37°C, with bottle cap loosened under anaerobic condition before kept in refrigerator (0 – 5°C) as stock culture.

DNA extraction from samples

Processed product samples, 1:10 diluted in Ringer solution, were centrifuged at 13,000 rpm for 10 min after which the supernatant was removed and the remaining pellet was subjected to DNA extraction (Podar et al., 2007; Yi-Ting et al., 2007) using four different extraction protocols which were evaluated in this study.

DNA extraction via phenol-chloroform method

Briefly, the pellet was dissolved with 467 µl TE buffer (Sigma, USA), added with 30 µl of 10% SDS (IBD, UK) and 3 µl 20 mgml^{-1} proteinase K (Sigma, USA). After incubation for 1 h at 37°C, 50 µl phenol: chloroform: isoamyl alcohol (Merck, Germany) was added and mixed by gentle inversion. Aqueous phase was transferred to another new tube and added with 0.1 ml of 3 M sodium acetate (Sigma, USA), 0.6 ml of isopropanol (Merck, Germany) and mixed slowly until DNA precipitated and DNA was spooled with pasteur pipette. DNA was dried and washed by dipping the end of the pipette into 1 ml 70% ethanol (Merck, Germany) for 30 s before dissolving in 150 µl TE buffer (Kochl et al., 2005).

DNA extraction via modified heat shock/boiled-cell method

Briefly, 1 ml sterile distilled water was added to the pellet, vortexed and subjected to heating temperature of 100°C for 20 min. The suspension was then cooled immediately to -20°C for 20 min and centrifuged at 13,000 rpm for 3 min before the supernatant was kept in freezer (0 - 5°C) (Keegan et al., 2005).

DNA extraction via Kimchi modified method

Briefly, the pellet was mixed with universal DNA extraction buffer (Promerga, USA) and 2 µl proteinase K (20 mgml^{-1}) before shaking for 30 min at 37°C. A 300 µl of 20% SDS (IBD, UK) was added and mixture was incubated for 2 h at 65°C before centrifuged at 13,000 rpm for 3 min and the supernatant was mixed with equal amount 24:1 of chloroformamyl alcohol (Merck, Germany). The aqueous part was transferred to a new tube, and isopropanol and 70% ethanol (Merck, Germany) were added to wash the pellet obtained before 100 µl TE buffer (Sigma, USA) was added to dissolve DNA (Podar et al., 2007).

DNA extraction via the Wizard® Genomic DNA Purification Kit

The total genomic DNAs from samples were extracted using Wizard® Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instruction with some modifications. 10 ml of the 1:10 diluted sample pellet were centrifuged at 13,000 g for 3 min, washed and re-suspended in 700 µl of glucose-Tris-EDTA buffer (50 mM glucose, 25 mM tris-HCl from Sigma, USA and 10 mM EDTA from Merck, Germany, at pH 8.0). Lysozyme (Sigma, USA) was added to final concentration of 20 mgml^{-1} and incubated in water bath (Reciprocal Water Bath Incubator Model, Certomat® WR) for 1 hr at 37°C before the suspension was centrifuged at 13,000 g for 3 min. Then, 600 µl of nuclei lysis solution were added to the pellet to lyse the cell membrane before incubating for 5 min at 80°C. After cooling at the room temperature, 3 µl of RNase solution (Promega, USA) were added and the tubes were inverted for 5 times before the mixture was incubated again for 1 h at 37°C.

200 µl of protein precipitation solution (Promega, USA) were added into the mixture to purify the genomic DNA and the reaction mixture was vigorously vortexed for 20 s. Afterwards, the mixture was incubated in ice for 5 min, and centrifuged at 13,000 g for 3 min. The supernatant was carefully transferred into a clean 1.5 ml micro-centrifuge tube containing 600 µl isopropanol (Merck, Germany) and the mixture was gently mixed by inverting the tube. The mixture was then centrifuged at 13,000 g for 3 min and the supernatant was discarded. The pellet was then washed with 600 µl of 70% ethanol (Merck, Germany) by centrifuging at 13,000 g for 3 min. Finally, the ethanol was discarded and the pellet containing the genomic DNA was re-hydrated by adding 100 µl DNA rehydration solution.

DNA extraction from standard bacteria and samples' enriched media

The total genomic DNAs from all standard bacteria strains (Table 1) and samples' enriched bacteria were extracted using the same Wizard® Genomic DNA purification kit (Promega Corporation, Madison, USA) used for the DNA extraction of samples.

Quality and yield of extracted nucleic acids

Different DNA extraction methods were evaluated on the basis of performance in agarose gel electrophoresis. Gel electrophoresis of extracted genomic DNA was conducted together with a ready-to-use VC 1 kbp Plus DNA Ladder as a molecular weight standard (Vivantis, Italy). The extracted DNA was also checked by using UV-Visible spectrophotometer (UV-1601 Shimadzu Model, Japan) at 260 nm and 280 nm. The quality of DNA was determined by A_{260}/A_{280} ratio value. DNA yield, in terms of DNA concentration, was being calculated. The formula for the calculation of DNA yield was as follows:

DNA concentration (µg µl-1) = (A260 in OD units x 50 µgml-1 x DF) / 1000.

DNA yield (µg) = DNA concentration (µgµl-1) x amount of DNA kept as stock (100 µl).

