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IDENTIFICATION OF LACTOBACILLUS IN FERMENTED CHEESE AND THE ANALYSIS OF THEIR ANTIBIOTIC RESISTANCE

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ABSTRACT

Probiotics are living microorganisms that can improve health by providing benefits beyond that of the traditional nutritional value when consumed in required amounts. They are present in various fermented foods, including cheese where Lactobacillus is one of the most commonly found species. This study was designed to isolate and evaluate the antibiotic resistance patterns of Lactobacillus present in ten commercially available Cheddar (n=5) and Gouda cheese (n=5) samples. The Lactobacillus from homogenized cheese samples were isolated on Lactobacillus specific MRS agar, and phenotypic identification was achieved by morphological and biochemical investigations such as colony morphology, gram's staining and catalase test. The genomic DNA from isolated Lactobacillus colonies were extracted from the Wizard genomic DNA purification kit and modified boiled cell method. Extracted DNA was quantified using the spectrophotometer. Results revealed that the DNA yields significantly varied between the two methods ($P < 0.05$). PCR based genotypic detection of Lactobacilli using genus-specific primers revealed the presence of bands in all samples. Furthermore, in a PCR assay for the detection of antibiotic resistance genes, the erm(B) gene was found in 1 sample (n=1) and no sample was positive for the tet(M) gene (n=0). In conclusion, the wizard method was confirmed as the most efficient extraction method since it indicated higher DNA yields and purity. Screening of antibiotic resistance of probiotics used in the food industry is essential for the safety of probiotic food products as resistance genes can be transferred to pathogens.

Keywords: Probiotics, Lactobacillus, Cheese, Antibiotic Resistance

INTRODUCTION

Probiotics are viable microorganisms which when administered in appropriate amounts exert beneficial effects on the consumer by improving the gastrointestinal flora in the GIT (Kechagia et al., 2013). These bacteria are non-toxic and are categorized as 'GRAS' organisms and can be safely used in the processing of food and clinical applications. They are highly used for fermenting food or added as dietary supplements to food, especially for dairy products as they able to effectively carry these probiotic microorganisms especially species of Lactobacillus and Bifidobacterium. Common food types that contain probiotic bacteria include fresh milk, and fermented food such as olives, pickles yoghurt, curd and kefir (Rezac et al., 2018). Some of these organisms reside in the human gut, naturally associated with the

gastrointestinal microbiota. These beneficial probiotic microorganisms promote digestion, strengthen the immune system, inhibit the growth of pathogenic bacteria, alter intestinal flora equilibrium and increase resistance to infection. In addition, probiotics is therapeutically proven to reduce blood cholesterol, prevent cancer, improve lactose tolerance, prevent or reduce the effects of Crohn's disease, acute diarrhoea, and constipation as well as candidiasis urinary tract infection (Reid, 1999). A study by Ghoneum and Gimzewski, (2014), found that Lactobacillus species in kefir possessed an apoptotic effect on cell lines of myeloid leukaemia. Yang et al., (2014), showed that the clinical improvement of children with atopic dermatitis is associated with probiotic supplements containing Lactobacillus species.

The genus Lactobacillus is gram-positive, catalase-negative, non-spore producing bacteria which are appeared as non-motile thin rods in different lengths (Rafieian-Kopaei et al., 2017). It is also known as a member of lactic acid bacteria as they produce lactic acid as a sole or primary product of carbohydrate metabolism. In addition, Lactobacillus is fermentative, facultative anaerobes or aerotolerant, aciduric or acidophilic bacteria with below 50% mol (33% - 50%) G + C content (Tannock, 2004; Slover and Danziger, 2008). Currently, more than 80 species of Lactobacilli have been identified and, L. casei, L. acidophilus, L. fermentum, and L. salivarius are the commonest Lactobacilli found in human GI tract (Walter, 2008). Probiotics confer beneficial effects on human health via several mechanisms. They contribute to the enhancement of the intestinal epithelial barrier, the only physical barrier against invasion of the tissue's underneath by potential pathogens. For instance, Lactobacilli regulate the genes encoding the proteins responsible for cell adhesion. Probiotics adhered to the intestinal mucosa causes alterations in intestinal mucins, to facilitate pathogen binding and also trigger the epithelial cells to secrete defensins, to fight against pathogenic bacteria. Moreover, the production of lactic and acetic acid from probiotic bacteria can result in environmental modification to make it less suitable to the competitive pathogens thereby, excluding pathogenic microbes (Bermudez-Brito et al., 2012). The major role is played by antibacterial substances including bacteriocins produced by the probiotic bacteria, which are generally considered alternative to antibiotics that act against the growth of several pathogenic bacterial strains (Eid et al., 2016). Thereby, can improve host health by fighting off pathogenic organisms and treating bacterial intestinal infections. Jin et al. (1996) reported that Lactobacillus species from chicken intestine inhibited strains of E. coli and Salmonella. Similarly, antimicrobial activity of bacteriocin produced by probiotics showed that, Lactobacillus species had strong antibacterial effects against pathogenic bacteria (Tambekar and Bhutada, 2010).

