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Methods for Precise Molecular Detection of Probiotic Microflora: Using Adjusted Molecular Biology Protocols, Primer Sets and PCR Assays

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Abstract: *Lactobacillus* sp. is probiotic bacteria for which many detection methods were envisaged. However, culture-based methods failed to achieve specific detection of this bacterium due to its presence in mixed bacterial complex communities. The PCR assay was optimized to detect and quantify *Lactobacillus* sp. specifically in complex microbial community of mixed bacteria. Four DNA extraction methods, DNA integrity, primers specificity and optimized PCR procedure were all tested. It was shown that extracted genomic DNA using Wizard® Genomic DNA Purification Kit showed the highest yield, quality and performance in gel electrophoresis. Moreover, the specificity of the primer set, Lacto-16S-F /Lacto-16S-R, specific for *Lactobacillus* sp. was checked and found highly specific. In conclusion, the best DNA extraction protocol, working specific primer set and working PCR assay were achieved for achieving efficient, specific and reliable molecular-based, culture-independent, method of detection of *lactobacillus* sp. in PCR-suppressor highly protein-complex environment of mixed bacteria community.

Key words: PCR, DNA extraction, *Lactobacillus* sp., probiotic

INTRODUCTION

Common microbiological methods to quantify microorganisms have traditionally required culture-based methods, which are labor intensive and time consuming (Iacumin *et al*., 2009). Variation in the method of inoculation, the choice of target cell and the type of culture medium often create some problems in identifying the target bacterial cells (Aliskan, 2008). Prior to 1980s and the advent of PCR, identification of microorganisms relied on bacteriological methods and subsequent biochemical tests to confirm the identity of the microorganism (Fairchild *et al*., 2006). Besides, culture-based tests, the biochemical tests such as gram-staining, oxidase and catalase tests, need to identify certain species of bacteria were also not precise or perfect (Pikkemaat, 2009).

On the other hand, PCR is a simple technique to quickly amplify specific sequences of target DNA from indicator organisms to an amount that can be viewed by human eye with a variety of detection devices (Klein, 2002). The development of a molecular culture-independent detection methods appear to be invaluable in the case of probiotics particularly *Lactobacillus* sp., since other available methods are not very efficient to enumerate this bacterium in Complex Microbial Communities (CMC) (Ray and Bhunia, 2007; Logan and Edwards, 2004).

Lactobacillus bacteria were found to be a valuable source for nutritional healthy supply and essential for proper function of intestine (Laiho *et al*., 2002; Umesaki and Setoyama, 2000). Therefore, *Lactobacilli* bacteria are extensively used as probiotics and technologically used as food-associated microorganism as they are generally recognized as safe (Lombardo, 2008; Salminen *et al*., 1998). Although, several studies on microflora of many CMC had been done, most of these studies focused on the conventional methods which are not specific and are time-consuming (Tanaka *et al*., 2006; Rabe and Hillier, 2003; Kang *et al*., 2003; Messi *et al*., 2001). Hence, there is evident shortage of suitable and precise detection methods of *Lactobacillus* sp. in CMC. Therefore, the purpose of this study was to optimize DNA extraction methods, scrutinize specific PCR primers and adjust the best molecular methods for precisely identifying *Lactobacillus* sp. bacteria using highly optimized and standardized PCR assay.

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MATERIALS AND METHODS

Samples and media: This study was conducted in the period from June 2007 to February 2009 in Selangor state in Malaysia. For securing stringent conditions of the current study's objectives, a very complicated microbial community that is full of PCR suppressing proteins, a typical CMC, was chosen as a model in this study, namely fermented fish sauce and shrimp sauce. Three commercial samples of fish sauce, fish sauce from Malaysia (CMC1), fish sauce from Thailand (CMC2) and fish sauce from China (CMC3) and three samples of shrimp sauce Malacca, Malaysia (CMC4), shrimp sauce from Cheras, Malaysia (CMC5) and shrimp sauce from Muar, Malaysia (CMC6) were obtained from local market. Growth media used in this study were MRS Broth (Difco, USA), 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.

Samples preparation and inoculums: A 10 g sample was taken aseptically from sample bottles and homogenized in 90 mL of sterilized Ringer solution. The mixture was homogenized in Stomacher bag. One mL of diluents was inoculated into 9 mL of MRS broth for *Lactobacillus* sp. before incubated anaerobically for 72 h at 37°C in an anaerobic jar, which contained Anaerocult ® A (Merck KGaA, 64271 Darmstadt, Germany). A 0.1 mL of the inoculum from MRS broth was spread onto the surface of MRS agar before incubated for 72 h at 37°C in anaerobic condition. The colonies observed on agar surface was picked and streaked onto nutrient agar slope in triplicate. 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 (Zoetandal *et al*., 2002).

