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COMPARISON BETWEEN TWO DNA EXTRACTION METHODS FOR THE DETECTION OF LACTOBACILLUS IN YOGHURT

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ABSTRACT

Probiotics are formulated into dairy products like yoghurt to enhance its functional properties. By definition probiotics are a group of live microorganisms, capable in providing health benefits to the host when delivered in adequate quantity. The genera most commonly used in this dietary preparation is Lactobacillus. The aim of this study was to evaluate the efficiency of the Lactobacillus DNA extraction from yoghurt using a commercial kit (PromegaTM WizardTM Genomic DNA purification kit) and an optimized boiled cell extraction method. A total of 5 isolates, obtained from 5 different commercial brands were characterized as Lactobacillus by considering physiological, morphological and biochemical characteristics. The DNA was then extracted by each of the two methods, and tested in genus-specific PCRs to confirm the presence of Lactobacillus. The DNA quantity and quality was determined by spectrometry. The most efficient method of extraction was the kit based method, in which a substantial DNA yield with purity was generated. The DNA yields from both extractions were further subjected to one-way analysis of variance (ANOVA) using SPSS (Version 21, IBM) statistical software, in which a significant difference [P (0.019) 0.05] was observed. In conclusion, the study emphasize on the use of appropriate DNA extraction protocol as the DNA purity and the quantity contributes to quality biological analyses such as polymerase chain reaction (PCR).

Key words: Yoghurt, Lactobacillus, DNA yield

INTRODUCTION

Functional foods are considered a dietary requirement due to supplementary health benefits to an individual's health, in addition to its provision of incorporated nutrients. Among many other additives used in functional food production, probiotics are the most frequently used, precisely in fermented dairy products (Granto et al, 2010). Probiotics by definition are a group of live microorganisms, capable in providing health benefits to the host when delivered in adequate quantity. The term "probiotic'' was Greek originated and said to be invented by Ferdinand Vergin in 1954, which was further addressed in articles by Lilly and Stillwell (1965), Fuller (1989), Guarner and Schaafsma (1998) (Sanders, Merenstein, Merrifield and Hutkins, 2018).

The lactic acid bacteria (LAB) is one of the large group of microorganisms commonly preferred as potential pathogens in food industry. LABs ferment carbohydrate either homofermentatively or heterofermentatively to produce lactic acid as the major end product in which by-products contribute to the enhancement of

organoleptic properties of the food. LAB comprises of 16 genera, in which 12 are industrially exploited as starter cultures, including Lactobacillus, Streptococcus, Bifidobacterium, Carnobacterium, Aerococcus, Leuconostoc and Enterococcus (Zielińska and Kołożyn-Krajewska, 2018).

The largest genus within the LABs are Lactobacillus, which belongs to phylum Firmicutes and family Lactobacillaceae. They are known as gram positive, rod shaped, catalase-negative, non-spore forming anaerobic bacteria. Depending on the carbohydrate metabolism pathway, the bacterium is classified as obligate homofermentive, facultative heterofermentive or obligate heterofermentive. Lactobacillus share a diverse gram stain morphology like short plump rods, long slender rods, chains or palisades due to its species variations. Furthermore colony morphology varies on the cultured medium, like gray colonies on blood agar and white, mucoid colonies on MRS (Man, Rogosa, and Sharpe) (Karami et al., 2017; Goldstein, Tyrrell and Citron, 2015).In addition to its presence in commercial products, some acid-tolerant Lactobacillus are naturally found in the human gut. Therefore, its products are stabilized by the bile and acidic PH. Probiotic actions of Lactobacillus include strengthening of epithelial barrier, production of antimicrobials, immune system modulation, the inhibition of pathogen adhesion by strong adherence to the intestinal mucosa and concomitant elimination of the pathogens (Bull, Plummer, Marchesi and Mahenthiralingam, 2013). Intestinal barrier integrity is said to be stabilized by the modulation of the genes encoding tight junction proteins like cadherin and catenin, further more cytokine induced apoptosis is inhibited by activating anti-apoptotic proteins thereby, preventing intestinal disorders like inflammatory bowel disease. Presence of mucus adhesion-promoting proteins mediate the adhesion of the bacteria with the mucus. Furthermore, the stimulation of the release of mucin and defensins from the epithelial cells inhibit the adherence of gastrointestinal pathogens. The productions of antimicrobials also known as bacteriocins, bacteriocins induce pore formation in the pathogen's cell membrane, causing destabilization of the membrane, resulting cell lysis or involve in the inhibition of the pathogenic cell wall synthesis (Bermudez-Brito et al., 2012). In the regard of the immunomodulation, down regulation of toll-like receptors, inhibition of NF-kB signaling in the innate immune system and up regulation of NLR expression involve in apoptosis, contributes to the regulation and suppression of intestinal inflammation (Wells,2011).