Cheese is the most widely consumed milk product worldwide. Various Lactobacilli are used in cheese making. They aid in the development of the cheese flavor when used as adjunct cultures (Stefanovic et al., 2018). Lactobacilli rely on other potential sources of energy such as metabolizing peptides, amino acids, sugars released from enzymatic hydrolysis of casein, and products of degrading starter bacteria in order to sustain growth in cheese depleted of fermentable carbohydrates (Peterson and Marshall, 1990). The high-fat content and buffering capacity of the cheese provides additional advantages for the survival of probiotics in the gastric environment (Gomes da Cruz et al., 2009).

Lactobacilli resistance to antibiotics is achieved through acquired and intrinsic mechanisms. Intrinsic resistance is the

innate ability of the bacteria to tolerate the antibiotic activity through its inherent structural or functional characteristics. Vancomycin resistance of Lactobacilli is the best example. It inhibits peptidoglycan, in the bacterial cell wall, thus, inhibiting growth (Anisimova and Yarullina, 2018). Studies on L. acidophilus and L. reuteri species demonstrated that all L. reuteri species were resistant to vancomycin whereas, only a few strains of L. acidophilus were vancomycin resistant. Acquired resistance of bacteria is either due to the occurring of spontaneous gene mutations or horizontal gene transfer, which exerts a threat on the non-pathogenic strains. In the case of intrinsic antibiotic resistance, antibiotic resistance genes in Lactobacilli are not transferred to the intestinal microflora unlike the acquired resistance, which can result in the exchange of resistance genes from Lactobacilli to the intestinal flora and virulence genes vice versa. This can completely modify the genotypic profile of the microbes (Jose et al., 2015). Furthermore, in Lactobacillus species, genes conferring resistance to tetracycline and erythromycin through various mechanisms have been detected, of which tet(M) and erm(B) genes are the two commonly found genes (Gevers et al., 2003). These genes are considered potentially transferable to plasmids and integrons in pathogenic microbes within the host. The emergence of such bacteria can pose a serious global threat (Peterson and Kaur, 2018).

Lactobacilli have been observed to be resistant to a wide range of antibiotics. Thereby, has a high possibility of transferring the resistance to the consumers' intestinal flora and be horizontally transferred to a pathogen. The current study was aimed to characterize the most efficient method of DNA extraction and assess the antibiotic resistance, so as to evaluate the safety of the cheese samples to be consumed.

METHODOLOGY

Sample Collection and Preparation

Five cheddar cheese samples (A-E) and five gouda cheese samples (F-J) of different brands were purchased from Colombo and under aseptic conditions, 10g of each cheese samples were homogenized in 10ml of autoclaved distilled water in beakers.

Isolation of Lactobacillus on MRS Agar

20 ml of prepared MRS agar was poured into the petri dishes and allowed to solidify. Then, under aseptic conditions loop full of samples were inoculated on solidified MRS agar plates according to the quadrant streaking method. The petri plates were incubated at 370C for 48 hours.

Gram Staining

After 48 hours, colony morphology of the bacterial growths was observed, and bacterial smears were prepared on glass slides. The heat fixed smear was flooded with crystal violet for 1 minute, Gram's iodine for a minute, decolorizer for 3 seconds and safranin for 45 seconds. After each step of flooding, the slide was washed with distilled water.

Catalase Test

Bacterial smears were prepared using marked colonies on the glass slides and added a drop of 3% H2O2 on the smears.

Sub-culturing of pure colonies

The gram-positive and catalase negative colonies were sub-cultured in 10 mL Lactobacillus MRS broth, incubated at 37°C for 2 days and stored at 4 °C.