The total genomic DNA was stored at 0 - 5°C for further

Table 1. Standard bacterial strains used in this study.

Bacterial strains	Sources
B. pseudocatenulatum G4 *B. pseudocatenulatum* F81 *B. longum* BB536	Morinaga Milk Industry, Japan
B. longum JCM 1260 *B. longum* JCM 1210 *B. longum* JCM 1217 *B. breve* ATCC 15700 *B. breve* strain Yakult	Isolated from Yakult B1 Seichoyaku Product, Japan
B. infantis ATCC 15697 *B. animalis* ATCC 27672 *L. brevis* ATCC 14869 *L. gallinarium* ATCC 33199 *L. reuteri* ATCC 23272 *L. casei* strain Shirota	Isolated from Yakult® Drink, Japan
Lactobacillus acidophilus	Isolated from Vitagen® Drink
L. rhamnosus GG ATCC 53103 *E. faecalis* JCM 5803 *E. faecalis* S 256 *S. choleraesuis* JCM 6977 *S. choleraesuis* ATCC 14028 *S. enteritidis* (Group D) ATCC 13076 *S. typhimurium* S917 *B. ovatus* ATCC 8483 *B. uniformis* ATCC 8492 *B. vulgates* ATCC 8482 *B. thetaiotaomicron* ATCC 29741 *E. coli* JM 109 *E. coli* K-12 *E. coli* E1	Laboratory strain provided by Institute of Medical Research, Kuala Lumpur, Malaysia

analysis. The best yield and quality of the extracted DNA determined which method would then be used to continue for PCR assays.

Standard bacterial strains and growth conditions

The standard bacterial strains used in this study were composed of six different genera covering 29 strains. Two LAB genera cover six strains of *Lactobacillus* and two strains of *Enterococcus fecalis* and four non-LAB genera cover ten strains of *Bifidobacterium,* four strains of *Salmonella*, four strains of *Bacteroides* and three strains of *E. coli* (Table 1). The strains of probiotic bacteria, *Lactobacillus* and *Bifidobacterium* were grown anaerobically in MRS and TPY broths, respectively. On the other hand, the strains of *Salmonella*, *Enterococcus fecalis* and *E. coli* were grown in aerobic condition in nutrient media while *Bacteroides* spp. were grown in anaerobic condition in nutrient media. All bacteria were incubated for 24 – 72 h at 37°C.

Primers

Two different genus-specific primer sets were used in this study, (g-Bifid-F/g-Bifid-R) for *Bifidobacterium* and (Lacto-16S-F / Lacto-16S-R) for *Lactobacillus* (Table 2). These primers were used to detect and quantify the species-specific highly conservative region of 16S rRNA gene. g-Bifid-F/g-Bifid-R primer set (Promega, USA) was customized according to a previous study (Matsuki et al., 2004). On the other hand, Lacto-16S-F/Lacto-16S-R primer set was designed and customized from the nucleotide sequence of *Lactobacillus* brevis ATCC 14869 (accession no. EU194349) using Primer-BLAST program (NCBI, GenBank, BLAST). The primer set was synthesized in the First Base Laboratories, Shah Alam,

Table 2. Primer sets used in this study.

Target organism	Primer Set	Sequence (5' to 3')	Product Size (bp)	T_a (°C), time (s)	Reference
Lactobacillus genus	Lacto-16S-F Lacto-16S-R	GGA ATC TTC CAC AAT GGA CG CGC TTT ACG CCC AAT AAA TCC GG	216	56, 10 s	This study This study
Bifidobacterium genus	g-Bifid-F g-Bifid-R	CTC CTG GAA ACG GGT GG GGT GTT CTT CCC GAT ATC TAC A	562 (549 - 563)	61, 20 s	Matsuki et al. (2004) Matsuki et al. (2004)

Malaysia and was named Lacto-16S-F and -R. Since the specificity of Lacto-16S-F/Lacto-16S-R was not evaluated before, two levels of specificity checkup was thoroughly undertaken. Firstly, the specificity of Lacto-16S-F/Lacto-16S-R primer set was proven by using Primer-BLAST program (NCBI, GenBank, BLAST). BLAST-Primer program was set to (nr) parameter where All GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequences are included and the database of unintended gene sequences was unlimited to certain organism to increase the range of specificity checking. It was found that Lacto-16S-F and –R was highly specific to all *Lactobacillus* species where complete complementarily was found. On the other hand, nucleotide sequences rather than that of *Lactobacillus* spp. turned to be uncomplimentary. In addition, the specificity of Lacto-16S-F/R primers was checked in laboratory. Eight strains from LAB genera, *Lactobacillus* and *Enterococcus* and 21 strains from non-LAB genera, *Salmonella*, *Bifidobacterium*, *Bacteroides* and *E. coli*, (Table 1) were subjected for recognition by Lacto-16S-F / Lacto-16S-R primer set using PCR assay.

PCR reaction

After determining the most efficient method of DNA extraction, PCR reaction for DNA extracted from samples, samples' enriched bacteria and standard bacteria, in duplicates, was carried out in a total volume of 25 µl with a reaction mixture containing 2.5 µl of 10 x PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 1.65 µl of 15 µM forward and reverse primers of g-Bifid-F / g-Bifid-R and Lacto-16S-F / Lacto-16S-R, 0.125 µl of 5uμl^{-1} Taq DNA polymerase, 3 µl of genomic DNA (~10 ng) and 14.075 µl of sterile distilled water. All the reaction mixtures were obtained from Promega Corporation, Madison, USA.