Probiotics delivery by non-conventional food based products like yoghurt, cheese and chocolate as a method of supplementation of intestinal favorable bacteria is preferred over conventional pharmaceutical formulations due to easy availability, convenience and the providence of accurate physiological conditions for the bacterial survival (Govender et al., 2013). Among commercial products, yoghurt have been commonly used for the probiotic delivery. The yoghurt contain a mixture of milk, water, protein, fats, carbohydrates and bacterial cultures. The synergistic relationship between the components of the dairy product and probiotic cultures marks fermented dairy products as a good candidate. The ability of the yoghurt to deliver probiotics is demonstrated by Hemsworth, Hekmat and Reid, 2011; where the incoporated Lactobacillus strain count elevated upon administration of the yoghurt. Furthermore, Marafon et al., in 2011 demonstrated the methods of optimization of the yoghurt as a delivery system. Probiotic yoghurt usually contain a combination of Lactobacillus and

streptococcus or Bifidobacterium and Lactobacillus as starter cultures (Routray and Mishra, 2011). A study evaluated the combined effects of Bifidobacterium and Lactobacillus in yoghurt which produced satisfactory results in high caries individuals upon short-term consumption (Bafna et al., 2018). Similarly the efficacy of the two probiotics in lowering serum cholesterol level was studied in which demonstrated positive results, providing evidence of the Lactobacillus in the health management (Rerksuppaphol and Rerksuppaphol, 2015). This study evaluate the efficiency of the two DNA extraction methods for Lactobacillus and emphasize on the necessity of high purity and yield of DNA for successful PCR.

METHODOLOGY

Sample collection and preparation

Five yoghurt samples (A-E) from different brands were obtained. Approximately 10g of each sample was aseptically transferred into the beaker. The samples was then gently stirred to concoct a homogenous mixture. The above step was carried out for all the samples.

Culturing of the bacteria on MRS agar

A loop full of sample was aseptically obtained and streaked on agar (refer appendix) using quadrat technique. The petri plates was then incubated at 37□C for 24-48 hours before observing the morphological features.

Gram staining

A loop full of distilled water was placed on the glass side and an isolated bacterial colony was introduced aseptically into the water drop. The bacteria was evenly dispersed to form a smear. The smear was then air dried and heat fixed before proceeding with the staining. The smear was initially stained with crystal violet for 1 minute, followed by Gram's iodine for 1 minute, decolorizing agent for 3seconds and safranin for 1 minute. After each step of flooding the slide with the reagents and its incubation, the slide was thoroughly washed in gentle stream of water and blotted and air dried before observing under 100X magnification.

Catalase test (Slide test)

Small amount of bacterial colony was aseptically transferred to a glass slide using a loop. A drop of 3% H2 O2 was introduced to the bacteria on the slide, and then observed for fizzing (formation of bubbles)

Sub culture

Into the prepared broth (refer appendix), pure bacterial colony was introduced and incubated at 37□C for 24-48 hours. The aforementioned step was repeated for all the five samples. DNA extraction with Modified boil cell method by Perera and Weerasooriya, 2019.

5ml of bacterial broth was centrifuged at 4000rpm for 15minutes. Into the pellet 100µl of TE buffer was added and left in the water bath for 20 minutes, followed by quick freezing at -20□C for 20 minutes. The sample was then centrifuged at 4000rpm for 10 minutes. Supernatant was transferred into a new tube and 60µl of 10mg/ml lysozyme was added along with 5µl of 10mg/ml proteinase K and 20µl of 10% SDS. After the sample incubation at 37□C for 20 minutes, 0.5ml of saturated NaCl solution was added and mixed vigorously followed by a centrifugation at 13000rpm for 3 minutes. The supernatant was carefully obtained and transferred into a new microcentrifuge tube. 100µl of cold 100% ethanol was added and quick spinned. Supernatant was discarded, into the pellet 200µl of 70% ethanol was added to wash the pellet. The aforementioned step was repeated twice. The tubes was then allowed to air dried thoroughly in order to evaporate all the ethanol. DNA pellet was dissolved in 100µl of TE buffer and stored at -20ºC in the refrigerator.