Optimized extraction of DNA: Four DNA extraction methods were evaluated in this study. For protocol (1), (2) and (3), the overnight culture broth was vortexed and subjected to centrifugation at 13,000 g for 3 min after which the supernatant removed and the remaining pellet was subjected to different extraction protocols:

- The phenol-chloroform method, where the pellet was dissolved with 467 μL TE buffer, added with 30 μL of 10% SDS and 3 μL 20 mg mL^{-1} proteinase K. After incubation for 1 h at 37°C, 50 μL phenol: chloroform: isoamyl alcohol 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, 0.6 mL of isopropanol and mixed slowly until DNA precipitated and DNA was spooled with pasteur pipette. DNA was dried and washed by dipping end of pipette into 1 mL of 70% ethanol for 30 sec before dissolved in 150 μL TE buffer (Parayre *et al*., 2007)
- The heat shock/ boiled-cell method, where the pellet was added with 1 mL sterile distilled water, being vortex and subjected to heating temperature of 100°C for 20 min. The suspension was transferred 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) (Pandey *et al*., 2007)
- The Kimchi modified method, where the culture broth was mixed with DNA extraction buffer and 2 μL proteinase K (20 mg mL^{-1}) before shaking for 30 min at 37°C. A 300 μL of 20% SDS 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. Aqueous was transferred to another new tube and isopropanol, 70% ethanol was added to wash the pellet obtained before 100 μL TE buffer was added to dissolve DNA (Hur *et al*., 2000)
- The Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA), according to the manufacturer's instruction, with some modifications

The total genomic DNAs of all standard bacteria strains are listed in Table 1 were extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA), according to the manufacturer's instruction with some modifications. Ten milliliter of

Table 1: Bacterial strains used in this study

Bacterial strains	Sources
Lactobacillus brevis ATCC 14869	Isolated from Yakult® Drink, Japan
Lactobacillus gallinarium ATCC 33199	
Lactobacillus reuteri ATCC 23272	
Lactobacillus casei strain Shirota	
Lactobacillus acidophilus	Isolated from Vitagen® Drink
Lactobacillus rhamnosus GG ATCC 53103	Laboratory strain provided by Institute of Medical Research, Kuala Lumpur, Malaysia
Enterococcus faecalis JCM 5803	
Enterococcus faecalis S 256	
Salmonella choleraesuis JCM 6977	
Salmonella choleraesuis ATCC 14028	
Salmonella enteritidis (Group D) ATCC 13076	
Salmonella typhimurium S917	
Bacteroides ovatus ATCC 8483	
Bacteroides uniformis ATCC 8492	
Bacteroides vulgates ATCC 8482	
Bacteroides thetaiotaomicron ATCC 29741	
E. coli JM 109	
E. coli K-12	
E. coli E1	

Table 2: Primer set used in this study

Target organism	Primer set	Sequence (5' to 3')	Product size (bp)	T_a (°C)	Time (sec)	Reference
Lactobacillus genus	Lacto-16S-F	GGA ATC TTC CAC AAT GGA CG	216	56	10	Present
	Lacto-16S-R	CGC TTT ACG CCC AAT AAA TCC GG				Present

bacterial culture 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 and 10 mM EDTA, pH 8.0). Lysozyme (Sigma, USA) was added to the bacterial suspension with the final concentration of 20 mg mL^{-1} and incubated in water bath (Reciprocal Water Bath Incubator Model, Certomat® WR) for 1 h at 37°C before the suspension was centrifuged at 13,000 g for 3 min. The pellet of bacteria was then added with 600 μL of nuclei lysis solution to lyses the cell membrane before incubating for 5 min at 80°C. After cooling at the room temperature, 3 μL of RNase solution were added and the tubes were inverted for 5 times before the mixture was incubated again for 1 h at 37°C.

Two hundreds micro litters of protein precipitation solution was added into the mixture to purify the genomic DNA and the reaction mixture was vigorously vortexed for 20 sec. 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 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 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.

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 done, together with a ready-to-use of VC 1 kbp plus DNA ladder as a molecular weight standard (Vivantis, Shah Alam, Malaysia), according to the protocol described later under heading: gel electrophoresis. The extracted DNA was also checked by using UV-Visible spectrophotometer (UV-1601 Shimadzu Model, Japan) at 260 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 total genomic DNA was stored at 0-5°C for further analysis. The DNA extracted with the best method would then be used to continue for the PCR-based assay (Edwards, 2004).

