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EVALUATING THE ANTIBIOTIC RESISTANCE OF LACTOBACILLUS BACTERIA ISOLATED FROM FRESH MILK SAMPLES.

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ABSTRACT

Probiotic bacteria are bacteria which confer health benefits to a host when obtained in an adequate quantity. They are present in various foods, including fresh milk, where Lactobacillus is among the most commonly found species. Assessing presence of Lactobacillus in food and characterization of their antibiotic resistance could be a valuable tool in manipulation of said food, in order to provide a high nutritive value with added benefits of probiotics to the consumer. In the current study, five fresh milk samples obtained from cows raised by small scale farmers were cultured on Lactobacillus specific MRS media, and biochemical tests were done for identification of Lactobacillus. DNA was extracted from these cultures using three different boiled cell methods. No significant difference was observed in the amount of DNA obtained from each method (p value>0.05). This DNA was quantified using spectrophotometry to assess the DNA concentration in each sample, which was found to not be significantly different among each other (p value>0.05). PCR-based detection of Lactobacilli at a genus-level confirmed their presence in 3 samples (n=3). Further, from these samples, using a PCR-based assay, a tet(M) gene was detected in 1 sample (n=1). No sample contained erm(B) gene (n=0). In conclusion, Lactobacillus bacteria is present in fresh milk samples obtained directly from cows in a considerable number. All three extraction methods could be used to extract DNA from Lactobacillus cultures for further analysis. Further, some of these bacteria contain antibiotic resistance genes, which should be considered when utilizing benefits of Lactobacillus bacteria.

Keywords: Lactobacillus, Fresh milk, DNA extraction, PCR, Antibiotic resistance.

INTRODUCTION

Probiotic bacteria are live bacteria that confer health benefits and help the functions of normal microbiota in the GIT, when administered to the host in appropriate levels (Hunsche et al., 2018). Most probiotic bacteria belong to the Lactobaciilus or Bifidobacter genera, and some Streptococcus genera as well (Jensen et al., 2014). In order to exploit the beneficial effects, of probiotics, they must be present in an easily administrable method. Thus, probiotics are usually incorporated into various food types, most commonly dairy products, as live microbial food supplements (Arief et al., 2015). Common food types that host probiotic bacteria include yoghurt, curd and fermented food such as olives, kefir and also in fresh milk (Ascone et al., 2017; Eid et al., 2016). The normal human gut hosts a vast number of bacteria of various species. Alterations to the balance of normal to pathogenic microorganisms could result in a dysbiosis state, leading to various pathological conditions including diarrhoea, Crohn's disease and Inflammatory Bowel Disease and many more.

Probiotics confer their health benefits to the host via several mechanisms. One of the major modes of probiotic bacterial function is through restoring the balance of normal flora in states of dysbiosis. Probiotic bacteria produce certain peptides called bacteriocins, which act as natural antibiotics against pathogenic bacterial strains (Eid et al., 2016). These will help to reduce the pathogenicity of the gut microbiota induced by other invading pathogenic bacteria. Probiotic bacteria also competitively exclude the growth of microbes. These thereby promote improved gut, and thus, host health. Probiotic bacteria are also capable of performing immunomodulatory functions, which may assist in eliminating pathogenic microbes within the body, via playing a role in regulating the function of the white blood cells in the gut-associated lymphoid tissue (GALT). Another interesting feature of probiotic bacteria is that they tend to proliferate within tumour-cells. This paved way to a relatively more recent development in the therapeutic use of probiotics. It involves utilizing them as tumour-specific activators of prodrugs by production of enzymes involved in the bioconversion (Lehouritis et al., 2016).

Lactobacillus is a gram-positive catalase-negative rod-shaped bacterium which is generally recognized as safe (GRAS), and is therefore found incorporated into many food products (Eid et al., 2016). It is also present as a major probiotic in raw unprocessed cow's milk (fresh milk), among other bacterial genera (Ali, 2011). Therefore, consumption of raw milk containing these Lactobacilli would provide a high nutritive value along with the added advantages conferred by the probiotic organisms.