The reaction mixture in micro-centrifuge tube was amplified in a thermocycler PCR system (PTC-110^{TM} Model, MJ Research, Inc., USA). For *Bifidobacterium* spp., the initial denaturation was performed at 95°C for 3 min and the target DNA was amplified in 40 cycles. Each cycle consisted of denaturation (95°C, 30 s), annealing (57°C, 30 s) and extension (73°C, 60 s). The final extension step was performed at 73°C for 5 min and the holding temperature was 10°C. For *Lactobacillus* spp., the same PCR cycle and conditions were pursued except for the annealing temperature which was performed at 61°C for 30 s. It is noteworthy to mention that negative control, master mix devoid of genomic DNA and positive control, a positive sample taken from previous experiments, were used simultaneously in duplicates.

Gel electrophoresis

The amplified PCR products were checked for the expected size on 1.5% (w/v) agarose gel (LE analytical grade, Promega, Madison, USA). Ten µl of each PCR amplified product and 3 µl of 6 x Loading Dye were loaded into agarose gel and run in 1 x TBE buffer (0.089 M Tris-HCl, 0.089 M Boric acid, 0.002 M EDTA, pH 8.3) (Promega, USA). A ready-to-use VC 100 bp Plus DNA Ladder - molecular weight standard (Vivantis, Italy) was used along with positive control, negative control and PCR amplified products. The PCR products were separated by an electrophoresis system at a constant voltage of 80 V for 50 min. Then, the gel was stained in ethidium bromide (Sigma, USA) staining (0.5 µgml^{-1}) for 5 min and followed by washing with distilled water for about 30 min. Finally, the gel was visualized under UV transilluminator (Vilber Lourmat, Cedex, France) and the photos were taken using gel documentation system (Bio Rad Gel Doc 2000 Model Imaging System).

Standard curve for real-time PCR

In order to quantify *Lactobacillus* and Bifidobacterium spp. in the tested samples, serially diluted DNA of standard *Lactobacillus* brevis ATCC 14869 and the standard Bifidobacterium longum strain JCM 1260 were used to generate a standard curve for the mathematical conversion of Ct values into bacterial cell number. Ct value is the cycle number where the reaction fluorescence exceeds background fluorescence. Two different standard curves for *Bifidobacterium* spp. and *Lactobacillus* spp. were generated. Stock plasmid DNA was prepared for these two different bacteria. For the preparation of plasmid DNA, the purified PCR product of interest was prepared first. Ligation of PCR product of interest into pGEM®-T Easy Vector (Promega, Madison, USA) which

consisted of a mixture of 2 x rapid ligation buffer, pGEMTR Easy Vector, purified PCR product, T4 DNA ligase and sterile distilled water. *E. coli* competent cells of strain JM 109 were used to carry pGEM®-T Easy Vector (Promega, Madison, USA) that had been ligated with the target sequence from each bacterial genus. The screening of plasmid insert was done, where the white colony indicated that the PCR product gene of interest was successfully cloned and transformed. The white colony was inoculated into Luria broth supplemented with ampicillin. The plasmid DNA of interest was then extracted from the cell according to the manufacturer's instruction using Wizard® Plus SV Minipreps (Promega, Madison, USA). The initial concentration of the plasmid DNA of interest was 10.6 ug/ml. The plasmid with the correct insert was then 10-folds serially diluted into six dilutions. Since the molecular weight of the plasmid DNA is known, the concentration of these dilutions were then transformed into the log copy number which was plotted against the threshold cycle (Ct) to generate the standard curve used for the absolute quantification of real-time PCR.

Quantitative real-time PCR

After the conventional PCR, the genomic DNA obtained either directly from the tested samples or from standard bacterial strains was used for the real-time PCR amplification. Genomic DNA of standard *Bifidobacterium* and *Lactobacillus* strains were included in the real-time PCR assay as a positive control while PCR mixture solution devoid of genomic DNA was used as negative control. Real-time PCR amplification reaction was performed with Rotor-Gene™ 3000 (Corbett. Research, Australia) using fluorescent dye SYBR Green (Qiagen™ QuantiTect® SYBR Green PCR kit). The reaction was performed in a total volume of 25 µl (2.5 µl of 10 x PCR buffer), with the composition of the reaction mix per sample as follows: 1.8 µl of 25 mM $MgCl_2$, 0.75 µl of 10 mM dNTP, 9.5 µl SYBR Green, 1.0 µl of 25 µM each reverse and forward primers (g-Bifid-F / g-Bifid-R and Lacto-16S-F / Lacto-16S-R), 0.125 µl of 5uμl^{-1} Taq DNA polymerase, 5.0 µl DNA template (~16 ng) and 5.82 µl RNase free water. All the reaction mixtures were obtained from Promega Corporation, Madison, USA. The serially diluted bacterial standard and samples were simultaneously assayed in real-time amplification.