DNA extraction with PromegaTM WizardTM Genomic DNA purification Kit

1ml bacterial broth was centrifuged at 13000rpm for 2 minutes and supernatant was discarded. 480µl of 50mM EDTA was then added along with 120µl of lytic enzyme and mixed gently using the pipette, followed by an incubation at 37□C for 45 minutes and centrifugation at 13000rpm for 2 minutes. The supernatant was then discarded. The sample was re-suspended with 600µl of nuclei lysis solution and incubation was carried out at 80□C for 5 minutes. After allowing to cool down to room temperature, 3µl RNase solution was added and mixed by inverting the tube several times. The sample was then incubated at 37□C for 60 minutes, after cooling down to room temperature, 200µl of protein precipitation solution was added and vigorously vortexed for 20 seconds. The sample was incubated on ice for 5 minutes followed by a centrifugation at 13000rpm for 3 minutes. Supernatant was carefully obtained and transferred into a new tube. 600µl of isopropanol was added and mixed by inverting the tube until thread-like DNA strands were visible. The sample was then centrifuged at 13000rpm for 2 minutes and the supernatant was discarded. 600µl of 70% ethanol was added and centrifuged at 13000rpm for 2 minutes. The ethanol was left to aspirate and air dried overnight. 100µl of rehydrating solution was added and incubated at 65□C for 1 hour. The solution was periodically mixed and stored at -20□C.

Quantification of the extracted DNA

10µl of both boil cell and kit based extracted DNA was diluted in 2990µl of TE buffer. The absorbance was measured at 260nm, 230nm and 280nm in triplicates (Refer appendix).

DNA concentration, Yield and purity was calculated using below equations (Refer appendix);

DNA concentration (µg/µl) = absorbance at 260nm x dilution factor x 50µg/µl

DNA yield (µg) = DNA concentration (µg/µl) x DNA stock volume (100µl)

DNA purity = A260/A280 and A260/A230 A=absorbance

Genus specific identification of Lactobacillus using PCR

Boil cell and kit based extracted DNA was amplified using genus specific primers (Table 1) and the PCR mix was prepared accordingly (Table 2).

Table 2. PCR genus-specific primers sequences

Primer	Sequence (5'-3')	Expected size of the amplicon	Reference
LactoF	TGGAAACAGRTGCTAATACCG	233bp	Byun *et al.*, 2004
LactoR	GTCCATTGTGGAAGATTCCC		

Table 2. Reagents and volumes required for the preparation of the PCR mixture for Lactobacillus identification

Reagents	**Volume for 1 reaction (µl)**	**Final Concentration**	**Volume for 8 reactions (µl)**
5X PCR buffer	5µl	1X	40µl
25Mm $MgCl_2$	1.5µl	1.5mM	12µl
10mM dNTPs	0.5µl	0.2mM	4µl
2µM Lacto F primer	2.5µl	0.2µM	20µl
2µM Lacto R primer	2.5µl	0.2µM	20µl
5U/µl Taq DNA polymerase enzyme	0.25µl	0.05U/µl	2µl
Sterilized water	11.75µl		94µl
DNA	1µl	100 ng/µl	
Total volume	25µl		200µl

The reaction mixture of 25µl was prepared for 8 reactions, 5 samples (A-E), positive and negative controls. The PCR was carried out according to the cyclic conditions given in table 3.

Table 3. Cyclic conditions for the PCR

Process	**Temperature**	**Time**	
Initial denaturation	94◦C	5 minutes	
Denaturation	94◦C	1 minute	35 cycles
Annealing	62◦C	1 minute	
Extension	72◦C	2 minutes	
Final extension	72◦C	12 minutes	
Final hold	4◦C	∞	

Visualization of PCR products

Into 2% agarose gel, 2µl of 50bp DNA ladder was loaded into the first slot separately followed by addition of 7µl of each PCR products (A-E, and N) consecutively. Positive control was loaded adjoining to the negative control (N). The gel was initially allowed to run at 45V for 35 minutes and then switched to 50V for 25minutes. Finally the gel was observed under the UV light.

DATA ANALYSIS

DNA yield obtained from modified boiled cell and kit based methods was compared using one way ANOVA, and the P value was calculated at 0.05 significance level.