DNA extraction via Modified Boiled cell method

5ml of broth from each sample were taken into flacon tubes (15ml) and centrifuged at 4000rpm (15 minutes). Then, the supernatants were discarded and added 100µl of TE buffer. Thereafter, the falcon tubes were kept in the water bath at 1000C (20 minutes) and quickly froze at -200C (20 minutes). After that, the frozen

samples were thawed and centrifuged at 4000rpm (10 minutes). Then the supernatants were transferred to another Eppendorf tubes (1.5ml) and added 60µl of lysozyme (10mg/ml), 5µl of proteinase K (10mg/ml) and 20µl of 10% SDS. After that, incubated at 370C (1hour) and added 0.5ml of NaCl. Then, the Eppendorf tubes were shaken vigorously and centrifuged at 13000rpm (3 minutes). Thereafter, transferred the supernatants into new Eppendorf tubes and added 100µl of cold 100% ethanol. Then the samples were mixed and spun for few seconds, and supernatants were removed. After that, added 200µl of 70% ethanol and spun and removed the supernatants (repeated 2-3 times). After that DNA pellets were airdried and added 100µl of TE buffer. Finally extracted DNA samples were stored at -200C.

DNA extraction via Wizard® Genomic DNA Purification Kit

1 mL broth of sample A was added into an eppendorf, centrifuged at 13,000 rpm for 2 minutes and the supernatant was discarded. To it, 480 µl EDTA and 120 µl lysozyme was added, incubated for 60 minutes at 37°C, centrifuged at 13,000 rpm for 2 minutes and the supernatant was discarded. 600 µl nuclei lysis solution was added and incubated at 80°C for 5 minutes. After cooling at RT, 3 µl RNase was added, inverted a few times and incubated at 37°C for 1 h. To it, protein precipitation solution of 200 µl was added, vortexed for 20 seconds, incubated on ice for 5 minutes and centrifuged at 13,000 rpm for 3 minutes. The supernatant was transferred into an eppendorf containing 600 µl isopropanol, gently mixed, centrifuged at 13,000 rpm for 2 minutes and the supernatant was removed. The pellet was washed with 600 µl of 70% ethanol. Ethanol was aspirated after centrifuging at 13,000 rpm for 2 minutes. The pellet was left to air dry and was re-hydrated by adding 100 µl DNA rehydration solution. The mixture was then incubated at 65°C for 1 hour and the DNA extracted was stored at 2 - 8°C. The above procedure was repeated for the rest of the samples.

Quantification of Extracted DNA

10µl of extracted DNA samples were diluted in 2990µl of TE buffer and measured the absorbance at 260nm, 230nm, and 280 nm in triplicates. Then, calculated the DNA purity, concentration, and DNA yield, using below equations for both extraction methods.

DNA Purity = A260/280 and A260/230

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

DNA yield (µg) = DNA concentration (µg/µl) x amount of DNA kept as stock

Then, DNA samples were diluted up to 100ng/µl by adding adequate amount TE buffer for the usage of PCR

Identification of genus Lactobacillus by PCR

The extracted DNA from both methods were amplified using genus-specific primers (Table 02). The PCR master mix was prepared according to the below table (Table 01)

Table 01. Materials required for preparation of PCR master mix for five samples, negative control, and positive control.

Component	Stock Concentration	Working Concentration	Volume (µl)	
			Per one reaction	Per 16 reactions
PCR buffer	5X	1X	5.0	80.0
$MgCl_2$	25 mM	1.5 mM	1.5	24.0
Forward primer	2 µM	0.2 µM	2.5	40.0
Reverse primer	2 µM	0.2 µM	2.5	40.0
10 mM dNTP	10 mM	0.2 mM	0.5	8.0
Taq polymerase	5 U/µl	0.05 U/µl	0.25	4.0
DNA Template	-	100 ng/µl	1.0	16.0
Autoclaved distilled water	-	-	11.75	188.0
Total volume	-	-	25.0	400.0

Master mix was prepared for sixteen reactions (10 samples, 2 negative and 2 positive controls) and aliquoted 12.25µl of master mix into PCR tubes. Thereafter, DNA samples, Taq polymerase, and distilled water were added into the corresponding PCR tubes. 1µl of distilled water was added to the negative control instead of DNA. Then PCR was carried

out according to the cyclic conditions given in Table 03.

Table 02. Lactobacillus genus specific primer sequences.

