GLOBAL ACADEMIC RESEARCH INSTITUTE

COLOMBO, SRI LANKA

GARI International Journal of Multidisciplinary Research

ISSN 2659-2193

Volume: 04 | Issue: 07

On 15th January 2019

http://www.research.lk

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GARI Publisher | Food Science | Volume: 04 | Issue: 07

Article ID: IN/GARI/05ICHM/2018/115 | Pages: 96-108 (12)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 16.12.2018 | Publish: 15.01.2019

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GARI Publisher | Food Science | Volume: 04 | Issue: 07
Article ID: IN/GARI/05ICHM/2018/115 | Pages: 96-108 (12)
ISSN 2424-6492 | ISBN 978-955-7153-00-1
Edit: GARI Editorial Team | Received: 28.01.2019 | Publish: 01.03.2019

THE IDENTIFICATION OF *LACTOBACILLUS* IN COMMERCIAL YOGURT PRODUCTS AND ANALYSIS OF THEIR ANTIBIOTIC RESISTANCE

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ABSTRACT

Probiotic bacteria confer numerous health advantages to humans when consumed in adequate amounts and are widely used in starter cultures of fermented dairy products such as yogurt. The main probiotic for yogurt starter cultures consists of *Lactobacillus,* which eventually colonise the human gastrointestinal tract (GIT) after their consumption. Although lactobacilli are 'generally regarded as safe' (GRAS) to humans, they can transfer antibiotic resistance genes to pathogens in the GIT. Antibiotic resistance transmitted by food rather than antibiotic selective pressure itself, raises serious public health concerns. The purpose of this study was to identify *Lactobacillus* in 5 brands of commercial yogurt products and assess their antibiotic resistance. Identification of *Lactobacillus* was performed by colony morphology, Gram staining and polymerase chain reaction (PCR). Deoxyribose nucleic acid (DNA) extraction was performed by boiled cell and Cetyl trimethylammonium bromide (CTAB) methods. The DNA extracts were quantified by spectrophotometry and their concentration and yields were compared. Detection of *tet*(M) and *erm*(B) resistance genes were performed by PCR. The results of this study revealed all 5 samples contained *Lactobacillus,* and its DNA yield depended on the brand and method of extraction. Overall, boiled cell method yielded higher concentrations of DNA. The *tet*(M) resistance gene was contained in 40% of the isolates, while no isolates tested positive for the *erm*(B) gene. This study highlights that lactobacilli in yogurt contain antibiotic resistance determinants. Therefore, pre-production screening of lactobacilli is necessary to minimise the risk of transmission of these determinants to pathogens.

Keywords: Yogurt, Lactobacillus, antibiotic resistance, PCR, *tet*(M)

INTRODUCTION

Probiotics are defined as live microorganisms conferring benefits to health, when ingested in adequate amounts. They are generally rendered harmless to humans. Since the discovery of probiotics in 1908 by Elie Metchnikoff, a spectrum of studies has been performed to assess their diverse functions in immune system modulation to evade diseases and raise concern regarding their detrimental effects associated with antibiotic resistance (Bermudez-Brito *et al*., 2012; Sanders, 2008).

General overview of *Lactobacillus*

Probiotics can be classified into specific lactic acid bacteria (LAB) for their natural ability to anaerobically ferment lactose in milk to synthesize lactic acid. They naturally inhabit soil, water, and colonize the mucous

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membranes of gastrointestinal (GI) tract and urogenital regions in humans. Most commonly found LAB in the human GIT are *Bacteroides*, *Lactobacillus*, *Clostridium*, and *Bifidobacterium* (Collado et al., 2008). However, *Lactobacillus* and *Bifidobacteria* have been recognized for their predominant genera in the intestine. The largest genus within the LAB group is acquired by *Lactobacillus* and is the primary interest of this study (Gueimonde et al., 2013).

Lactobacilli are non-spore forming, facultative anaerobes and their colonial appearance may present as white mucoid colonies on selective Man Rogosa and Sharpe (MRS) agar (***Figure 1A***). They are catalase negative and Gram positive in nature (***Figure 1B***) and occur as short rods either in chains or in single form (Shyu et al., 2014).

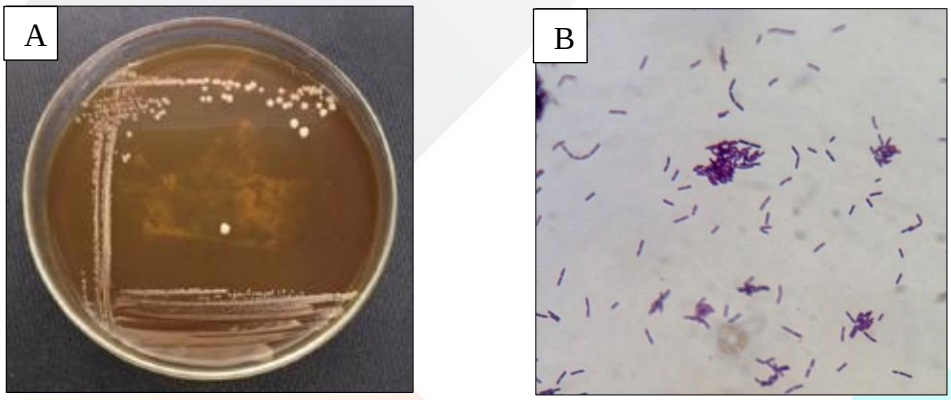

Figure 1: A: Mucoid white colonies of Lactobacillus on MRS agar, B: Gram stain morphology, appearing as short rods taking on a purple stain (Gram positive) (Shyu et al., 2014).

Starter cultures of commercial food products and pharmaceuticals use purified lactobacilli. Yogurt, being the media tested in this study, commonly constitutes of *Lactobacillus* species and *Streptococcus thermophilus* and the production should ensure a minimum of 10^7 viable cells/ g of the probiotic when reaching the GIT, to fulfil its chronicled nourishment status (Ranasinghe and Perera, 2016; Casarotti et al., 2014; Granato et al., 2010; Parvez et al., 2006).