The PCR program consisted of an initial denaturation step, amplification step (40 cycles) and a melting-curve determination step. The condition for the amplification was as same as the conventional PCR. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°Cs^{-1} increments from 60 - 99°C, with continuous fluorescence collection. Analysis of PCR amplification and melting curves were done by Rotor-Gene Real-Time Data Acquisition and Analysis Software version 1.7 (Corbett Research, Australia). Measurement of the SYBR Green fluorescence was performed at the end of each amplification step and continuously during the melt-curve analysis (Masco et al., 2007). A melting curve would be generated at the end of amplification for monitoring specificity of PCR reaction.

RESULTS

Quality and yield of extracted DNA

The purity and quality of DNA extracted from complex microbial samples are important for proper PCR-based detection assays. Thus, in the experimental design, A_{260}/A_{280} ratio of extracted DNA from tested samples was evaluated. The quality of the extracted DNA was determined by agarose gel electrophoresis too, where the sharpness of the DNA band was visualized. Besides quality, the extracted DNA yield was also important for a subsequent analysis of PCR. Table 3 showed the results of the quality and yield of the extracted DNA using four different extraction methods. Results for DNA quality showed that all DNAs extracted from fish sauce and shrimp sauce using phenol-chloroform method, boiling method and kimchi method gave an A_{260}/A_{280} ratio less than 1.5. However, all the DNAs extracted from fish sauce and shrimp sauce using Wizard protocol had an A_{260}/A_{280} ratio of more than 1.5 which showed that the quality of DNA produced by Wizard protocol was the best among the four methods.

A good DNA extraction method should not give only high DNA purity, but also high DNA yield. The results in Table 3 showed that the DNA extracted with Wizard protocol produced the highest yield compared to the other three methods. The trend was almost similar to the A_{260}/A_{280} ratio of DNA quality. The DNAs extracted using four methods were observed for degradation by agarose gel electrophoresis. It was observed that all DNAs extracted from fish sauce and shrimp sauce by Wizard protocol produced bands at the uppermost part of the gel. For the other three methods, the results of agarose gel electrophoresis revealed that some of the bands were not detected which showed that not all DNAs were liberated during the DNA extraction from each fish sauce and shrimp sauce (Figure 1).

Primer specificity

The specificity of the used primer set, Lacto-16S-F/Lacto-16S-R, was tested before conducting PCR and real-time PCR assays on the complex microbial samples to ensure

Table 3. Quality and yield of the extracted DNAs using four different protocols.

Sample	Phenol-chloroform extraction	Boiling method	Wizard protocol	Kimchi method
DNA quality (A_{260}/A_{280})				
Fish sauce A	1.1083	1.1875	1.6549	1.012
Fish sauce B	1.0135	1.1683	1.5828	1.0132
Fish sauce C	1.1613	1.0348	1.5175	1.0500
Shrimp sauce D	1.0230	1.0825	1.6783	1.0238
Shrimp sauce E	1.0390	1.1375	1.5620	1.1467
Shrimp sauce F	1.0762	1.1735	1.5840	1.0465
DNA yield (µg)				
Fish sauce A	66.5	66.5	93.5	42.0
Fish sauce B	37.5	59.0	119.5	38.5
Fish sauce C	46.5	59.5	108.5	42.0
Shrimp sauce D	44.5	52.5	120.0	43.0
Shrimp sauce E	40.0	45.5	94.5	43.0
Shrimp sauce F	56.5	49.0	99.0	45.0

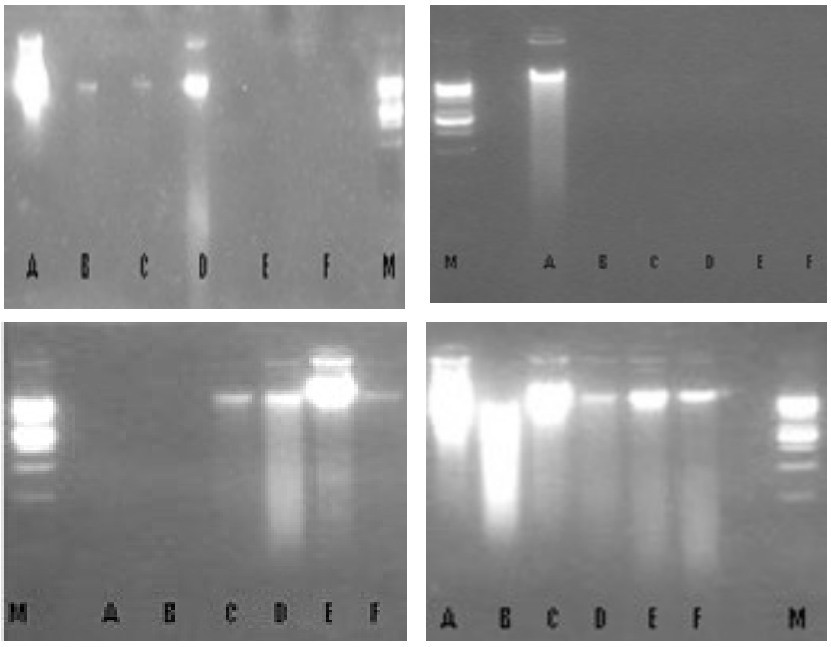

Figure 1. Agarose gel electrophoresis of genomic DNA extracted from fish sauce and shrimp sauce samples with four different methods. Upper left = Phenol-chloroform method; Upper right = Kimchi method; Lower left = Boiling method; Lower right = Wizard protocol. Lane M = 1 Kb DNA ladder Marker; Lane A - C = Fish sauce A, B, and C; Lane D - F = Shrimp sauce D, E and F.