Primer	Primers (5' – 3')	Amplicon Size	Reference
Forward primer	5' TGGAAACAGRTGCTAATACCG 3'	233bp	Byun *et al.*, (2004)
Reverse primer	5' GTCCATTGTGGAAGATTCCC 3'		Byun *et al.*, (2004)

Table 03. Thermal cycle for Lactobacillus primers

Steps	Temperature (ºC)	Time	Cycle No
Initial Denaturation	$94^{0}C$	5minutes	
Denaturation	$94^{0}C$	1 minute	
Annealing	$62^{0}C$	1 minute	35 cycles
Extension	$72^{0}C$	1minute	
Final extension	$72^{0}C$	12 minutes	
Infinite hold	$4^{0}C$	œ	

Detection of antibiotic-resistant genes

Lactobacillus positive samples were amplified using tet(M) and erm(B) specific primers (Table 05).

The PCR master mix was prepared following the same procedure mentioned in Table 02 and PCR was performed using tet(M) and erm(B) primers (Table 04).

Table 04. Primers for PCR detection of tet(M) and erm(B) antibiotic resistance genes.

Gene	Primers (5' – 3')	Amplicon Size	Reference
erm(B)	Forward: 5'CATTTAACGACGAAACTGGC 3' Reverse: 5' GGAACATCTGTGGTATGGCG 3'	405 bp	Gad, Abdel-Hamid, and Farag, (2014).
tet(M)	Forward: 5'GGTGAACATCATAGACACGC 3' Reverse: 5'CTTGTTCGAGTTCCAATGC 3'	401 bp	Gad, Abdel-Hamid, and Farag, (2014).

Table 05. Thermal cycle for erm(B) primers

Steps	Temperature (ºC)	Time	Cycle No
Initial Denaturation	$94^{0}C$	5minutes	
Denaturation	$94^{0}C$	1 minute	
Annealing	$55^{0}C$	1 minute	35 cycles
Extension	$72^{0}C$	1minute	
Final extension	$72^{0}C$	12 minutes	
Infinite hold	$4^{0}C$	œ	

Table 06. Thermal cycle for tet(M) primers

Steps	Temperature (ºC)	Time	Cycle No
Initial incubation	$94^{0}C$	5minutes	
Denaturation	$94^{0}C$	1 minute	
Annealing	$52^{0}C$	1 minute	35 cycles
Extension	$72^{0}C$	1minute	
Final extension	$72^{0}C$	12 minutes	
Infinite hold	$4^{0}C$	œ	

Agarose gel electrophoresis

The PCR products were visualized using prepared 2% agarose gel electrophoresis. 50 bp ladder (2µl), PCR products (A-J) (7µl), negative and positive controls (7µl) were loaded into the wells according to the mentioned order and gel was run under 45V for 35 minutes and 50V for 25 minutes. Finally, bands were visualized under UV illuminator

DATA ANALYSIS

The DNA yields obtained from both the extraction methods were compared using one-way ANOVA, and P-value was calculated using SPSS version 25.

RESULTS

Isolation of Lactobacillus in MRS agar

The morphological features of the bacterial cultures incubated for 48 hours are shown in figure 01.

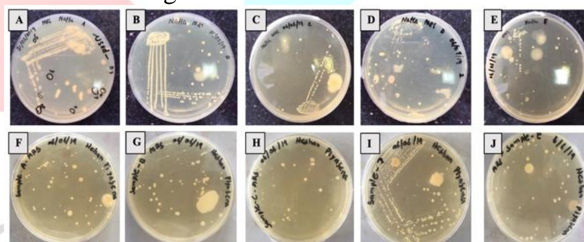


Figure 01. Colony morphology on the bacteria cultured on MRS agar from cheddar cheese (A-E) and Gouda cheese (F-J) samples after 48 hours incubation.

The shiny whitish cream color, mucoid, and rounded colonies with wavy margins were observed from all ten samples. Some contaminations were observed from several samples.

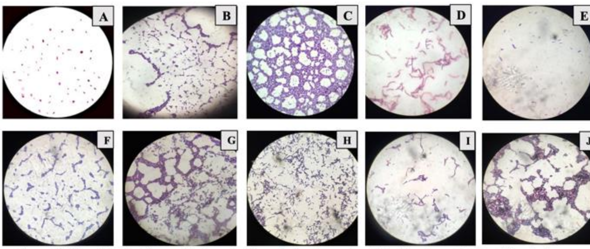


Figure 02. Gram Staining for possible Lactobacillus colonies from cultured cheddar cheese (A-E) and Gouda cheese (F-J) samples.

The gram staining of the selected colonies from all the samples indicated the appearance of purple color, rod-shaped (bacilli) bacteria of varied lengths in chains and palisades, reveling the presence of gram-positive bacteria.