Function of *Lactobacillus* against intestinal putrefaction

The mucosal surface of the intestines is one of the primary interfaces for a plethora of exogenous antigens. Part of the defense line in the GIT is accounted by lactobacilli by endorsing mucous secretion to increase adhesion in tight junctions on gastrointestinal brush border surfaces preventing pathogens interaction (Bermudez-Brito et al., 2012). Additionally, production of secretory immunoglobulin A by the probiotic, which abrogates the adhesion of the pathogen to the mucosal surface (Kang et al., 2011; Kaji et al., 2010). Literature suggests additional mechanisms by which lactobacilli abrogate certain diseases (***Table 1***).

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Table 1: Lactobacillus spp. and their cure against disease, with the mechanism of action identified in previous studies adapted from (Makino et al., 2015; Druart et al., 2014; Jenq et al., 2012; Carol et al., 2006).

Bacteria involved	Cure against	Mechanism	Reference
Lactobacillus casei	Inflammatory Bowel Disease	Reduce the number of highly activated T cells and decrease intraluminal pH	Carol et al., 2006
Lactobacillus plantarum	Graft Versus Host Disease	Mechanism under research	Jenq et al., 2012
Lactobacillus gasseri	Obesity	Reduction in adipocyte hypertrophy and mass of viscera visceral adipose tissue	Druart et al., 2014
Lactobacillus delbrueckii spp. bulgaricus	Common cold	Production of an exopolysaccharide which is immunostimulatory	Makino et al., 2015

Antibiotic Resistance

Antibiotics are used as a selective target against a specific spectrum of invasive pathogens. Their regular consumption over time has led to a strong selective pressure, exhibiting resistance in pathogens they target, and the constant need for new antibiotics to be developed (Marinaki et al., 2016; Kastner et al., 2006).

Studies reveal that lactobacilli become resistant to antibiotics and restore their colonization in the GIT after antibiotic consumption. However, this serves as a pre-requisite for the transfer of the resistant genes to other pathogenic species occupying the same niche (Shao et al., 2015; Kastner et al., 2006). Mechanisms of resistance may be innate or acquired. Innate resistance is most common to vancomycin, ciprofloxacin and gentamycin by *Lactobacillus* species, but is rendered safe due to no evidentiary mechanisms of gene transfer to opportunistic pathogens (Kšicová et al., 2013; Mayrhofer et al., 2010; Ammor et al., 2008; D'Ammino et al., 2007).

Studies show that acquired resistance (mutations, plasmids, transposons and incorporation of new genes), can pass by horizontal gene transfer between species of the same and different kinds. In support of this, studies showed the transfer of the erythromycin [*erm*(B)] resistant gene on a plasmid to opportunistic pathogens such as *Enterococcus faecalis* and *Listeria monocytogens*, and the tetracycline [*tet*(M)] resistance gene which was possessed by Shigella, thus posing a risk to humans (KyselkovÃ¡ et al., 2015; Toomey et al., 2009; Ouoba et al., 2008). Since the presence of *tet*(M) and *erm*(B) resistance genes have been documented in most Lactobacillus species, these genes will be the main concern in this study (Flórez et al., 2007). The study by Jose and colleagues showed prevalence of acquired antibiotic resistance to tetracycline and erythromycin in lactobacilli to be less than that of innate resistance, but roughly equal in prevalence to each other (***Figure 2)***.

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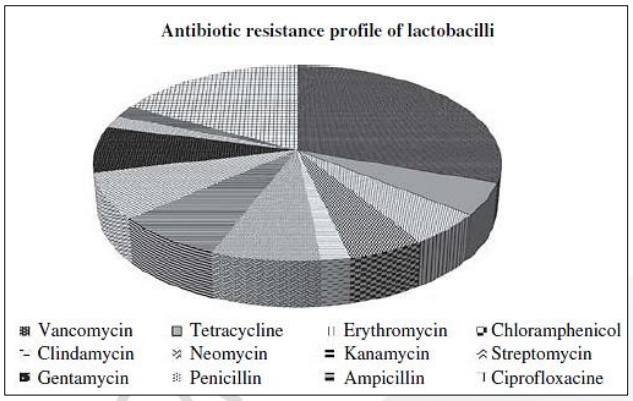

***Figure 2: Antibiotic** resistance in Lactobacillus for 12 antibiotics most commonly described in literature. The antibiotics read from left to right, correlate with the profile when analyzed in a clockwise direction, showing highest resistance to vancomycin and minimal resistance to ampicillin/penicillin (Jose et al., 2014).*

With increasing consumption of yogurt for perceived health benefits, antibiotic resistance transmission to pathogens from *Lactobacillus*, remains an imperative problem which could overrule the benefits of yogurt. Recent advancements by PCR and microarray analysis have helped characterize resistant genes in dairy supplements. The objective of this study was to identify *Lactobacillus* in five different brands of commercial yogurt products and assess antibiotic resistance of the *Lactobacillus* isolates for the *tet*(M) and *erm*(B) resistance genes, by the gold standard method of PCR. Furthermore, to extract DNA by boiled cell and CTAB methods and compare DNA yields and assess if it depends on the brand and method of extraction.

MATERIALS

Reagents

Tris (hydroxy methyl) aminomethane, Ethanol, Isopropyl alcohol, Acetic acid, Man Rogosa and Sharpe (MRS) agar (HiMedia), CTAB powder (HiMedia), immersion oil, Crystal Violet , Gram's Iodine, Gram's decolouriser, Safranin, PCR Reagents (5X PCR green buffer, 10 mM dNTP , 5U/ µL Taq Polymerase and 25 mM $MgCl_2$) (PROMEGA), Ethidium bromide (PROMEGA), Agarose powder (Sigma-Aldrich), all primers (Integrated DNA Technologies), 50 bp DNA ladder (Applied Biological Materials) and distilled water.

Sample Collection

Commercial yogurt products of 5 different brands (A, B, C, D and E), were collected from local markets in Sri Lanka. The samples were refrigerated until use.

METHODOLOGY

Isolation of *Lactobacillus*

The samples were stirred until a uniform, thick paste was formed. A loopful of the sample was transferred onto the MRS agar using the quadrant streaking method. The plates were incubated at 37 ºC for 72 hours (Goyal et al., 2012).

Phenotypical identification of isolates

Individual colonies were identified by their phenotypic appearance. Morphological and cultural examination of the identified colony was performed by Gram staining (Holt, 1994).

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Gram positive rod shape bacteria were subjected to DNA extraction.