Lactobacillus is a bacterial genus present normally within the human gut. This makes the utilization of Lactobacillus in order to achieve probiotic effects a much better choice over other strains of bacteria since they contain adaptations which allow better survival within the host system. These adaptations facilitate retention of their viability in the conditions encountered within the body once ingested, determining the probiotic potential of Lactobacilli.

When taken into the body, the probiotics should be stable at the low pH of the stomach (about 2.5), where they must be able to thrive in order to interact with the gut microbiota an exert their therapeutic effects. This pH stability of Lactobacilli has been reported by several studies (Reale et al., 2015). It has been observed that pre-treatment of Lactobacilli to acidic stress promotes their survival in acidic conditions by several defensive strategies (Srisukchayakul, Charalampoulos and Karatzas, 2018). Lactobacilli are also capable of exhibiting antimicrobial effects on several pathogenic bacteria, such as enteropathogenic Escherichia coli, Staphylococcus aureus, Salmonella typhi and Proteus spp. (Kang et al., 2017; Jabbari et al., 2017, Goudarzi et al., 2017).

Certain probiotic bacteria may exhibit antibiotic resistance, through acquired and intrinsic mechanisms. This is a growing concern, particular since this could lead to reduced efficacy of antibiotic medication. Genes coding for antibiotic resistance, such as the tet and erm genes may play a role. In bacteria, the tet and erm genes are involved in conferring resistance to tetracycline and erythromycin respectively (Gad et al., 2014). There are various tet and erm genes, which confer resistance via different mechanisms, of which tet(M) and erm(B) are two commonly found genes in probiotic bacteria including Lactobacillus.

The significance of these genes lies in the fact that they may be transferred to pathogenic microorganisms within the host body, making management of conditions caused by such pathogens much more challenging. This mobilization of antibiotic resistance genes is immensely facilitated by the presence of the said

genes within plasmids and transposons, which are often exchanged among bacterial species (Flórez et al., 2014).

Lactobacilli obtained from various dairy and pharmaceutical products have been observed to be resistant to a wide array of antibiotics including nalidixic acid, vancomycin, kanamycin, tetracycline and erythromycin (Sharma et al., 2016). Characterization of the antibiotic resistance of Lactobacillus is of great importance in clarifying the molecular basis behind the antibiotic resistance effect of probiotic bacteria, which would allow better understanding of the mechanisms of antibiotic resistance transfer to pathogenic microorganisms and ways to minimize this transfer.

METHODOLOGY

Sample collection

The fresh milk samples were obtained from 4 different small-scale farmers in the Kalutara district of Sri Lanka. These were labelled as S1, S2, S3, S4 and S5.

Culturing of the samples

Using aseptic techniques, 2mL of each fresh milk sample was cultured in Lactobacillus-specific MRS broth medium and incubated at 36.9°C for 24 hours. Under aseptic conditions, the bacteria in the broth cultures were sub-cultured in Lactobacillus-specific MRS agar media and were incubated at 36.9°C for 48 hours.

Biochemical tests for the cultures

Initial identification of Lactobacillus was by Gram staining and catalase tests, carried out according to the method described by Abdulamir et al. (2010).

Sub-culturing of pure colonies

The gram-positive, catalase-negative colonies were sub-cultured into Lactobacillus-specific MRS broth as described above.

DNA extraction

Boiled Cell Method 1

9ml of sub-culture broth was centrifuged at 4000rpm for 15 minutes. The supernatant was discarded and 100µL of TE buffer was added to the pellet, followed by placing in a water bath at 99.9°C for 20 minutes. After incubation, it was transferred to a freezer to incubate at -20°C for 20 minutes. Following this, it was centrifuged at 13,000rpm for 3 minutes. The supernatant was then stored at -4°C.

Boiled Cell Method 2

The same procedure was followed using the second set of sub-cultures, until separation of the supernatant after final centrifugation. 5µL of 10mg/mL Proteinase K and 20µL of 10% SDS were added to the supernatant and was placed in the water bath at 37°C for 20 minutes. Next, 0.5mL of saturated NaCl solution was added, and shaken vigorously. Following 3-minute centrifugation at 13,000rpm, the supernatant was separated and 100µL of 100% ethanol was added. The tube was observed for precipitate formation. The supernatant was discarded, and the precipitate was washed by adding 135µL of 70% ethanol. The extracted DNA was left to dry overnight. 100µL of TE buffer was added to dissolve the pellets, and these were stored at -4°C.