RESULTS

Culturing of the bacteria on MRS agar

The bacterial culture was incubated at 37□C for 24-48 hours and the colony morphology was observed as below (Figure 1)

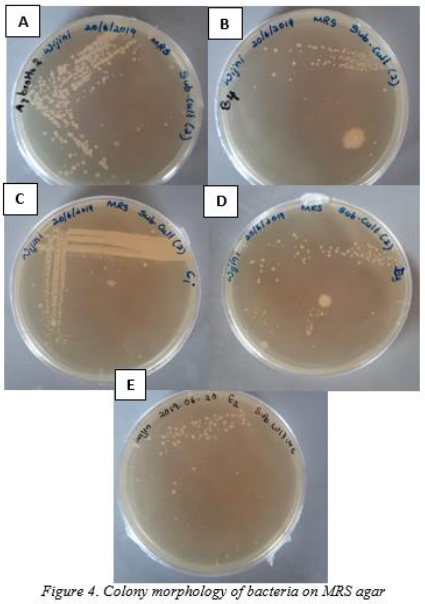


Figure 4. Colony morphology of bacteria on MRS agar

Colonies with creamy grey colour, round in form, entire in margin and raised in elevation was noted on the surface of the medium in all the five samples. Contamination was observed in samples B and D

Gram staining

The morphological features of the isolated bacteria were further examined by Gram staining as shown below (Figure 2)

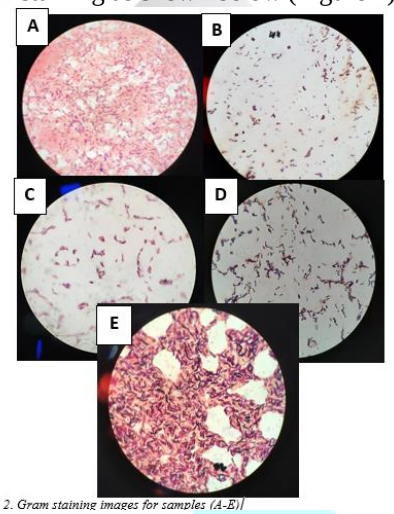


Figure 2. Gram staining images for samples (A-E)

Rod shaped, purple coloration was observed in all the five samples indicating the presence of Gram-positive bacillus bacteria

Catalase Test

The presence of catalase enzyme in the bacterial isolate was determined by the catalase test (Figure 3)

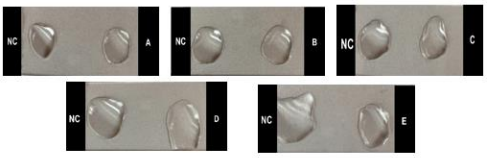
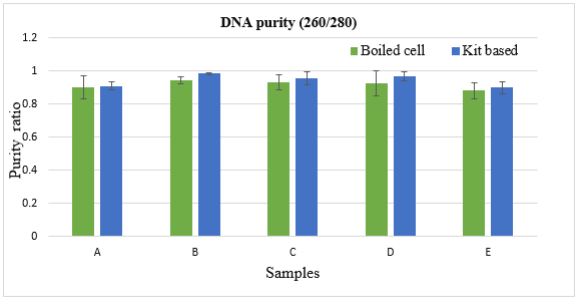


Figure 3. Slide method for catalase test

The mixture in all the five samples produced no effervescence, thus, identifying the bacteria as catalase-negative.

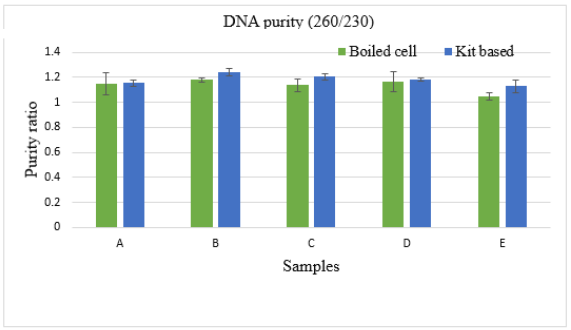
DNA purity (260/280)



Graph 1. DNA purity (260/280) obtained from the two extraction methods

The mean values of the ratio A260/A280 was high in kit based method. The sample B depicts the highest purity and Sample E with lowest purity in both the extraction methods

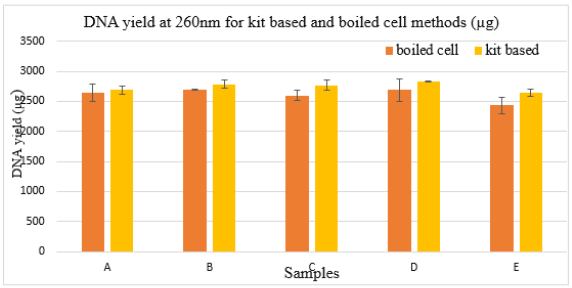
DNA purity (260/230 Ratio)



Graph 2. DNA purity (260/230) obtained from the two extraction methods

The mean values of the ratio A260/A230 was high in kit based method. The sample B depicts the highest purity and sample E with lowest purity in both the extraction methods

The DNA yield



Graph 3. DNA yield obtained from the two extraction methods.