DNA extraction

Boiled cell method

A volume of 2.5 mL of each subculture was obtained and centrifuged at 4000 rpm for 30 minutes. The supernatant was discarded, and 1 mL of Tris-Ethylenediaminetetraacetic acid (TE) was added, briefly vortexed, and incubated at 100 ºC for 20 minutes before cooling at -20 ºC for 20 minutes. The contents were centrifuged at 4000 rpm for 10 minutes. The pellet was discarded, and supernatant was stored at 0-5 ºC. The protocol was modified from (Abdulamir et al., 2010).

Cetyl trimethylammonium bromide (CTAB) method

A volume of 2.5 mL of each subculture was obtained, mixed with 2.5 mL of CTAB solution, incubated at 65 ºC for 30 minutes and centrifuged at 4000 rpm for 30 minutes. The supernatant was precipitated with 1 volume of isopropanol and centrifuged at 4000 rpm for 30 minutes. The supernatant was discarded, and the pellet was rinsed with 2.5 mL of 70% ethanol. A quick-spin was performed, and excess ethanol was removed. The pellet was dried overnight and dissolved thereafter, in 500 µL of TE buffer. It was kept at 4 ºC for 24 hours and later stored at -20 ºC. The protocol was modified from (Gad et al., 2014).

DNA quantification by spectrophotometry

To quantify DNA in the extracts, the absorbance was measured at 230 nm, 260 nm, and 280 nm, using a spectrophotometer. The DNA concentration and yield were calculated for each extract using the equations shown below.

DNA concentration = Optical density at 260 nm x 50 x dilution factor (Abdulamir et al., 2010; Collado and Hernández, 2007).

DNA yield = DNA Concentration x sample volume (Abdulamir et al., 2010).

The ratios of optical densities at 260/230 and 260/280 were recorded for each extract.

Genus specific identification of *Lactobacillus* isolates by PCR

Genus specific identification of *Lactobacillus* from both extraction methods, was executed using genus specific primers (***Table 2***).

***Table 2**: Primer sequences used for genus specific identification of Lactobacillus (Byun et al., 2004).*

Genus specific primer	Primer Sequence (5' – 3')	Expected amplicon size (bp)
Forward	TGG AAA CAG RTG CTA ATA CCG	230
Reverse	GTC CAT TGT GGA AGA TTC CC	

Total volume of PCR mastermix was 25 µL and consisted of 5x PCR buffer, 10 mM dNTP, 2 µM forward primer, 2 µM reverse primer, 25 mM $MgCl_2$ and 5 U/µL Taq polymerase, autoclaved distilled water and 100 ng bacterial DNA was used.

The following PCR thermocycling conditions were set (***Table 3***), and the PCR products were run on a 2% agarose gel.

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Table 3: PCR thermocycler conditions for PCR identification of Lactobacillus isolates adapted from (Gad et al., 2014).

Step	Temperature (°C)	Time (Minutes)	Repetition
Initial Denaturation	94	5	-
Denaturation	94	1	
Annealing	62	1	X 35 cycles
Extension	72	1	
Final Extension	72	12	-
Final Hold	4	∝	-

Genotypic confirmation of antibiotic resistance genes [*tet*(M) and *erm*(B)] in *Lactobacillus* isolates

Genotypic identification of *tet*(M) and *erm*(B) genes for *Lactobacillus* isolated from boiled cell method of DNA extraction (since this method produced better yield), were performed separately, using primers specific to *tet*(M) and *erm*(B) genes (***Table 4***). A volume of 25 µL of PCR mastermix was prepared as afore mentioned.

Table 4: PCR primers for tet(M) and erm(B) resistant genes (Werner et al., 2003; Jensen et al., 1999).

Antibiotic resistance gene primer	Primer Sequence (5' – 3')	Expected amplicon size (bp)
Forward *tet*(M)	GGT GAA CAT CAT AGA CAC GC	401
Reverse *tet*(M)	CTT GTT CGA GTT CCA ATG	
Forward *erm*(B)	CAT TTA ACG AAA CTG GC	405
Reverse *erm*(B)	GGA ACA TCT GTG GTA TGG CG	

The following conditions were set for *tet*(M) gene detection (***Table 5***), and *erm*(B) gene detection (***Table 6***).

Table 5: PCR thermocycler conditions for PCR identification of Lactobacillus isolates for tet(M) gene identification adapted from (Gad et al., 2014).

Step	Temperature (°C)	Time (Minutes)	Repetition
Initial Denaturation	94	5	-
Denaturation	94	1	
Annealing	52	1	X 35 cycles
Extension	72	2	
Final Extension	72	12	-

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Final Hold	4	∝	-

Table 6: *PCR thermocycler conditions for PCR identification of Lactobacillus isolates for erm(B) gene identification adapted from (Gad et al., 2014).*

Step	Temperature (ºC)	Time (Minutes)	Repetition
Initial Denaturation	94	5	-
Denaturation	94	1	
Annealing	55	1	X 35 cycles
Extension	72	2	
Final Extension	72	12	-
Final Hold	4	∝	-

Expected PCR products were detected by agarose gel electrophoresis as mentioned previously.

Statistical interpretation by Analysis of Variance (ANOVA)

Statistical analysis was performed using the SPSS software (edition 21). One-way ANOVA was performed to analyse the significant difference between DNA yields produced by different brands and methods of DNA extraction. The p value was used to compare the 0.05 significance level, and $p < 0.05$ was rendered significant.

RESULTS

Phenotypic identification of *Lactobacillus* isolates

Colony appearance on agar after 72 hours are shown in ***Figure 3***. The Gram staining images respective to the specific colony picked are denoted from ***Figure 4***.