Standard bacterial strains and growth conditions: Six strains of *Lactobacillus* sp. were used from different sources along with other 11 different bacterial species (*Salmonella*, *Bacteroides, Enterococcus* and *E. coli*) (Table 1). The bacterial strains were grown anaerobically in MRS Broth, respectively. The strains of *Salmonella* sp., *Escherichia faecalis* and *E. coli* were grown in aerobic condition in nutrient media. For *Bacteroides* sp., they were grown in anaerobic condition in nutrient media. All bacteria were incubated for 24-72 h at 37°C.

Primers: *Lactobacillus*-specific primer set was being used in this study, Lacto-16S-F/Lacto-16S-R primer set, which was obtained from First Base Laboratory, Shah Alam (Table 2). The specificity of Lacto-16S-F / Lacto-16S-R was evaluated by using many strains from three LAB genera (i.e. *Lactobacillus* and *Enterococcus*) and 11 strains from three non-LAB genera (i.e., *Salmonella*, *Bacteroides* and *E. coli*) (Table 1).

PCR reaction: The PCR reaction 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, 0.125 μL of 5 u μL^{-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 genomic DNA was extracted by using the most efficient method used in this PCR reaction. The reaction mixture in micro-centrifuge tube was amplified in a thermocycler PCR system (PTC-110™ Model, MJ Research, Inc.). For *Lactobacillus* sp., 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 sec), annealing (61°C, 30 sec) and extension (73°C, 60 sec). The final extension step was performed at 73°C for 5 min and the holding temperature was 10°C (An *et al*., 2006).

Gel electrophoresis: After PCR amplification, 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). A ready-to-

use VC 100 bp Plus DNA Ladder-molecular weight standard (Vivantis, Shah Alam, Malaysia) and a positive control were run together with the 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 staining (0.5 μg mL^{-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) (Parayre *et al*., 2007).

RESULTS

Quality and yield of extracted DNA: The purity and quality of extracted DNA was important and constituted the prerequisites in PCR-based detection assays. Thus, in the experimental design, A_{260}/A_{280} ratio of extracted DNA from the CMC enrichment 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. The formula for the calculation of DNA yield was as follows:

$$\text{DNA concentration } (\mu g\ \mu L^{-1}) = \frac{A_{260} \text{ in OD units} \times 50\ \mu g\ mL^{-1} \times DF}{1000}$$

Where:

DNA yield (μg) = DNA concentration (μg μL^{-1})×Amount of DNA kept as stock (100 μL)

Table 3 shows the results of the quality and yield of the extracted DNA using four different methods. Results for DNA quality showed that DNA extracted from CMC using phenol-chloroform method, boiling method and kimchi method gave an $OD_{260/280}$ ratio of less than 1.5. However, DNA extracted from CMC using Wizard protocol had an $OD_{260/280}$ 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 findings of the current study showed that DNA extracted with Wizard protocol produced the highest yield compared to the other three methods (Table 3). The trend was almost similar to the A_{260}/A_{280} ratio of DNA quality. The DNA extracted using four protocols were observed for the degradation using agarose gel electrophoresis. It was observed that all DNAs extracted from CMC by Wizard protocol produced integral band 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 CMC (Fig. 1).

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

Primer specificity: Primer specificity was tested before performing PCR assay on CMC samples to ensure a proper and specific amplification process. The specificity of the PCR primer set Lacto-16S-F /Lacto-16S-R was tested by PCR assay with strains other than *Lactobacillus* sp., including *Enterococcus* sp. and non-LAB strains (Fig. 2). All the *Lactobacillus* strains used in this study were PCR positive to the *Lactobacillus* genus specific primer set Lacto-16S-F /Lacto-16S-R while other bacteria proved to be negative. Therefore, this primer set proved to be highly specific even when it was used for DNA extracted from CMC, which is full of mixed bacteria, fragmented DNA and PCR suppressor proteins. The specificity of the primer set Lacto-16S-F /Lacto-16S-R for the 16S rRNA gene fragment represents a strain-specific DNA for almost all *Lactobacillus* sp. Under the described PCR conditions, the six reference strains of *Lactobacillus* sp. generated the expected PCR product at molecular weight of 216 bp (Fig. 2).