a proper and specific amplification process. The The specificity of the PCR primer set Lacto-16S-F/Lacto-16S-R was tested by PCR assay with strains other than *Lactobacillus* spp. and most commonly found in complex microbial samples, including *Bifidobacterium* spp., *Enterococcus* spp., Salmonella, *E. coli*, and bacteroides. All used strains of *Lactobacillus* were PCR positive to the *Lactobacillus* genus specific primer set Lacto-16S-F/Lacto-16S-R while all other bacteria proved to be negative. Therefore, in conjunction with specificity check via Primer-BLAST program, the specificity of Lacto-16S-F/Lacto-16S-R has been proven for the 16S rRNA gene fragment which is a strain-specific DNA for almost all *Lactobacillus* spp.

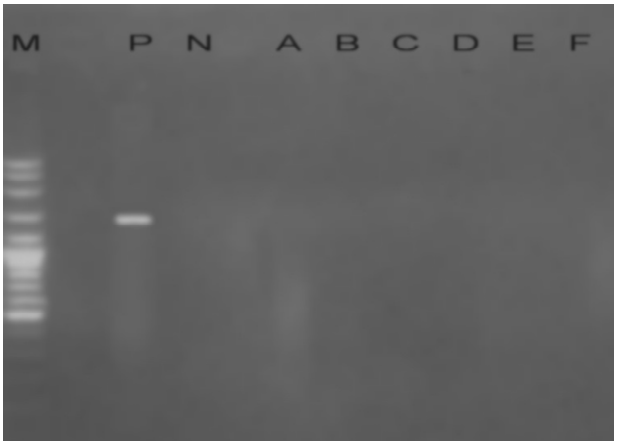

Figure 2. Agarose gel electrophoresis of PCR product amplified from Bifidobacterium spp. in fish sauce and shrimp sauce samples. Lane M = 100bp DNA ladder Marker; Lanes P and N are positive and negative controls respectively; Lane A – C = fish sauce A, B and C; Lane D – F = shrimp sauce D, E and F. No positive PCR band at 562 was seen at any of tested samples.

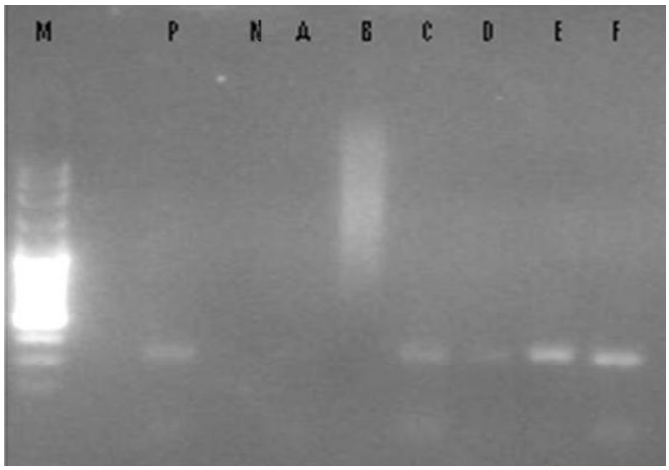

Figure 3. Agarose gel electrophoresis of PCR product amplified from Lactobacillus spp. in fish sauce and shrimp sauce samples. Lane M = 100bp DNA ladder Marker Lanes P and N are positive and negative controls respectively; Lane A – C = fish sauce A, B and C; Lane D – F = shrimp sauce D, E and F. Positive PCR bands at 216 bp were only found in C, D-F samples.

PCR detection of *Bifidobacterium* spp. and *Lactobacillus* spp.

The molecular identification of target *Bifidobacterium* spp bacteria using DNA extracted directly from tested samples for PCR indicated that, even though specific band with molecular weight of 562 bp was found at the lane of the positive control, there was no specific bands with molecular weight of 562 bp observed in all the six tested samples (Figure 2). This indicated that, in the preliminary testing, *Bifidobacterium* spp. was absent in all of the samples. On the contrary, DNA of *Lactobacillus* spp. extracted directly from tested samples was detected in one of the three fish sauce samples and in all three shrimp samples (Figure 3) by observing a specific band, PCR product with molecular weight of 216 bp. However, the band for shrimp sauce D was hardly seen (Figure 3). On the other hand, DNA extracted from samples' enriched bacteria in MRS and TYP media revealed that *Lactobacillus* spp., unlike DNA extracted directly from samples, was detected in all 6 samples including fish sauce A and B while for *Bifidobacterium* spp the same findings were found that all samples were negative [data not shown].

Real-time PCR Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp.

By incorporating the standard curve for the absolute quantification of bacteria in terms of Log CFU ml^{-1} via inversely proportioning the amount of the positively amplified bacteria to Ct value, it was shown that all samples of fish and shrimp sauce yielded positive *Lactobacillus*. Moreover it was shown that fish sauce A had the lowest amount of *Lactobacillus* spp., while shrimp sauce F had the highest amount of *Lactobacillus* spp (Table 4). On the contrary, real-time PCR for *Bifidobacteria* did not reveal any sign of amplification indicating the possibility of the absence of this bacteria or inability of its detection. For DNA extracted from samples' enriched bacteria, the findings were similar; the detection of Bifidobacterium spp. was again negative and the detection of *Lactobacillus* spp. was positive in all samples but with bacterial load 3 to 4 Log CFU ml^{-1} higher than that detected directly from the tested samples.