Catalase test

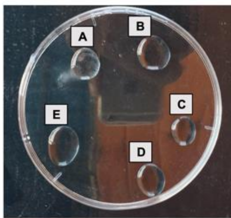


Figure 03. Results of the catalase for selected colonies from cultured cheddar cheese sample

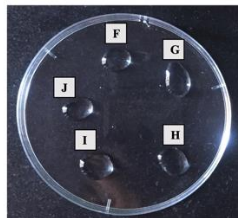
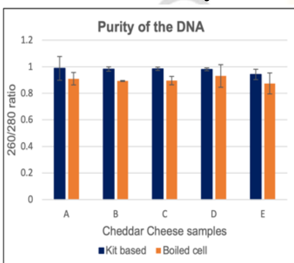


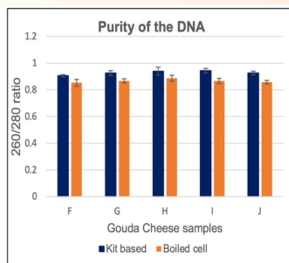
Figure 04. Results of the catalase for selected colonies from cultured gouda cheese sample

No air bubble formation observed in any sample indicating the presence of catalase negative bacteria in the selected colonies from each sample.

Purity of the DNA (260/280 Ratio)



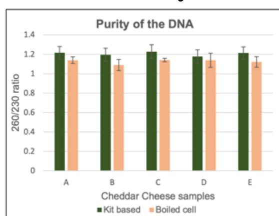
Graph 01. The comparison of the DNA purity (260/280) of the extracted DNA from cheddar cheese samples via kit-based method and modified boiled cell method.



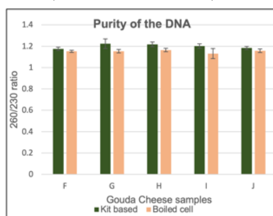
Graph 02. The comparison of the DNA purity (260/280) of the extracted DNA from Gouda cheese samples via kit-based method and modified boiled cell method.

The DNA samples extracted from both methods indicated lower purity. However, the DNA purity of the samples, extracted from the kit-based method were higher than the modified boiled cell method

Purity of the DNA (260/230 Ratio)



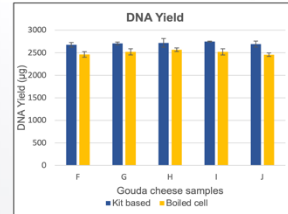
Graph 03. The comparison of the DNA purity (260/230) of the extracted DNA from cheddar cheese samples via kit-based method and modified boiled cell method.



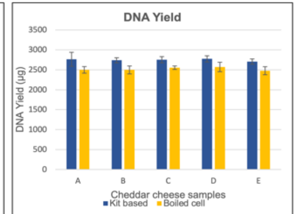
Graph 04. The comparison of the DNA purity (260/230) of the extracted DNA from Gouda cheese samples via kit-based method and modified boiled cell method.

The DNA purity, which was measured by the A260/230 ratio of extracted DNA samples from both methods, also indicated lower purity. However, the purity of the DNA samples extracted from kit-based method was higher compared to the boiled cell method.

DNA Yields



Graph 05. The comparison of the DNA yields of the extracted DNA from cheddar cheese samples via kit-based method and boiled cell method.



Graph 06. The comparison of the DNA yields of the extracted from Gouda cheese samples via kit-based method and boiled cell method.

All DNA samples extracted from kit-based method were indicated the higher DNA yields compared to the modified boiled cell method and DNA yields of kit-based method appeared within the same range.

Statistical analysis of DNA yields

Table 07. Statistical analysis for the differences of DNA yields of cheddar cheese samples between two extraction methods by SPSS using one-way ANOVA (Significance level = 0.05).

ANOVA

DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	388513.200	1	388513.200	50.133	.000
Within Groups	216988.800	28	7749.600		
Total	605502.000	29			

Table 08. Statistical analysis for the differences of DNA yields of gouda cheese samples between two extraction methods by SPSS using one-way ANOVA (Significance level = 0.05).

ANOVA

DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	309676.800	1	309676.800	84.617	.000
Within Groups	102472.500	28	3659.732		
Total	412149.300	29			

The significance value (P=0.000) was less than 0.05 for both the cheddar and gouda cheese samples. Therefore, the differences in DNA yields between DNA samples, extracted via two methods was statistically significant.