Boiled Cell Method 3

The same procedure was followed using the third set of sub-cultures, until separation of the supernatant after final centrifugation. Into this, 50µL of 10mg/mL lysozyme was added, followed by Proteinase K. The same procedure was repeated from that point onwards, and they were stored at -4°C.

Quantification of DNA

Into a quartz cuvette, 3ml of TE buffer was added and used to calibrate the spectrophotometer. Into a falcon tube, 2mL of TE buffer and 30µL of DNA was added. and transferred to the cuvette, and triplicates of absorbance measurements were taken at 230nm, 260nm and 280nm. This was repeated for all 5 DNA samples from all 3 extraction methods. The DNA concentrations and yields of each sample from each extraction method were calculated as shown in equations (1) and (2) respectively.

DNA concentration (µgµL-1) = ([OD]_260 × 50µgml-1 × Dilution Factor)/1000 (1)

DNA yield (µg) = DNA concentration (µgµL-1) × amount of DNA kept as stock (100µL)

(2)

PCR-based detection of Lactobacillus

DNA extracted from all three methods was subjected to PCR amplification using genus-specific primers (Table 1).

Table 1: Primers and sequences used for the PCR-based detection of Lactobacillus.

Primer	Primer sequence	Expected PCR product size	Reference
LactoF	TGGAAACAGRTGCTAATACCG	231-233bp	Byun *et al.* (2004)
LactoR	GTCCATTGTGGAAGATTCCC		

Reverse Primers and 5U/ µL Taq Polymerase. 0.5µL of DNA was added to each reaction mixture. The total volume of each reaction was 25µL. PCR was carried out under cyclic parameters as shown in Table 2. The PCR products were visualized in a 2% agarose gel.

Table 2: Cyclic parameters for PCR

Step	Temperature	Time duration
Initial denaturation	94°C	5 minutes
Denaturation	94°C	1 minute
Annealing	62°C	1 minute
Elongation	72°C	2 minutes
Final elongation	72°C	12 minutes
End	4°C	∞

PCR-based detection of antibiotic resistance

The Lactobacillus-positive samples were subjected to PCR amplification using tet(M) and erm(B) primers (Table 3).

Table 3: Primers and sequences used for the PCR-based detection of antibiotic resistance genes (adapted).

Primer	Primer sequence	Expected Amplicon size	Reference
tet(M) forward	GGTGAACATCATAGACACGC	401	Werner *et al.*, 2003
tet(M) reverse	CTTGTTCGAGTTCCAATGC		
erm(B) forward	CATTTAACGACGAAACTGGC	405	Jensen *et al.*, 1999
erm(B) reverse	GGAACATCTGTGGTATGGCG		

PCR-based detection of antibiotic resistance

The *Lactobacillus*-positive samples were subjected to PCR amplification using *tet*(M) and *erm*(B) primers (*Table 3*).

Table 3: Primers and sequences used for the PCR-based detection of antibiotic resistance genes (adapted).

Primer	Primer sequence	Expected Amplicon size	Reference
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tet(M) reverse	CTTGTTCGAGTTCCAATGC		
erm(B) forward	CATTTAACGACGAAACTGGC	405	Jensen *et al.*, 1999
erm(B) reverse	GGAACATCTGTGGTATGGCG		

The PCR reaction mixture was prepared using 5X PCR buffer, 25mM MgCl2, 10mM dNTP's, 2µM Forward and Reverse Primers and 5U/ µL Taq Polymerase. 0.5µL of DNA was added to each reaction mixture. The total volume of each reaction was 25µL.

PCR was carried out under cyclic parameters as shown in Table 4 and Table 5. The PCR products were visualized in a 2% agarose gel.

Table 4: Cyclic parameters for tet(M) PCR.