On the other hand, by using the melting curve analysis during the real-time PCR reaction, it was observed that T_m value for the serially diluted standard *Bifidobacterium* strains was located about 89°C. The findings showed that there was no specific peak at T_m = 89°C for the DNA extracted directly or via enrichment step from all samples of the fish and shrimp sauce. Therefore, in addition to the conventional PCR and quantitative real-time PCR, melting curve analysis provided extra evidence that *Bifidobacterium* spp. were most likely absent in all tested samples.

On the other hand, the T_m value for the serially diluted standard *Lactobacillus* strains was located about 85.5°C. It was shown, for DNAs extracted directly from samples or via bacterial enrichment, that the melting curve for *Lactobacillus spp*. in three fish sauce (A, B and C) and three shrimp sauce (D, E and F) had a specific peak at T_m = 85.5°C for each of the six samples, pointing out that all the tested samples contained *Lactobacillus* spp. Therefore, the *Lactobacillus* spp. sharp peak in the

Table 4. Comparison of Ct value and *Lactobacillus* spp. in fish sauce and shrimp sauce by Real- Time PCR.

Sample	Ct value	Real-Time PCR (Log CFUml^{-1})
Fish sauce A	24.44	5.53
Fish sauce B	24.17	5.67
Fish sauce C	22.67	6.41
Shrimp sauce D	23.97	5.77
Shrimp sauce E	22.12	6.68
Shrimp sauce F	22.28	6.60

** The efficiency of the standard curve was R^2 = 0.99959.

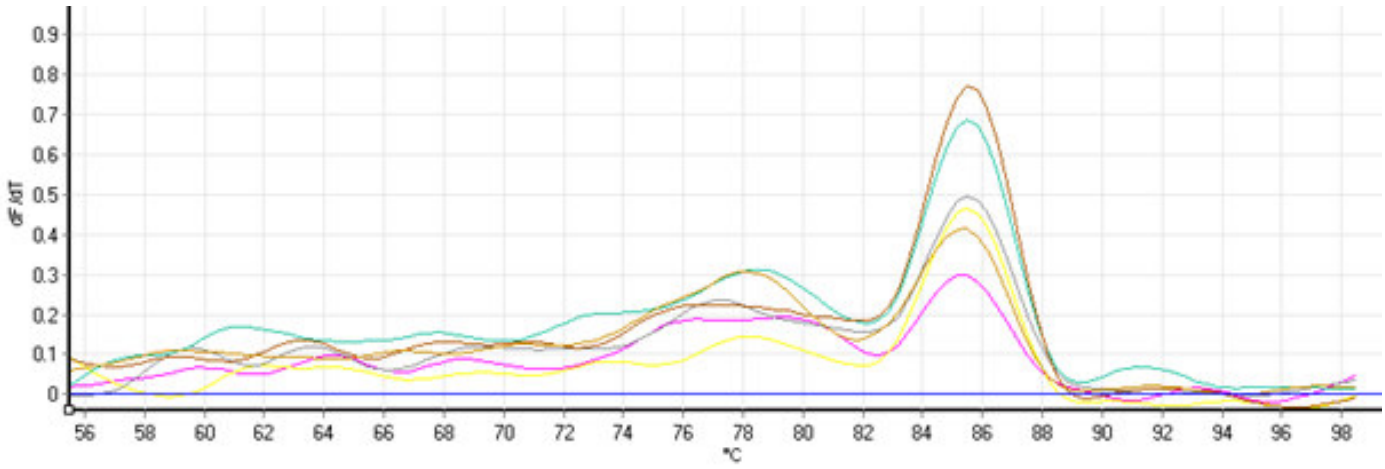

Figure 4. Melting curve analysis of *Lactobacillus* spp. for three fish sauce and three shrimp sauce samples. The peak (T_m = 85.5°C) is specific to the genus *Lactobacillus* spp. (The peak from top to bottom: shrimp sauce E, shrimp sauce F, fish sauce C, shrimp sauce D, fish sauce B, fish sauce A).

melting curve was a further confirmation of the conventional PCR result (Figure 4).

In addition, it was important to confirm that there were no products of non-specific amplification, including primer dimers that contributed to the signal. This was detected by using the melting curve analysis too. In this study, there was just a weak signal appearing at a lower temperature than the T_m for both the *Bifidobacterium* spp. and *Lactobacillus* spp. in all fish sauce and shrimp sauce samples, which was identified as primer-dimers. The presence of these primer-dimers might be due to the insufficient primer annealing time, or the inappropriate annealing temperature.

DISCUSSION

The main species believed to have probiotic characteristics are *Lactobacillus* spp. and *Bifidobacterium* spp. This study attempted to work out two problems facing the microbial analysis of probiotics from complex microbial samples. First, the reliability of many enumeration procedures of probiotic bacteria is compromised by the lack of suitable media for the selective isolation of these organisms from probiotic products. Secondly, the microbial analysis of some bacteria in fermented products mostly was done using culture-dependent methods which were imprecise and time-consuming (Martin et al., 2006). Therefore DNA amplification methods, PCR, are more intended and used for their invaluable preciseness. However, PCR assays need a lot of optimization and standardization prior to any valid testing especially for detecting microflora in highly mixed complex environments.