PCR detection of *Lactobacillus* sp.: After the gel was visualized under UV transilluminator (Vilber Lourmat, Cedex, France), photos were taken using gel documentation system (Bio Rad Gel Doc 2000 Model Imaging System) (Fig. 3). The molecular identification of target *Lactobacillus* sp. was detected in all CMC samples except CMC1 and CMC2, which represents 66.6% of tested samples (Fig. 3). The results from gel electrophoresis confirmed that the molecular weight of the amplicon for *Lactobacillus* sp. was the correct size (216 bp), which indicated that the primers were specific

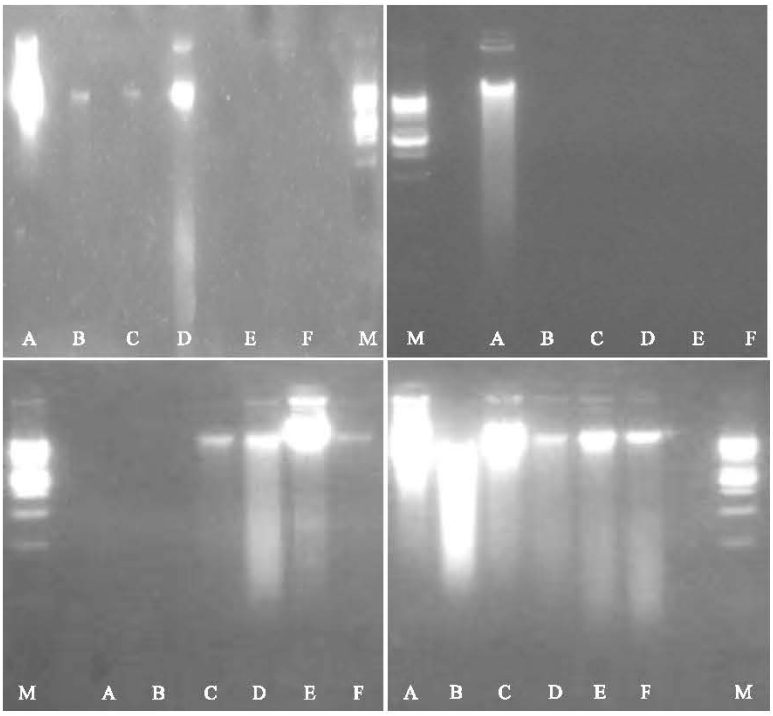

Fig. 1: Agarose gel electrophoresis of genomic DNA extracted from fish sauce and shrimp sauce samples with four different methods. Upper left = Phenol-chloroform method; Upper right = Kimchi method; Lower left = Boiling method; Lower right = Wizard protocol. Lane M = 1 Kb DNA ladder Marker; Lane A-C: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

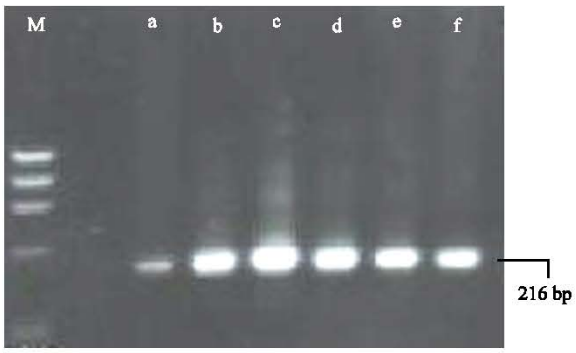

Fig. 2: Amplified PCR products from *Lactobacillus* strains with primer set Lacto-16S-F /Lacto-16S-R. Lane m: 100 bp ladder; Lane a-f: PCR products amplified from six *Lactobacillus* strains

(Fig. 2). Since, single band of PCR was found, this confirmed that no products of non-specific amplification, including primer dimers, were contributing to the signal. Moreover, these results revealed that the optimized DNA extraction, the specific primers used and the used PCR protocol were successful in amplifying the diagnostic target gene of *Lactobacillus* sp. which is present in genomic DNA came from a very PCR-hostile CMC.

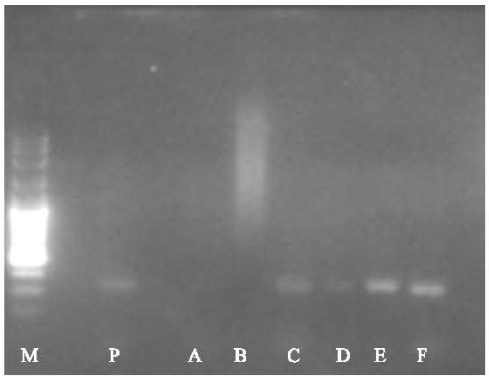

Fig. 3: Agarose gel electrophoresis of PCR product amplified from *Lactobacillus* sp. in fish sauce and shrimp sauce samples. Lane M = 100 bp DNA ladder Marker; Lane P = positive control; Lane A-C: Fish sauce A, B and C; Lane D-F: Shrimp sauce D, E and F. Positive PCR bands at 216 bp were found in C and D-F samples

DISCUSSION

The culture-based methods are labor-intensive, imprecise and time-consuming when used for CMC;

therefore DNA amplification methods, PCR, are being 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, CMC. This study aimed at standardizing thoroughly a series of molecular steps from DNA extraction till PCR reaction. This standardization was absent in the previous studies. Moreover, the previous studies did not test the specificity of PCR primers among large set of competing bacteria.