Step	Temperature	Time duration
Initial denaturation	94°C	5 minutes
Denaturation	94°C	1 minute
Annealing	52°C	1 minute
Elongation	72°C	2 minutes
Final elongation	72°C	12 minutes
End	4°C	∞

Table 5: Cyclic parameters for Erm(B) PCR

Step	Temperature	Time duration
Initial denaturation	94°C	5 minutes
Denaturation	94°C	1 minute
Annealing	55°C	1 minute
Elongation	72°C	2 minutes
Final elongation	72°C	12 minutes
End	4°C	∞

DATA ANALYSIS

Statistical analysis was conducted using one-way ANOVA via SPSS Statistics version 21 software. DNA concentrations obtained from different methods were compared to evaluate if there was a significant difference between them. The p-value was calculated at 5% level of significance. P-values<0.05 were considered as statistically significant.

Result

Streaks were not obtained as expected on Lactobacillus-specific MRS agar media. All cultures exhibited growths only until the third streak, with isolated colonies present. Cream-white smooth circular

colonies with an entire margin were observed.

The Gram-stain showed the presence of relatively short purple coloured rods with round edges, often present in chains of two or three, for all 5 samples (Figure 1). These were short Gram-positive bacilli. No bubbles were observed on the colonies selected from all 5 samples for the catalase test, indicating catalase negativity.

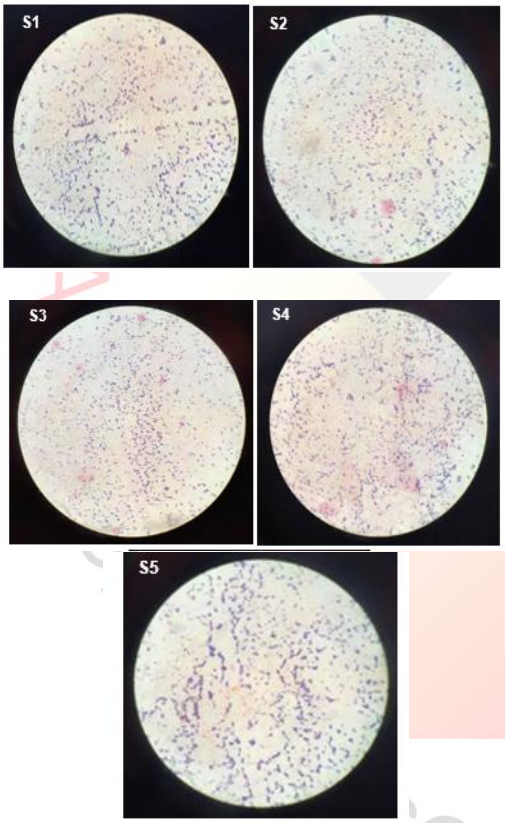

Figure 1: Gram staining of the samples, as observed on 100X of the light microscope.

DNA Quantification

The mean DNA concentrations obtained by each extraction method (Figure 2) shows extraction method 1 with highest concentration in all samples except samples 2 and 4. The highest concentration was obtained from sample S2 by extraction method 2. However, for all other samples, extraction method 2 shows the lowest DNA concentration.

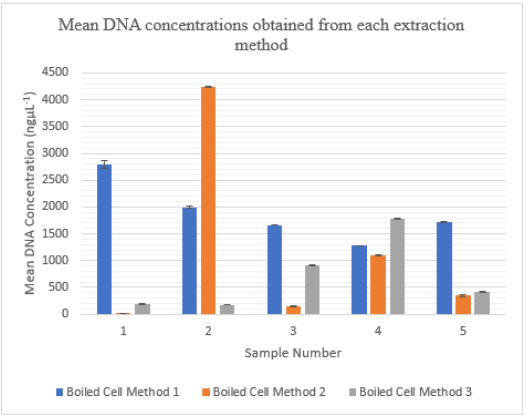

Figure 2: Mean DNA concentrations obtained by each extraction method.

Statistical Analysis

The p-value < 0.05 in the one-way ANOVA for comparison of DNA content in the samples (Table 6), indicating that there is a significant difference between DNA concentration obtained from each method. However, the p-values > 0.05 for each extraction method compared with other methods (Table 7), except for boiled cell methods 1 and 3. This highlights that, apart from methods 1 and 3, there is no significant difference in the yields of DNA obtained from each method when compared with the others.

When comparing samples, Table 8 and Table 9 indicate that there is no significant difference between the amounts of DNA obtained from each sample (p value>0.05).

Table 6: One-way ANOVA for the extraction methods.