Identification of *Lactobacillus* by PCR – Modified boiled-cell method

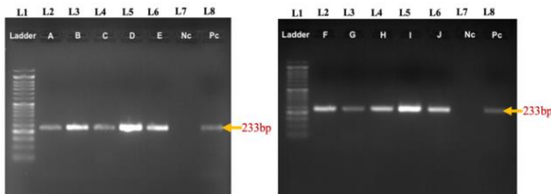


Figure 05. 2% Agarose gel electrophoresis of PCR products amplified from extracted Lactobacillus DNA from cheddar cheese samples by modified Boiled cell method. Lane 1: 50bp ladder; Lane 2: Sample A; Lane 3: Sample B; Lane 4: Sample C; Lane 5: Sample D; Lane 6: Sample E; Lane 7: Negative Control; Lane 8: Positive Control.

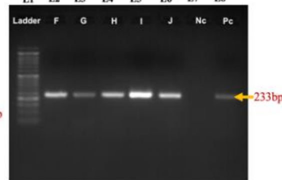


Figure 06. 2% Agarose gel electrophoresis of PCR products amplified from extracted Lactobacillus DNA from gouda cheese samples by modified Boiled cell method. Lane 1: 50bp ladder; Lane 2: Sample F; Lane 3: Sample G; Lane 4: Sample H Lane 5: Sample I; Lane 6: Sample J; Lane 7: Negative Control; Lane 8: Positive Control.

Identification of *Lactobacillus* by PCR – Kit based method

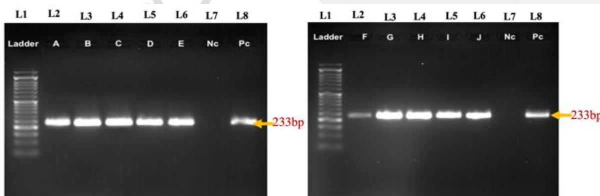


Figure 07. 2% Agarose gel electrophoresis of PCR products amplified from extracted Lactobacillus DNA from cheddar cheese samples by kit-based method. Lane 1: 50bp ladder; Lane 2: Sample A; Lane 3: Sample B; Lane 4: Sample C; Lane 5: Sample D; Lane 6: Sample E; Lane 7: Negative Control; Lane 8: Positive Control.

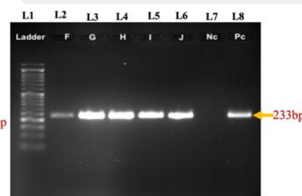


Figure 08. 2% Agarose gel electrophoresis of PCR products amplified from extracted Lactobacillus DNA from gouda cheese samples by kit-based method. Lane 1: 50bp ladder; Lane 2: Sample F; Lane 3: Sample G; Lane 4: Sample H Lane 5: Sample I; Lane 6: Sample J; Lane 7: Negative Control; Lane 8: Positive Control.

PCR products of band size 233bp were observed for DNA extracted from all samples by the two methods, coinciding with the positive control band and the negative control did not indicate any bands. Variations in the band intensities were observed.

Identification of *erm(B)* antibiotic resistance gene by PCR

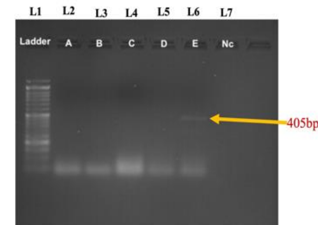


Figure 09. 2% Agarose gel image for the identification erm(B) gene of Lactobacillus isolates from cheddar cheese. Lane 1: 50bp ladder; Lane 2: Sample A; Lane 3: Sample B; Lane 4: Sample C; Lane 5: Sample D; Lane 6: Sample E; Lane 7: Negative Control.

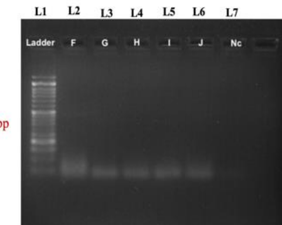


Figure 10. 2% Agarose gel image for the identification erm(B) gene of Lactobacillus isolates from gouda cheese. Lane 1: 50bp ladder; Lane 2: Sample F; Lane 3: Sample G; Lane 4: Sample H; Lane 5: Sample I; Lane 6: Sample J; Lane 7: Negative Control.

A band of 405bp was observed in sample E, indicating resistance to erythromycin. This band seemed to be very faint in intensity. The negative control showed no bands. Moreover, primer dimers were observed.