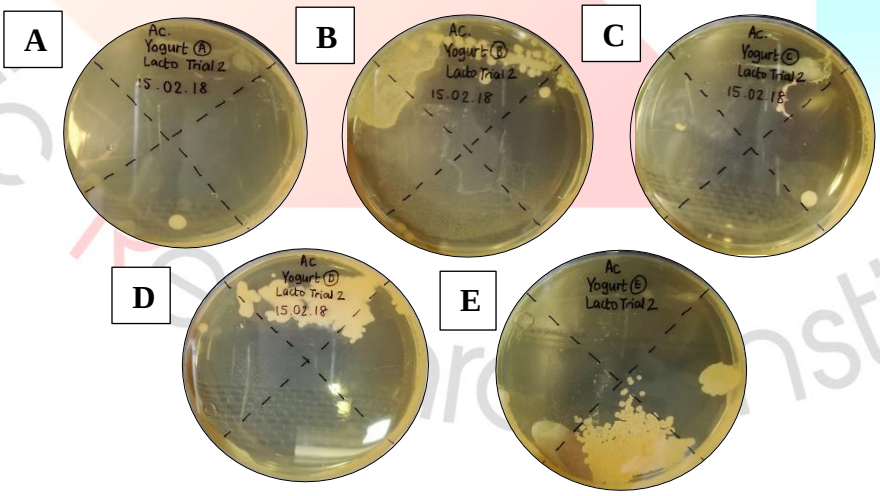

Figure 3: *Mucoid white colony appearance on agar after 72 hours for the respective yogurt samples, A, B, C, D and E.*

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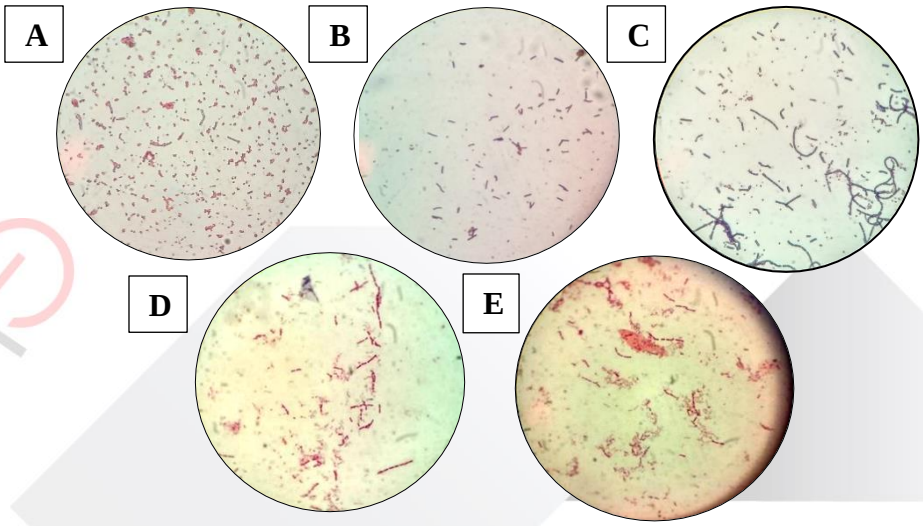

Figure 4: *Photomicrograph of Gram staining of yogurt samples A, B, C, D and E under 100X magnification. Presence of Gram positive (purple) rods seen.*

DNA quantification by spectrophotometry

The DNA yields for both extraction methods were calculated and compared (***Figure 5***).

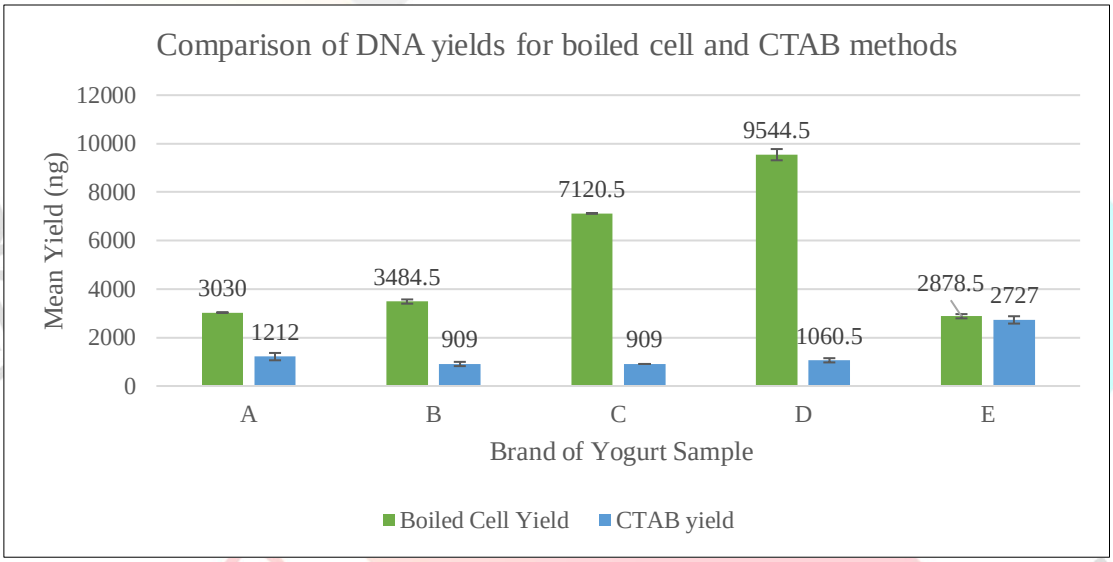

Figure 5*: Representation of the comparison of mean yields for the same samples extracted using boiled cell and CTAB methods. The green columns denote boiled cell method, while the blue columns denote CTAB method. Error bars indicate the standard deviation of the mean.*

Boiled cell method yields the highest DNA for all the samples tested (***Figure 5***), in comparison with CTAB method. Statistical analysis by one-way ANOVA is denoted by the Test-between subjects (***Table 7***) and POST-HOC test (***Figure 6***).