ANOVA

DNA Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10862134.444	2	5431067.222	4.883	.012
Within Groups	46710063.333	42	1112144.365		
Total	57572197.778	44			

Table 7: Multiple compartsons analysts of the extraction methods.

Multiple Comparisons

Dependent Variable: DNA Concentration

Tukey HSD

(I) Boiled Cell 1, Boiled Cell 2 and Boiled Cell 3	(J) Boiled Cell 1, Boiled Cell 2 and Boiled Cell 3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Boiled Cell Method 1	Boiled Cell Method 2	723.000	385.079	.158	-212.55	1658.55
	Boiled Cell Method 3	1194.667*	385.079	.009	259.12	2130.21
Boiled Cell Method 2	Boiled Cell Method 1	-723.000	385.079	.158	-1658.55	212.55
	Boiled Cell Method 3	471.667	385.079	.445	-463.88	1407.21
Boiled Cell Method 3	Boiled Cell Method 1	-1194.667*	385.079	.009	-2130.21	-259.12
	Boiled Cell Method 2	-471.667	385.079	.445	-1407.21	463.88

*. The mean difference is significant at the 0.05 level.

Table 8: One-way ANOVA for the different samples.

ANOVA

DNA Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10448831.111	4	2612207.778	2.217	.084
Within Groups	47123366.667	40	1178084.167		
Total	57572197.778	44			

Table 9: Multiple comparisons analysis of the different samples.

Multiple Comparisons

Dependent Variable: DNA Concentration

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1136.111	511.661	.193	-2597.46	325.24
	3	91.111	511.661	1.000	-1370.24	1552.46
	4	-386.667	511.661	.942	-1848.02	1074.68
	5	172.222	511.661	.997	-1289.13	1633.57
2	1	1136.111	511.661	.193	-325.24	2597.46
	3	1227.222	511.661	.137	-234.13	2688.57
	4	749.444	511.661	.591	-711.90	2210.79
	5	1308.333	511.661	.098	-153.02	2769.68
3	1	-91.111	511.661	1.000	-1552.46	1370.24
	2	-1227.222	511.661	.137	-2688.57	234.13
	4	-477.778	511.661	.882	-1939.13	983.57
	5	81.111	511.661	1.000	-1380.24	1542.46
4	1	386.667	511.661	.942	-1074.68	1848.02
	2	-749.444	511.661	.591	-2210.79	711.90
	3	477.778	511.661	.882	-983.57	1939.13
	5	558.889	511.661	.809	-902.46	2020.24
5	1	-172.222	511.661	.997	-1633.57	1289.13
	2	-1308.333	511.661	.098	-2769.68	153.02
	3	-81.111	511.661	1.000	-1542.46	1380.24
	4	-558.889	511.661	.809	-2020.24	902.46

Purity of the DNA

The highest 260/230 ratio was observed from the DNA of sample 1, extracted using Boiled Cell Method 1, and the lowest was also obtained from the DNA of sample S1, but extracted using Boiled Cell Method 2 (Figure 3). The highest 260/280 ratio was from sample S1, by extraction method 2, while the lowest from sample S4, by extraction method 3 (Figure 4).

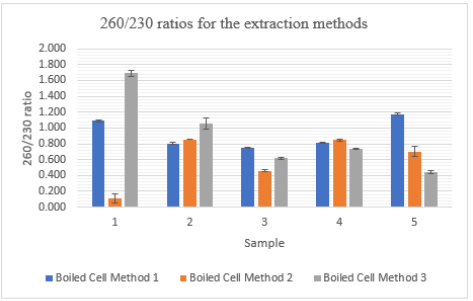

Figure 3: 260/230 ratios obtained by each extraction method.

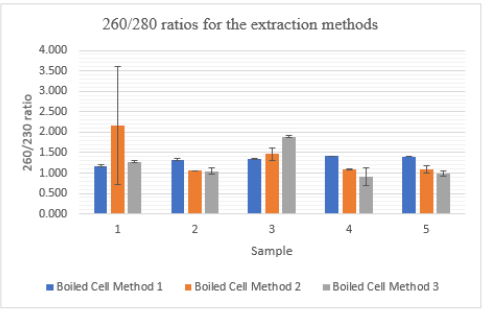

Figure 4: 260/280 ratios obtained by each extraction method.