A higher DNA was obtained from kit based than the modified boiled cell method. Sample D depicts the highest yield among all the samples in kit based whereas, sample B for modified boiled cell method.

DATA ANALYSIS

DNA yield from the two extraction methods was compared using one way ANOVA generated by SPSS (Table 4)

ANOVA : DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	96787.200	1	96787.200	6.185	.019
Within Groups	438185.100	28	15649.468		
Total	534972.300	29			

Table 4. Statistical analysis for the DNA yield with the extraction method

P value (0.019) is less than 0.05.Therefore, there is a significant difference between DNA yields obtained from the two extraction methods.

Genus specific identification using PCR

The DNA from both extraction methods was amplified using genus specific primers and the gel images was obtained as below (Figure 4 and Figure 5).

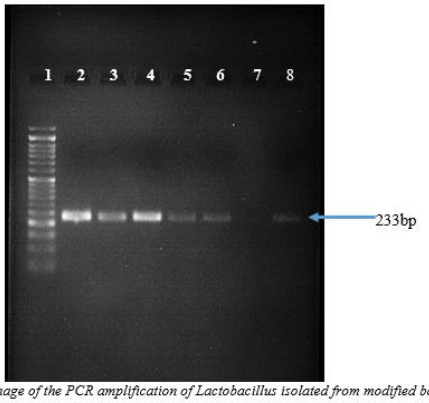


Figure 4. Gel image of the PCR amplification of Lactobacillus isolated from modified boiled cell method (L1- 50bp DNA ladder, L 2-L6-Samples, L7-Negative control, L8-Positive control)

All the produced amplicons of 233bp band size which was similar to the positive control and the negative control was free from any bands. Furthermore variations in band intensities was noted.

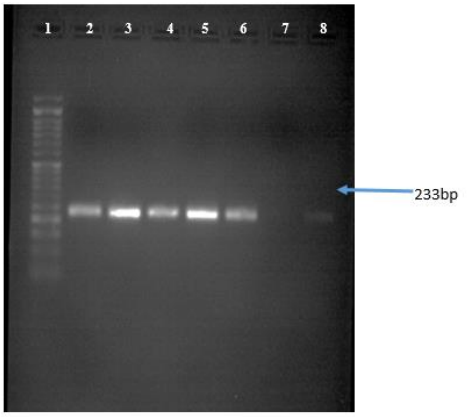


Figure 5. Gel image of the PCR amplification of Lactobacillus isolated from kit based method (L1- 50bp DNA ladder, L 2-L6-Samples, L7-Negative control, L8-Positive control)

All the produced amplicons of 233bp band size which was similar to the positive control and the negative control was free from any bands. Furthermore variations in band intensities was noted.

DISCUSSION

Probiotics are a group of live microorganisms, defined by their genus, species and strain designations. Upon administration of adequate quantity, they are capable in providing health benefits to the host by restoring the gut floral balance (Sanders, Merenstein, Merrifield and Hutkins, 2018). The aim of this study was to isolate Lactobacillus from yoghurt by two extraction methods and evaluate the DNA generated with regards to yield and purity

The isolates of each yoghurt sample was phenotypically identified. All samples displayed creamy-grey mucoid colonies on MRS medium and exhibited a colony morphology of round in form, entire in margin, raised in elevation as observed in Lactobacillus. Even though MRS is a selective culture medium designed to encourage Lactobacillus growth, Streptococci growth was noted with colonies of creamy white in colour, round in form, entire in margin and convex in elevation. The streptococcus growth could to be inhibited by lowering the PH level of the medium. Similar results was obtained by Guevarra and Barraquio, 2016. Fungal growth was noted in samples B and D which may be due to the sample originated contamination. This could be prevented by the addition of antifungal compounds like cycloheximide (Karami et al., 2017) (Figure 1). Considering the gram staining (Figure 2), the bacteria in all five samples appeared bacillus in shape with bluish-purple stain. The gram positive rod shaped bacterial isolates were determined as representative of genus Lactobacillus (Qian et al., 2018). Furthermore, the catalase test (Figure 3) was negative as no bubbles was evolved, thereby confirming the presence of Lactobacillus with the aid of the results obtained by Islam et al., 2016.