Quality and yield of extracted DNA are primary requirements for PCR-based detection assays in CMC and the selection of suitable extraction method was determined for a successful and valid PCR analysis. The main limitation associated with PCR application for the detection of microorganisms in CMC 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 this study, the tested CMC samples were highly complex matrices with high numbers of PCR inhibitors and background microflora (Rossen *et al*., 1992) which would interfere with the ability of PCR to detect *Lactobacillus* sp. Thus, prior to PCR amplification, the quality and the yield of the extracted genomic DNA had to be determined.

In addition, the purity of extracted DNA from CMC samples was indicated by $OD_{260/280}$ ratio, where $OD_{260/280}$ 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). The usual contaminant during DNA purification is protein when a lower A_{260}/A_{280} ratio is encountered. 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 by protein precipitation solution, to remove the protein in DNA extracted from CMC samples. Martin *et al.* (2006) stated that DNA isolation done by Wizard protocol from fermented microbial matrices allowed an increase in the amount of the purified DNA sample added to PCR without inhibitory consequences. These stringent conditions pursued in the current study regarding DNA extraction and purity of the extracted DNA of *lactobacillus* sp. rendered this study deviate from the failing molecular detection methods have been done before.

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 highly processed food (Tung-Nguyen *et al*., 2008). Although, the used CMC samples were fermented products which might affect DNA integrity, the high molecular weight single band obtained for all 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), DNA could be used in the subsequent PCR detection of lactobacillus bacteria because of the intact DNA (Fig. 1) and higher DNA yield (Table 3) compared to the other three methods. A previous study revealed that one of the main obstacles for detecting probiotic bacteria in CMC is the fragmentation of DNA (Kropf *et al*., 2004). On the other hand, the current study has standardized and verified the integrity of the extracted DNA from CMC samples.

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.

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 CMC is very important prior to the use of PCR-based assay. Therefore, the specific primer set of *lactobacillus* sp., Lacto-16S-F / Lacto-16S-R, was subjected to a thorough testing. It was found that Lacto-16S-F / Lacto-16S-R is highly specific for *lactobacillus* sp. rather than other tested bacteria, namely, *Bacteroides*, *Enterococcus*, *E. coli* and *Salmonella*. This grants validity for using this primer set in the subsequent PCR-based assays and abolishes the main obstacle of using PCR in CMC, meaning, the possibility of cross reaction and non-specificity. Moreover, this study is the first one to scrutinize and prove the specificity of Lacto-16S-F / Lacto-16S-R primers among bacterial species isolated from different CMC samples.

After standardizing PCR adequately, which is the main aim of this study, the findings of detecting *Lactobacillus* sp. can be discussed in confidence. The current study found that the optimized steps of DNA extraction and PCR reaction were successful in detecting

and identifying specifically *lactobacillus* bacteria in 4 out of 6, 66.6%, CMC samples. The absence of *Lactobacillus* sp. in two CMC samples might be due to the variation in chemical composition and quality characteristic of the CMC samples. And this could be related to the method of processing the raw material used. Moreover, the presence of PCR inhibitor in the PCR reaction, due to the high protein and fat content of such CMC, might present a barrier for the successful amplification and subsequent detection of target bacteria. Nevertheless, the impreciseness and difficulty of detecting *lactobacillus* microflora in CMC samples makes the current results of this study highly interesting and practicable.

CONCLUSIONS

Taken together, in this study, culture-independent methods, PCR, were used to investigate the presence of *Lactobacillus* in complex microbial communities, CMC. 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. Using traditional PCR proved to be superior for the detection and quantification of *Lactobacillus* bacteria in highly crude and PCR-suppressor- contaminated samples. The optimized molecular steps of DNA extraction, primer specificity checking and standardized PCR reaction indicated that *Lactobacillus* sp. were successfully detected in most of CMC samples at molecular weight band of 216 bp. Hence, optimized molecular detection methods might be more specific and sensitive than traditional culture-based methods for the detection of probiotic bacteria in highly crude and PCR-suppressor-contaminated samples.

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