Quality and yield of the extracted DNA are primary requirements for the PCR-based detection assay. The selection of suitable extraction method is essential for a successful and valid PCR analysis. The main limitation associated with PCR application for the detection of microorganisms in complex matrices is the presence of inhibitory substances that are co-extracted with DNA,

causing failure in the amplification reaction which leads to false negative results. In fermented fish products which contain a high number of inhibitory substances such as proteins, a suitable DNA extraction method seems to be very important (Podar et al., 2007). Thus, prior to PCR amplification, the quality and yield of the extracted genomic DNA had to be determined.

The purity of DNA was indicated by A_{260}/A_{280} ratio, where the value from 1.8 to 2.0 was considered as high purity. Nevertheless, the extracted DNA is considered of adequate purity if A_{260}/A_{280} is > 1.5 (DNA Quantification: Spectrophotometry, 10/2004). In this study, only the extracted DNA by Wizard protocol had a quality more than 1.5. This might be due to the additional step of protein precipitation in the protocol. Martin et al. (2006) stated that DNA isolation done by Wizard protocol from fermented sausage and meat allowed an increase in the amount of the purified DNA sample added to the real-time PCR without inhibitory consequences.

Regarding DNA integrity, the detection of bacteria using PCR method is dependent on the ability to extract intact DNA from food samples. If an appropriate method was used, the efficiency to recover DNA could be maximized even for complex and highly processed matrices (Tung et al., 2008). Although fish sauce and shrimp sauce are fermented products which might affect DNA integrity, the high molecular weight single band obtained for all of the four methods at the uppermost gel indicated that DNA was not fragmented. Although the extracted DNA by Wizard protocol was not in the highest purity (A_{260}/A_{280} = 1.8 to 2.0), they could be used in the subsequent PCR detection of bacteria in fish sauce and shrimp sauce because of the most intact DNA and the highest DNA yield compared to the other three methods.

Regarding DNA yield, for Wizard protocol, the obtained high yield of DNA was probably attributed to the addition of lysozyme, a required pre-processing step to efficiently breakdown peptidoglycan in the cell wall of gram-positive bacteria. A previous study showed that the yield of extracted DNA was high when cell pellet was lysed with lysozyme (Pitcher et al., 1989). For Kimchi method and phenol-chloroform method, the low DNA yield was probably due to the quality of enzyme used for the lysis of cell, which was the proteinase K. Treatment with proteinase K was very dependent on the quality of the enzyme where lysis of cells can be affected at long term storage of proteinase K (Agersborg et al., 1997). For boiling method, the low DNA yield was probably due to the heat-resistance of some fastidious strains found in fermented samples.

Although Wizard protocol produced relatively high yield of DNA compared to other three methods, the extracted DNA was not in optimum. According to Velazquez et al. (1993), the readjustment of pH of lysis mixture to 8.0 after addition of lysozyme proved to be definitive in subsequent cell lysis of gram-positive bacteria. Omission of this step caused incomplete lysis of cell and subsequent low yield DNA being extracted. Thus, in this study, DNA extraction using Wizard protocol could be improved to maximize the yield of DNA obtained.

The other requirement for well optimized PCR is the specificity of the used primers. It is well known that primer specificity of any target bacteria in complex microbial community is very important prior to the use of PCR-based assay. Therefore, since the specificity of *Bifidobacterium* primer set, g-Bifid-F/g-Bifid-R, had been tested in a study done by Matsuki et al. (2004), the specific primer set of *Lactobacillus spp.*, Lacto-16S-F/Lacto-16S-R, was subjected to a thorough testing via both Primer-BLAST program and laboratory testing. It was found that Lacto-16S-F/Lacto-16S-R was highly specific for *Lactobacillus* sp rather than other known bacteria listed in Genbank database. In addition, to keep the optimal fidelity, a thorough search in BLAST program for any complimentary sequence in species other than bacteria which showed totally negative results. Moreover, this primer was specific for *Lactobacillus* spp. rather than other laboratory-tested bacteria, namely, *Bacteroides, Enterococcus, E. coli* and *Salmonella*. This grants validity for using this primer in the subsequent PCR-based assays and abolish the main obstacle of using PCR in complex microbial communities, which is the possibility of cross reaction and non-specificity.

After standardizing PCR adequately, which is the first aim of this study, the findings of detecting *Lactobacillus* spp. and *Bifidobacterium* spp. can be discussed in confidence. In conventional PCR, the absence of *Lactobacillus* spp. in fish sauce A and B might be due to the variation in chemical composition and quality characteristic of the fermented fish products. This could be related to the method of processing and the raw material used. Some of the strains of *Lactobacillus* were unable to grow in such a low pH condition of fermented fish products. According to Itoh et al. (1985), some of the fish sauces from Thailand which contained levulinic acid had low microfloral viable counts, because levulinic acid suppressed the growth of bacteria. However, there was possibility that some of the DNAs were lost during DNA extraction using Wizard protocol. The number of *Lactobacillus* spp. might be under the detection limit of this PCR condition, and thus the band was hardly to be seen. There was also a possibility that amplification was inhibited by the PCR inhibitor such as high protein or fat content found in fish sauce and shrimp sauce. However, fish sauce C, shrimp sauce A, B and C were found to contain *Lactobacillus* spp., which is tolerable to the high salt condition in fermented fish products which was believed to take part in the fermentation process. A study done by Ijong and Ohta (1995) on Indonesian fermented fish sauce ”bakasang”, among six genera of bacteria identified, *Staphylococcus* spp. and *Lactobacillus* spp. were the predominantly isolated microorganisms.