Identification of *tet(M)* antibiotic resistance genes by PCR

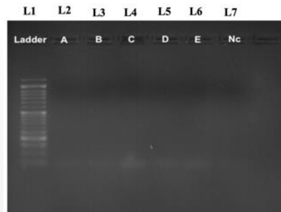


Figure 11. 2% Agarose gel image for the identification tet(M) gene of Lactobacillus isolates from cheddar cheese. Lane 1: 50bp ladder; Lane 2: Sample A; Lane 3: Sample B; Lane 4: Sample C; Lane 5: Sample D; Lane 6: Sample E; Lane 7: Negative Control.

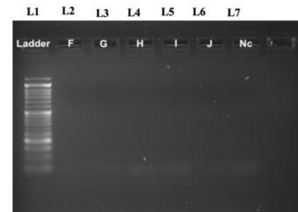


Figure 12. 2% Agarose gel image for the identification tet(M) gene of Lactobacillus isolates from gouda cheese. Lane 1: 50bp ladder; Lane 2: Sample F; Lane 3: Sample G; Lane 4: Sample H; Lane 5: Sample I; Lane 6: Sample J; Lane 7: Negative Control.

No bands were detected for the *tet(M)* gene in all ten samples, including the negative control. However, primer dimers were observed around 50bp range.

DISCUSSION

The consumption of probiotic foods, including curd, cheese, and yogurt promotes optimal health benefits and help to reduce risk of disease conditions (Ahmad et al., 2018). This study was performed to identify Lactobacillus, the commonest probiotic bacteria found in dairy products, from commercially available gouda and cheddar cheese samples and to evaluate the antibiotic resistance of these bacteria against selected antibiotics using molecular and microbiological techniques (Fijan, 2014). In order to identify Lactobacillus, phenotypical methods such as assessment of colony morphology and biochemical tests were carried out. Initially, the samples were cultured on MRS agar. The majority of the colonies observed in all ten samples were round, opaque, smooth, small and creamy white in color. This is consistent with the findings of Sharma and Goyal, (2015), Chakraborty and Bhowal, (2015) and Gad, Abdel-Hamid and Farag,

(2014), who observed similar colony morphologies for various Lactobacillus species. Therefore, the colonies observed in this study could be of the genus Lactobacillus. The bacterial growth in each sample was differed which could be possibly occurred due to the different bacterial concentration added in each of the brands tested. Although, Lactobacillus specific MRS broth and MRS agar is selective medium for the growth of Lactobacilli, it does not suppress the growth of other lactic acid bacteria such as Streptococcus. This was evident since certain isolated colonies which subjected to the catalase test were indicated positive results, due to the bubble formation and gram staining of these colonies revealed gram-positive cocci. Moreover, MRS agar with cycloheximide could inhibit the accompanying growth of yeasts and fungi molds preventing contamination of the cultured sample (Yang et al., 2018). With repeated gram staining of various colonies, purple colored rods, in varied lengths were observed in all ten samples indicating the presence of gram-positive bacteria (Figure 02). Similarly, Umemoto, Sato and Kito, (1978) reported the presence of short and long rods in ripened cheddar cheese. Varying length of the rods is associated with different strains of Lactobacillus species. In the present study, the rods obtained in two different lengths could indicate the presence of different strains of Lactobacillus. The purity of the extracted DNA is a critical factor in the sensitivity of the PCR as the inhibitory substances like proteins that are co-extracted with DNA could be present with the DNA samples. Those contaminants might be inhibited the PCR cycle resulting in false-negative results (Boesenberg- Smith, Pessarakli, and Wolk, 2012).

The A260/280 ratio can be used to judge DNA purity with regard to the protein contamination. High purity DNA have a A260/A280 of ~1.8 (Abdulamir et al., 2009) and the A260/A230 ratio, a secondary measure of DNA purity ranges in between 2.0-2.2 (Alrubaye et al., 2018). In this study, DNA extracted by the modified boiled-cell method and, the wizard protocol indicated A260/A280 purity values lower than the reference ranges confirming the presence of protein contaminants (Graph 1 and 2). Nevertheless, compared to the modified boiled cell method, the wizard protocol produced high DNA purity. Abed, (2013) was confirmed these findings by comparing four different extraction methods. This difference could be due to the addition of protein precipitation solution and RNase in the protocol which may have resulted in the removal of contaminants. Low purity of the DNA obtained via the boiled cell method could possibly be due to the lack of proper techniques for the removal of proteins in the boiled cell method (Abdulla, 2014). The mean A260/A230 for both the extraction methods were in between the values of 1.0 – 1.1, which was less than the standard values (Graph 3 and 4). This indicated the presence of polyphenols or organic compounds which absorb at 230 nm (Oliveira et al., 2014). Similarly, the low A260/A230 ratios could be caused due to EDTA contamination in both the methods as it absorbs at 230 nm (Lorenz, 2012). However, the DNA extracted via the two methods was not in the optimum purity. This could possibly occur due to the presence of contaminants during sample preparation since obtaining DNA with a higher purity is also dependent on sample quality and its preparation (Abed, 2013). However, PCR detection of Lactobacillus DNA was possible because of the dilution of DNA with TE buffer which could have diluted down the contaminating salts. Another reason could be the sensitivity of the PCR to low concentrations of DNA. According to graph 5 and 6, Wizard protocol resulted in the highest DNA yield in comparison to