Detection of Lactobacillus genus-specific sequence

Bands of 233bp were observed for DNA extracted from samples 1, 2 and 4 by all three methods, coinciding with the positive control band (Figures 5-7). The bands of samples S1 and S2 were bright and clear bands. However, the band for sample S4 was very faint for all three extraction methods. No bands were observed in the negative control.

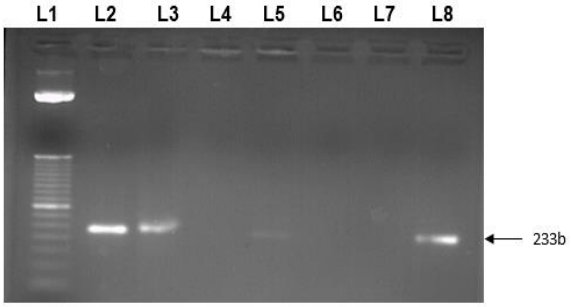

Figure 5: Gel image for PCR products of Boiled Cell Method 1 amplified with Lactobacillus genus-specific primers (L1- 50bp Ladder, L2 to L6- Samples S1 to S5, L7- Negative control, L8- Positive control).

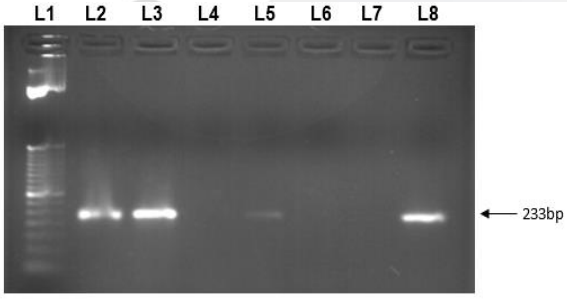

Figure 6: Gel image for PCR products of Boiled cell Method 2 amplified with Lactobacillus genus-specific primers (L1- 50bp Ladder, L2 to L6- Samples S1 to S5, L7-Negative control, L8-Positive control).

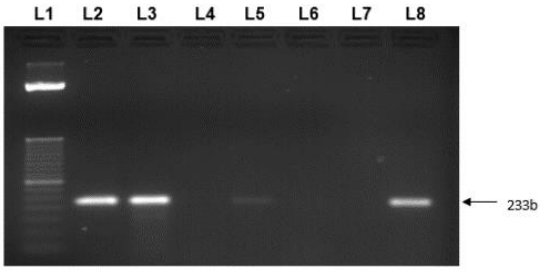

Figure 7: Gel image for PCR products of Boiled cell Method 3 amplified with Lactobacillus genus-specific primers (L1- 50bp Ladder, L2 to L6- Samples S1 to S5, L7-Negative control, L8- Positive control).

Detection of tet(M) in Lactobacillus

A single band of 401bp was observed in sample S4, in the PCR for tet(M) resistance (Figure 8). This band was of extremely faint intensity, despite the DNA used in the said PCR being the one having the highest concentration out of the three extraction methods. No band was observed in the negative control.

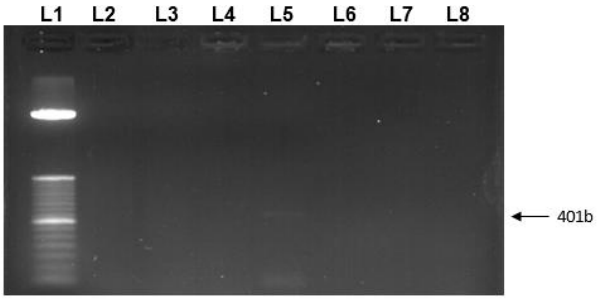

Figure 8: Gel image for PCR products of sample DNA amplified with primers for Tet(M) gene (L1- 50bp Ladder, L2- negative control, L3- Sample S1, L4- Sample S2, L5- Sample S4).

Detection of *erm*(B) in *Lactobacillus*

No bands were observed for DNA of any sample in the PCR for erm(B) resistance (Figure 9). No band was observed in the negative control.