Isolation of DNA with high yield and maximum purity are the two main features of a good extraction method. The purity was assessed by spectroscopy using both A260/A280 and A260/230 absorbance ratios (Graph 1 and 2). As a guideline, a good quality DNA should possess a value from 1.8-2.0 for A260/A280, the values above or below the reference range indicative of contamination with RNA and protein respectively. It has been reported that DNA absorption depends on the pH of solution, acidic pH (low ratio) and basic pH (high ratio). Therefore, lactic acid produced by Lactobacillus is suspected to cause lower A260/A280 ratio (Lucena-Aguilar et al., 2016). Whereas, in A260/A230 any value below the range 1.8-2.2 indicates contamination with phenol, salt, proteins or polysaccharides

(Olson and Morrow, 2012). Even though both extraction methods failed to achieve the criteria, upon value comparison, less contamination was noted in kit based extraction. This may be due to the additional step of protein precipitation after the RNase treatment both removing contaminants, especially proteins (Abed, 2013). Even though, the extracted DNA from both methods lacked purity, the DNA was used for the PCR to detect bacteria, in which reliable bands were obtained indicating the presence of intact DNA (Abdulamir, Yoke, Nordin and Baker, 2010).

The quality and yield of DNA is said to have a great influence on PCR process. The result in Graph 3, demonstrated the DNA extracted from kit protocol was higher than that of boiled cell. Moreover, the statistical analysis (Table 4) indicate a significant difference between the yield obtained by both extraction methods, as the p value <0.05 confirming the observation in the graph. According to Becker et al., 2016 Wizard genomic purification kit produced the highest yield among five commercially available kits for bacterial chromosome and plasmid DNA extractions. An adequate yield could be possible due to the addition of lysozyme in boiled cell method. Lysozyme is a best known muramidase, which efficiently hydrolyses 1, 4-beta glycosidic linkages present in the peptidoglycan layer of the gram-positive bacterial cell wall (Bag et al., 2016). A previous study conducted to determine the yield and quality of bacterial DNA extract from human oral rinse samples demonstrated a direct relationship between cell lysis method and the DNA yield. Here, zirconium beads cell lysis together with lysozyme was emphasized as an effective method for the bacterial DNA extraction (Sohrabi et al., 2016). However, in contrast, another similar study notify the application of enzymatic digestion (Lysozyme, mutanolysin and lysostaphin) alone than using bead-beading for greater DNA yield (Rosenbaum et al., 2019). The kit protocol of this study utilize both lysozyme and EDTA. According to Moore et al., 2004, lysozyme in combination with EDTA considered efficient in disrupting the bacterial cell wall. The modified boiled cell method produced better yield than the native boiled cell (Perera and Weerasooriya, 2019). Even though, the modified boiled cell method is a rapid, cost-effective, simple method for DNA isolation, due to the rigid nature of gram-positive bacterial cell wall, the yield generated was low (Ahmed, Asghar and Elhassan, 2014). However, the DNA extraction by boiling was sufficient for the PCR amplification of Lactobacillus DNA. Another study conducted to optimize and evaluate three Lactobacillus extraction methods confirm a higher yield from kit than boiled cell and phenol-chloroform methods, here an additional proteinase K step was included in the kit protocol which aided in further lysis of the cell (Abdulla, 2014).

A PCR was carried out to further confirm the results obtained from biochemical tests. The positive control containing lactobacillus produced an amplicon of 233bp (Byun et al., 2004). Similar bands at 233bp was observed for all the five samples each extracted from both the extraction methods. According to these results, all the samples were positive for Lactobacillus. Moreover, the negative control showed no band, indicating free from contamination. In this study, the PCR result of 233bp band, was in accordance with a study conducted by Senthilkumar et al., 2018).

CONCLUSION

In this study the microbiological and molecular techniques were used to determine the presence of Lactobacillus in a mixed microbial population. Among the

two extraction methods used, the PromegaTM WizardTM Genomic DNA purification kit was efficient over modified boiled cell by producing considerable DNA yield and purity. The variations in the DNA yield and the purity with regard to the boiled cell could be improved by further optimizing the aforementioned protocol.

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