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Table 7: *Tests between-subjects effects.*

Source	Sum of Squares	dF	Mean Square	F	Significance
Method	112814899.0	1	112814899.2	8673.882	.000
Brand	43451669.55	4	10862917.39	835.206	.000
Method and Brand	70581229.05	4	17645307.26	1356.676	.000

Multiple Comparisons

Dependent Variable: DNAyield

Tukey HSD

(I) Brand	(J) Brand	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
a	b	-126.2500	65.84394	.341	-323.2797	70.7797
	c	-1893.7500*	65.84394	.000	-2090.7797	-1696.7203
	d	-3181.5000*	65.84394	.000	-3378.5297	-2984.4703
	e	-707.0000*	65.84394	.000	-904.0297	-509.9703
b	a	126.2500	65.84394	.341	-70.7797	323.2797
	c	-1767.5000*	65.84394	.000	-1964.5297	-1570.4703
	d	-3055.2500*	65.84394	.000	-3252.2797	-2858.2203
	e	-580.7500*	65.84394	.000	-777.7797	-383.7203
c	a	1893.7500*	65.84394	.000	1696.7203	2090.7797
	b	1767.5000*	65.84394	.000	1570.4703	1964.5297
	d	-1287.7500*	65.84394	.000	-1484.7797	-1090.7203
	e	1186.7500*	65.84394	.000	989.7203	1383.7797
d	a	3181.5000*	65.84394	.000	2984.4703	3378.5297
	b	3055.2500*	65.84394	.000	2858.2203	3252.2797
	c	1287.7500*	65.84394	.000	1090.7203	1484.7797
	e	2474.5000*	65.84394	.000	2277.4703	2671.5297
e	a	707.0000*	65.84394	.000	509.9703	904.0297
	b	580.7500*	65.84394	.000	383.7203	777.7797
	c	-1186.7500*	65.84394	.000	-1383.7797	-989.7203
	d	-2474.5000*	65.84394	.000	-2671.5297	-2277.4703

Based on observed means.
The error term is Mean Square(Error) = 13006.275.
*. The mean difference is significant at the 0.05 level.

Figure 6: *Statistical analysis by the POST HOC test for statistical significance of the difference between brands by SPSS edition 21 using one-way ANOVA. The red boxes denote a p value greater than 0.05, indicating that there is no significant difference between brands A and B and their contribution to DNA yield.*

There is a significant difference between the brands and methods which contributes to the DNA yield, since the p value is < 0.05, for both variables tested (***Table 7***). The POST HOC revealed values of p > 0.05 for brands A and B, showing no significant difference but other

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brands had $p < 0.05$, suggesting that there is a significant difference between the brands, which contribute to different DNA yields (***Figure 6***).

To evaluate the purity of the DNA extracted for each method, the 260/230 and 260/280 absorbance ratios were calculated and compared (***Figure 7*** and ***Figure 8*** respectively).

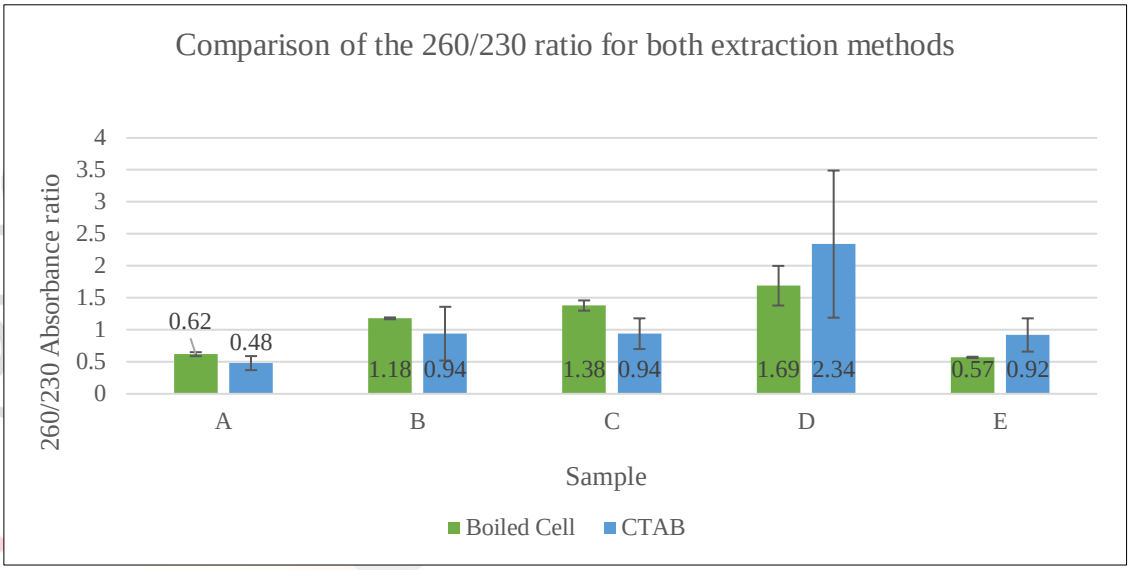

Figure 7: *Representation of purity (free from organic solvents) from the mean 260/230 ratio for DNA extracted by both methods. (High purity is denoted by a ratio more than 1.8). The green columns denote boiled cell method, while the blue columns denote CTAB method. Error bars represent the standard deviation of the mean.*

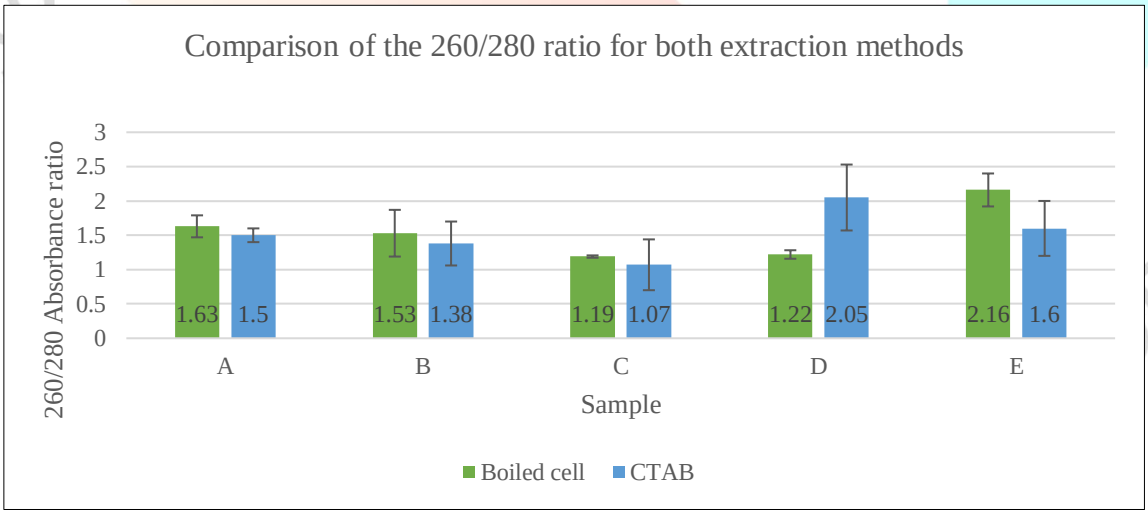

Figure 8: *Representation of purity (free from protein contaminants) from the mean 260/280 ratio for DNA extracted by both methods. (High purity is denoted by a ratio of 1.8). The green columns denote boiled cell method, while the blue columns denote CTAB method. Error bars represent the standard deviation of the mean.*

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Collectively, boiled cell method revealed higher purity for samples A, B, C and E, whilst CTAB method revealed higher purity for sample D for both ratios.