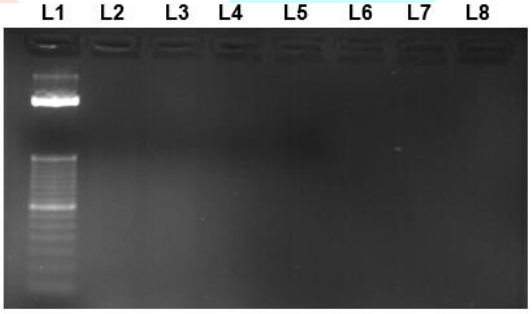

Figure 9: Gel image for PCR products of sample amplified with primers for Erm(B) gene (L1- 50bp Ladder, L2- negative control, L3- Sample S1, L4- Sample S2, L5- Sample S4).

DISCUSSION

Lactobacillus is a major probiotic bacterial genus known to have major health benefits. This study attempted isolation of *Lactobacillus* bacteria in fresh milk samples obtained from various small-scale farmers in the Kalutara district of Sri Lanka, using microbiological and molecular techniques. Once detected, antibiotic resistance of these bacteria against selected antibiotics was assessed using molecular techniques.

The initial identification of bacteria in samples was done by culture-based methods, which may be imprecise and thus affect reliability. The samples were cultured on *Lactobacillus*-specific MRS broth and agar media.

Cream-white smooth circular colonies with an entire margin were observed in all the samples but did not grow into clear streaks as expected. This may be due to low levels of bacteria present in the samples. This is consistent with the results of Ahmad *et al.* (2018), who observed similar colony morphologies for different *Lactobacillus* species. Therefore, the colonies observed in the current study could be *Lactobacillus* colonies.

Lactobacillus-specific MRS broth and agar media selectively promote the growth of *Lactobacillus* bacteria. However, this does not guarantee inhibition of growth of other bacteria such as *Streptococci*, which have also been observed to grow on *Lactobacillus*-specific MRS media. This, therefore, may result in the growth of multiple probiotic bacterial species, making isolation of *Lactobacillus* more challenging from microbially-complex samples (Sutula, Coulthwaite and Verran, 2012).

This was particularly observed by the catalase test, where certain isolated colonies from the agar medium were catalase positive. *Lactobacillus* is catalase negative, since it detoxifies hydrogen peroxide using peroxidase, which is non-oxygen evolving (Goyal *et al.*, 2012). This was also observed by Gram-staining, where the same colonies that gave out bubbles during the catalase test were observed to harbour both Gram-negative cocci (likely to be *Streptococcus*) in addition to Gram-positive rods (*Lactobacilli*).

The Gram-staining and catalase tests were repeated using various colonies on each MRS agar culture, until catalase negative colonies harbouring Gram-positive rods (***Figure 1***) were identified. The rods seen were purple-coloured, indicating that they were Gram positive. Even though the *Lactobacillus* in the samples were expected to be the characteristic long gram-positive rods, the ones that were observed were short. Various strains of *Lactobacilli* present as rods of various lengths. Peña *et al.* (2004) reported the presence of *Lactobacillus* with a similar morphology in murine intestines, belonging to the species *L. reuteri*. The short rods observed in the current study could therefore be a specific strain of *Lactobacillus* having such morphology. The pure colonies were sub-cultured into *Lactobacillus*-specific MRS broth to obtain a pure *Lactobacillus* culture, which was in turn assayed using PCR following DNA extraction.

PCR-based analysis was conducted for confirmation of the genus of bacteria isolated as *Lactobacillus* (Abdulamir *et al.*, 2010). PCR requires the DNA used in the assay to be of good quality in order to work best. It therefore follows that a suitable DNA-extraction procedure must be carried out.

In the current study, DNA from the cultured bacteria was extracted using the boiled cell method, as described by Abdulamir *et al.* (2010), and by two modified methods. For the purpose of the study, these three methods will be referred to as Boiled cell Methods 1, 2 and 3 respectively.

Usage of chaotropic detergents such as SDS assist in bursting of cells to release the DNA. In DNA extraction, Proteinase K serves the purpose of lysing the bacterial cell walls, which is especially useful with regards to Gram-positive bacteria, since they contain and thicker cell wall (Quigley *et al.*, 2012). Therefore, hypothetically the three methods should yield differing amounts of DNA, with Method 3 providing the highest and Method 1 the lowest yield.