the modified boiled cell method. Statistical analysis shows that DNA yield significantly differed depending on the DNA extraction method used ($P<0.05$)(Table 07 and 08). It was further confirmed in the studies conducted by Abu Bakar et al., (2010) and Abdulla, (2014) indicating higher DNA yields in kit-based method. It is conceivable that a higher DNA yield for Wizard protocol can be attributed to the use of lysozyme in combination with EDTA as it provides more efficient lysis of the peptidoglycan layer in gram-positive bacteria (Alimolaei and Golchin, 2016). Hence, use of lysozyme in combination with EDTA and use of additional lysis step using nuclei lysis solution might have contributed higher DNA yield in samples extracted from the kit-based method compared to the boiled cell method (Abed, 2013). However, in the boiled cell method, DNA yield could be lower due to the availability of heat resistant strains found in fermented food products and the use of lysozyme alone (Without EDTA). Hence, cell wall lysis is less efficient (Abdulamir et al., 2010). Nevertheless, the yields and concentrations of extracted DNA from both methods were much higher compared to the findings of Abdulamir et al., (2010). This could be occurred due to several reasons such as the presence of a large number of Lactobacilli in the homogenized samples (high microbial biomass), errors in the DNA extraction protocols or age and phase of growth of the microorganism (De et al., 2010).

Agarose gel images of boiled cell method (Figure 5 and 6) and wizard protocol (Figure 7 and 8) contain 233 bp bands for all 10 samples and the positive control which was the expected amplicons size for the primer confirming the presence of Lactobacillus DNA. Absence of band in the negative control indicates no contamination, no PCR inhibition and that the procedure has worked. No primer dimers and non-specific amplifications were observed in the gel images. Abed (2013) reported similar observations in his study. Successful amplification of the target gene indicated that the primers were specific to Lactobacillus genus. The faint bands seen in the images above could be due to loading errors which includes the insufficient amount of DNA loaded into the gel or preparation errors. Results obtained from PCR tallies with the biochemical tests done. PCR was used for the detection of tet(M) and erm(B) resistance genes in Lactobacillus positive samples. It was found that sample E harbors a 405-bp band for erm(B) gene (Figure 9) which correlates to the study conducted by Nawaz et al., (2011). Anisimova and Yarullina, (2018), has identified that both the erm(B) and erm(C) genes from Lactobacillus fermentum were responsible for erythromycin resistance. The rest of the samples did not contain any bands. No bands were detected for the tet(M) resistance gene (Figure 11 and 12). Nevertheless, presence of serval other genes or phenotypically resistant strains which may carries silent genes might be responsible for the tetracycline resistance in particular Lactobacillus strain (Gueimonde et al., 2013; Dec et al., 2017). The absence of bands in the negative control indicates no PCR contamination. Formation of primer-dimers can be minimized by proper primer designing and optimizing the protocol. However, Dec et al., (2017) reported that these genes can be passed on to the same or different bacterial species as they are carried on plasmids, transposons or integrons which can the play the role of a vector.

CONCLUSION

This study aimed at isolating Lactobacillus from commercially available cheddar and gouda cheese samples. PCR amplification with genus-specific primers revealed the presence of Lactobacillus in all samples. The DNA

yield obtained via the two extraction methods were statistically significant. Wizard protocol was found to be highly efficient compared to the modified boiled cell method as it resulted in a higher DNA yield and quality. The PCR assay for antibiotic resistance gene detection revealed an erm(B) gene in one sample. The presence of these genes in dairy products can pose a threat to the consumer as the resistance genes can be potentially transferable. Thus, it is necessary to include the screening of antibiotic resistance as a safety precaution prior to the use of Lactobacillus species as starter cultures or probiotics.

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