Genotypic identification of *Lactobacillus* isolates

Visualisation of the PCR products revealed that boiled cell method of DNA extraction contained *Lactobacillus* in all 5 extracts (230 bp DNA band) (***Figure 9***), whilst CTAB method confirmed the presence of *Lactobacillus* in extract B and E (***Figure 10***) (Byun et al., 2004).

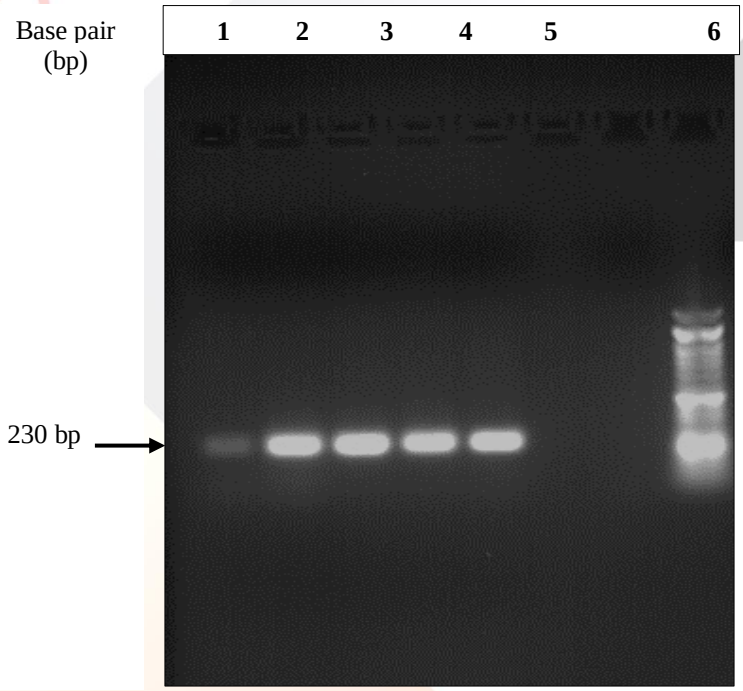

Figure 9*: 2% Agarose gel image showing identification of Lactobacillus isolates from boiled cell method of DNA extraction. All five samples were positive (230 bp band). Lane 1: Sample A, Lane 2: Sample B, Lane 3: Sample C, Lane 4: Sample D, Lane 5: Sample E, Lane 6: Negative Control and Lane 7: 50 bp DNA Ladder.*

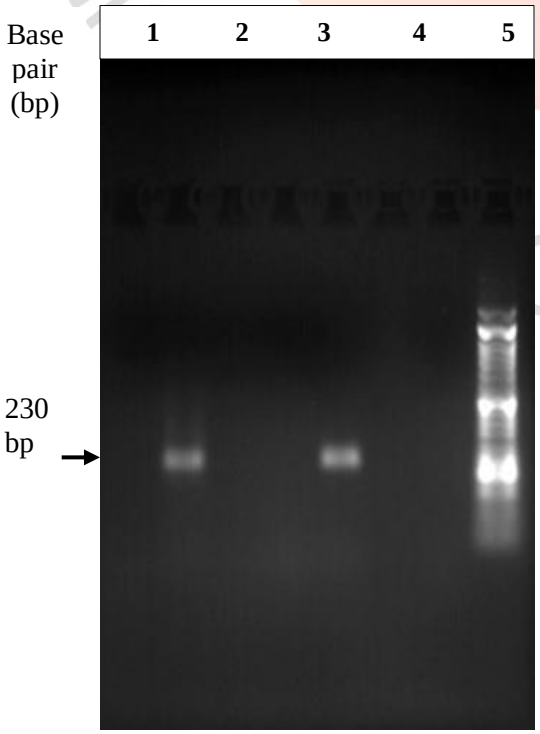

Figure 10*:*
2% Agarose gel image showing identification of Lactobacillus isolates from CTAB method of DNA extraction. Only two samples were positive, (230 bp band). Lane 1: Sample A, Lane 2: Sample B, Lane 3: Sample C, Lane 4: Sample D, Lane 5: Sample E, Lane 6: Negative Control and Lane 7: 50 bp DNA Ladder.

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Edit: GARI Editorial Team | Received: 28.01.2019 | Publish: 01.03.2019

Genotypic confirmation for antibiotic resistance genes [*tet*(M) and *erm*(B)] in *Lactobacillus* isolates

Boiled cell method extracts were obtained for PCR since due to higher purity. PCR products were visualised for *tet*(M) (***Figure 11***) and *erm*(B) genes (***Figure 12)*** respectively. Isolates from samples A and C contained the *tet*(M) resistance gene, but none of the isolates contained the *erm*(B) resistance gene.