According to ***Figure 2***, for all samples, except sample 2, the extraction method 2 shows the lowest DNA concentration. Extraction method 1 showed the highest concentration in all samples except samples 2 and 4 and was always higher than method 3.

The statistical analysis shows that there was a significant difference between the extraction methods, which tallies with the direct observations of the DNA concentrations. The difference is significant between extraction methods 1 and 3, which differ from each other with a p value<0.05.

This therefore suggests that the choice of the method of DNA extraction did have a considerable impact on the DNA concentrations obtained in the current study. However, the boiled cell method is very advantageous in several aspects. It takes less time, less reagents and less corrosive elements than most other conventional DNA extraction kits.

The ratios $\frac{OD_{260}}{OD_{230}}$ and $\frac{OD_{260}}{OD_{280}}$ were used to determine the purity of the extract, by level of RNA and protein contamination respectively (Psifidi *et al.*, 2015). This is important as the purity of the DNA is a critical factor affecting the PCR assay, as was mentioned above. Generally, a $\frac{OD_{260}}{OD_{230}}$ ratio >2.0 indicates contamination by RNA, and a $\frac{OD_{260}}{OD_{280}}$ ratio <1.8 indicates protein contamination. From ***Figure 3*** it could be seen that all the $\frac{OD_{260}}{OD_{230}}$ values were below 1.8, indicating protein contamination. This could be due to lack of proper protein removal techniques in the boiled cell methods. From ***Figure 4***, it is seen that the $\frac{OD_{260}}{OD_{280}}$ values are all less than 2.0, except for sample 2, by extraction method 1, indicating that there is no RNA contamination of any of the other samples.

Since there was a considerably high DNA concentration, only a smaller amount was used for the subsequent PCR, since higher levels could inhibit the PCR assay.

Agarose-gel images of all three extracts contain 233bp bands for samples 1,2 and 4, confirming presence of *Lactobacillus* genus-specific DNA in the said extracts (***Figures 5-7***). However, DNA extraction was performed using colonies containing Gram positive rods which were assumed to be *Lactobacilli*. Samples 2 and 5 not yielding bands in the PCR assay suggest that the rods that were observed were not in fact *Lactobacilli*, or the PCR amplification may not have taken place properly due to presence of certain inhibiting factors.

Agarose gel images for the PCR products testing the presence of antibiotic resistance genes showed the presence of *tet(M)* resistance only in Sample 4 (***Figure 8***), which correlates with another study conducted by Gad *et al.* (2014). No bands were observed for the remaining samples for *tet(M)* resistance gene. No bands were observed for any sample for *erm*(B) resistance gene (***Figure 9***), including sample 4. Lack of a band for negative controls indicated that there is likely no contamination the PCR.

These findings are consistent with previous studies, which show that *Lactobacillus* spp. exhibit resistance against a wide range of antibiotics, out of which resistance to tetracycline is considered to be the most common form of

acquired resistance in isolates of *Lactobacillus* (Sharma *et al*., 2016; Zago *et al.*, 2011; Temmerman *et al*., 2002). Gevers *et al.* (2003) reported that the gene for *tet(M)* resistance in *Lactobacilli* could be present on either plasmids or within the chromosome, depending on the species.

CONCLUSION

The present study aimed at isolating *Lactobacillus* bacteria from raw milk samples and extracting DNA using three methods. There was no significant difference in amount of DNA obtained using the three methods. This DNA was amplified by PCR using *Lactobacillus* genus-specific primers, and it was found that 3 samples contained *Lactobacillus* bacteria. From these three samples, another PCR was performed to assess the antibiotic susceptibility. Results indicated one sample to contain a *tet*(M) resistance gene.

Characterization of *Lactobacillus* found in commonly consumed products like fresh milk is an important avenue, allowing better understanding of ways to utilize and make better use of the beneficial properties of *Lactobacillus* bacteria. Understanding about the antibiotic properties of probiotic bacteria such as *Lactobacilli* present in fresh milk could pave the way for development of successful methods for inhibition of the antibiotic resistance genes present in the bacteria, and to minimize the transfer of the said genes to other bacterial species.

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