Regarding real-time PCR of DNA extracted from samples, it turned out to be more sensitive results than that of conventional PCR. Unlike conventional PCR, it succeeded to detect and quantify *Lactobacillus* spp., in all tested samples. However, for *Bifidobacterium* spp., like conventional PCR, real time PCR turned negative results. The result obtained for the number of *Lactobacillus* spp. in samples was between 4 to 6 log units indicating that *Lactobacillus* is present abundantly in fish and shrimp sauce. However, the quantification might be a bit inaccurate due to the fact that 16S rRNA gene can be present in multiple copies, possibly resulting in an overestimation of the number of *Lactobacillus* spp. in samples. Moreover, the formation of non-specific amplicons when using SYBR Green dye might cause overestimation of the number of *Lactobacillus spp*. In fact, the results obtained from the real time PCR of the samples' enriched bacteria were not different from that directed towards direct samples extracted DNA [data not shown]. In *Lactobacillus* spp. It was positive while Bifidobacterium spp. were absent in all samples' enriched bacteria. Nevertheless, the only difference was the enumeration of *Lactobacillus* as it was 3 - 4 log units higher than that obtained from DNA extracted directly from samples. This difference is expected as enrichment step amplified greatly the number of targeted bacteria.

The melting curve analysis was performed immediately after amplification by briefly denaturing PCR products followed by cooling to 5 - 10°C below the Tm of the detection probe given that Tm is a function of GC content (Lee et al., 2004; Edwards, 2004). Melting curves were useful for differentiating primer dimers from specific PCR products and to validate the identity of the amplified products where every PCR product has its own melting temperature. Hence, melting curve analysis confirmed the specific amplification for *Lactobacillus* spp., the absence of Bifidobacterium spp., and the very low level of primer dimers in DNA extracted from both direct samples and samples' enriched bacteria.

Accordingly, no *Bifidobacterium* sp. was found for all the six samples of fish and shrimp sauce via conventional PCR, quantitative real time PCR, melting curve analysis in both direct samples and samples' enriched bacteria. This might largely exclude the possibility that the negative results of *Bifidobacterium* spp., were due to either PCR inhibition or very low level of that bacteria because the samples' enriched bacteria did not reveal as well any sign for the presence of *Bifidobacterium* sp. Until now, no study has been done to detect the presence of this bacterium in fermented fish products and this was the first study to be conducted. There is a minor possibility that the absence of *Bifidobacterium* spp. might be due to the loss of DNA during the DNA extraction but this contradicts the successful DNA extraction done for *Lactobacillus* sp. which was present in the same samples. According to Cheng et al. (2008), the sensitivity for PCR detection limit of *Bifidobacterium adolescentis* in human stool using primers of bits- 1/bits- 2 was until 10°CFU/g, while for yogurt sample was until 10^2 CFU/ml. *Bifidobacterium* spp. was normally important in milk fermentation, and they are found in human milk as well. Their absence in fish sauce and shrimp sauce for this study might be possible because the condition of the fish sauce and shrimp sauce was suspected not to be suitable for *Bifidobacterium* spp. that needs a medium rich of all essential nutrients, for example, nitrogen sources. According to Gomes and Malcata (1999), *Bifidobacteria* tend to exhibit weak growth even in milk, and they need invariably long fermentation times and conditions of anaerobiosis. And it was shown that *Bifidobacteria* survival depends critically, more than other bacteria, on pH, the presence of competing microorganisms, the storage temperature, and the presence of microbial inhibitors (e.g. NaCl) in the food matrix (Kurmann and Rasic, 1991).

Taken together, in this study, culture-independent methods, traditional and real-time PCR, were used to investigate the presence of *Bifidobacterium* and *Lactobacillus* in complex microbial communities, fermented fish sauce and shrimp sauce. Among four of the DNA extraction methods being evaluated, Wizard® Genomic DNA Purification Kit was found to be the most efficient DNA extraction method for this study, in terms of DNA purity and yield. Detection by using traditional and real-time PCR proved to be highly efficient for the detection and quantification of *Lactobacillus* but not *Bifidobacterium*, most likely due to its absence, in highly crude and PCR-suppressor- contaminated samples. Quantitative real time PCR was shown to be more sensitive than traditional PCR for the detection of probiotic bacteria. Moreover, besides the inaccurate enumeration, the enrichment step for samples' bacteria did not yield better sensitivity for the detection of probiotic bacteria. Accordingly, quantitative real time PCR is highly recommended for the detection and enumeration of *Lactobacillus* spp., in highly complex microbial communities directly without conducting enrichment of samples' bacteria to save time, effort and cost when compared to culture- or PCR enrichment-based methods.

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