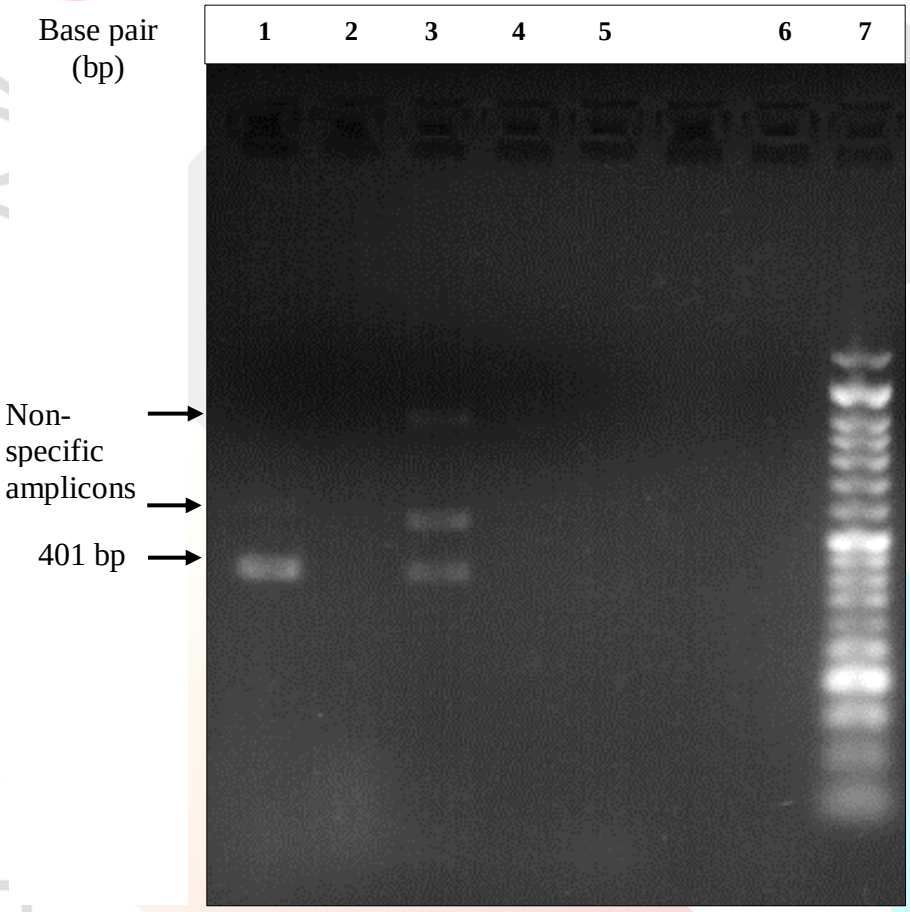

Figure 11*: 2% Agarose gel image showing identification of antibiotic resistance in tet(M) gene for Lactobacillus isolates. Sample A and C are positive, (401 bp band), and additionally sample C contains non-specific amplicons. Lane 1: Sample A, Lane 2: Sample B, Lane 3: Sample C, Lane 4: Sample D, Lane 5: Sample E, Lane 6: Negative Control and Lane 7: 50 bp DNA Ladder.*

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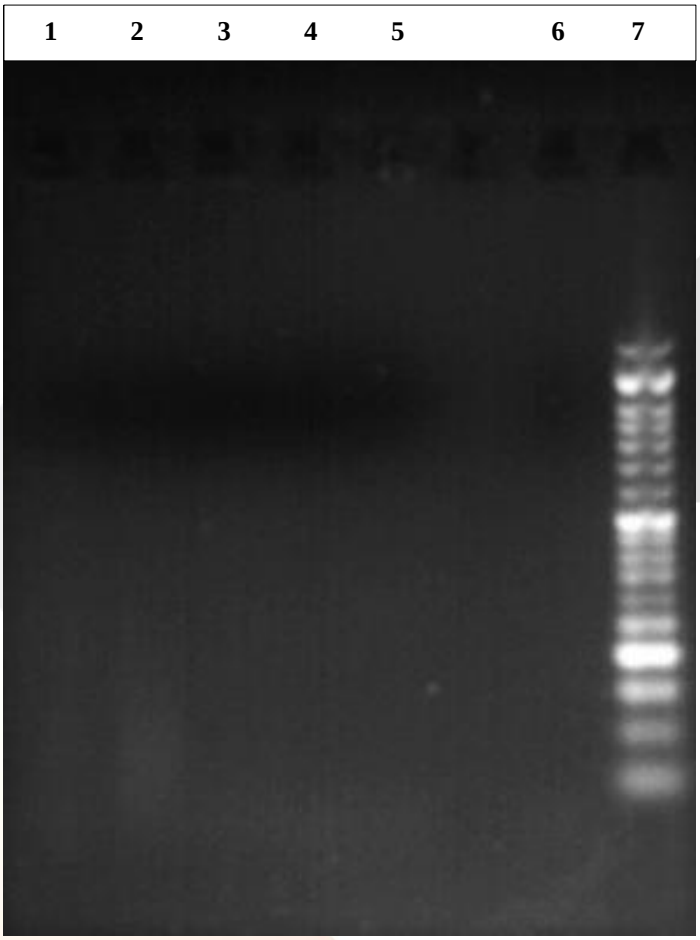

Figure 12*: 2% Agarose gel image for the identification of antibiotic resistance in erm(B)gene for Lactobacillus isolates. No samples are positive, (no bands visible). Lane 1: Sample A, Lane 2: Sample B, Lane 3: Sample C, Lane 4: Sample D, Lane 5: Sample E, Lane 6: Negative Control and Lane 7: 50 bp DNA Ladder.*

DISCUSSION

Yogurt starter cultures are predominantly known to contain *Lactobacillus* and standardized pre-market testing should ensure no transmission of antibiotic resistance to pathogens from the probiotic. This study aimed to identify if *Lactobacillus* was present, in five commercial yogurt products and analyse if the probiotic contained potential sources of antibiotic resistance. Identification of *Lactobacillus* by phenotypic traits were proceeded by assessing colonial appearance, and cellular morphology with Gram staining. All plates showed white-cream, opaque, mucoid colonies which were round in configuration, wavy in margin and existed on the surface of MRS agar which are characteristic of *Lactobacillus* (Jose et al., 2015). The growths differed in samples, with sample A exhibiting lowest number of colonies, in comparison to the others. The difference could be attributed to the difference in probiotic concentrations used in the different brands tested. There was contamination in plate E and addition of cycloheximide could prevent growth of other microbial flora (***Figure 3***) (Coeuret et al.,

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2003). Gram staining of colonies revealed purple rods, indicating the presence of Gram-positive bacilli for all samples, but required confirmation through PCR.

Boiled cell method exhibited higher DNA yield than CTAB method (***Figure 5***). This finding complies with a study that confirmed boiled cell method of extraction produced the highest yield when compared with three other extraction methods (Oliveira et al., 2014). It is worthy of note, that *Lactobacillus* peptidoglycan cell wall contains surface proteins, teichoic acids, and polysaccharides, making it difficult to lyse (Abdulla, 2014). The higher temperature used in boiled cell method compared to CTAB method, may have easily disrupted the peptidoglycan layer of the Gram-positive *Lactobacillus*, thus producing higher DNA yield (Junior et al., 2016). Further, the several centrifugations and multi-step processes of CTAB may have caused degradation of DNA (Ahmed et al., 2014).

The 260/280 depicts protein or RNA contamination, where 1.8 denotes pure DNA. *Lactobacillus* produces lactic acid on growth, hence the acidity affects the 260/280 ratios, and reduces the value by 0.2-0.3, hence why only a few samples in this study contain the ideal 260/280 range for pure DNA. Organic solvents/EDTA absorb at 230 nm, and a 260/230 ratio which is appreciably lower than 2.0, denotes the presence of these contaminants. Both extraction methods utilised EDTA, hence the 260/230 ratios for all samples were relatively low (Lucena-Aguilar et al., 2016; Ranasinghe and Perera, 2016).

Genotypic identification of *Lactobacillus* revealed 100% success rate with boiled cell extracts, but only 40% success rate with CTAB extracts (***Figure 9*** and ***Figure 10***). Interestingly in this study, the extracts required a minimum 260/230 ratio of 0.57 and 260/280 ratio of 1.19 for bands to be seen (***Figure 7*** and ***Figure 8***). These values are still very low in terms of DNA purity which usually requires a minimum 260/230 and 260/280 ratio of 1.8 for successful downstream applications. This finding portrays that a DNA purification step does not necessarily determine the performance of the PCR assay. Rather, the specificity of the primers towards the DNA present and tolerance to impurities, which undermine the successful amplification of DNA (Junior et al., 2016; Abdulamir et al., 2010).

Boiled cell method produced bands of 230 bp, which correlated with the Gram staining results, confirming the presence of *Lactobacillus* (Byun et al., 2004).

CTAB method sample A contained a 260/230 ratio of 0.48, which was less than the minimum 260/230 ratio needed to produce a band in this study (0.57). Hence the organic solvents in sample A, may have inhibited the reaction, thus exhibiting no DNA band. Sample C from CTAB method yielded a 260/280 ratio of 1.07 which was lower than 1.18, which was the value required to produce a band in this study. This meant protein contamination could have hindered PCR, and hence no DNA band was observed. Although Sample D from CTAB method yielded the highest ratios for 260/280 and 260/230 from the extracts of CTAB extraction, sample D did not produce a band, which could be due to the specificity of the primers to the DNA present, than the extraction method itself as afore mentioned.

Furthermore, a p value of 0.394 (>0.05), suggested there is no significance difference between the brands for DNA yield in samples A and B (***Figure 6***). Thus, the differences in the PCR results for samples A and B (***Figure 9*** and ***Figure 10***), solely relies on the extraction method. Both 260/280 ratios for sample A and B gave better purity from boiled cell method than CTAB, and this was in correlation with better DNA bands resulting from boiled cell method.

The results of this study comply with results of some studies which revealed that boiled cell yielded successful PCR assays (Junior et al., 2016;

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Abdulamir et al., 2010). A study confirmed that boiled cell method contained high sensitivity to that of a DNA extraction kit, (Junior et al., 2016). However, studies also contradict this study's findings, claiming that boiled cell method produced no DNA bands or that CTAB yields high quality DNA (Alimolaei and Golchin, 2016; Narwade et al., 2015; Oliveira et al., 2014; Abdulla, 2014).

The reason for the findings of this study can be justified primarily, by differences in the temperature used. CTAB method used a temperature of only 60 ºC, which may have not lysed cells adequately. Higher temperature used in boiled cell method than CTAB method, may have caused more protein denaturation and disruption of tertiary structures and solubility, allowing precipitation of the protein enhancing its removal which may otherwise have inhibited PCR. This notion is supported by a study confirming that proteins on the cell wall of *Lactobacillus* change their tertiary structures only at 80 ºC, thus will precipitate best at this temperature (Lighezan et al., 2012). Furthermore, organic molecules such as ethanol and isopropanol and additives such as mercapto-ethanol used in CTAB method are known to confer inhibition to PCR which further supports this study's findings (Demeke and Jenkins, 2010).

Among the inhibitors of protein synthesis, tetracycline resistance has been exclusively reported for its acquired resistance in *Lactobacillus* (Zago et al., 2012). In support of this statement, was the results of this study which showed 40% of the tested isolates conferred resistance to tetracycline (***Figure 11***) The mechanism of tetracycline resistance is assumed to be controlled by *tet* genes, which code for efflux or ribosomal proteins (Roberts et al., 2008). Acquired resistance by two most commonly witnessed resistance genes in *Lactobacillus* are *tet*(M) for and *erm*(B) resistance genes (Sharma et al., 2015). Although some samples did not render positive for the *tet*(M) antibiotic resistant gene tested, Instead, they could contain different tetracycline resistance genes [*tet*(W), *tet*(S), *tet*(O), *tet*(Q) and *tet*(Z)] (Klare et al., 2007). Non-specific bands were visualised for sample C, for *tet*(M) PCR and optimisation of PCR conditions could overcome this (Fox et al., 2007).

Erythromycin resistance in *Lactobacillus* has been proven to be equally common as tetracycline resistance. Reports suggest a 23S rRNA mutation which hinders erythromycin uptake in the cells, being a probable mechanism for resistance (Flórez et al., 2007). In this study, no isolates produced the resistant gene *erm*(B) (***Figure 12***), even when the DNA concentration of the sample was doubled, supporting findings that higher prevalence of *tet*(M) genes than *erm*(B) genes exist (Nawaz et al., 2011). The samples could give possible resistance to other resistant genes of erythromycin instead [*erm*(A), *erm*(C) and *erm*(T)] (Sharma et al., 2015; Mayrhofer et al., 2010).

CONCLUSION

The aim of the study was to identify *Lactobacillus* in commercial yogurt products and analyse their antibiotic resistance. The colony appearance, Gram staining and PCR results positively correlated with each other, confirming the presence of *Lactobacillus* in the five yogurt products. Yield of DNA is dependent on the yogurt brand and method of DNA extraction and boiled cell method produced highest yield in this study. The *tet*(M) resistance gene was found to be more prevalent than the *erm*(B) resistance gene in the samples tested, which highlights the need for pre-production screening of lactobacilli, to minimise the risk of transmission of these determinants to pathogens

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Edit: GARI Editorial Team | Received: 28.01.2019 | Publish: 01.03.2019

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