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IDENTIFICATION OF THE GENUS *BIFIDOBACTERIUM* IN COMMERCIALLY AVAILABLE YOGURT DRINK PRODUCTS AND DETERMINATION OF THEIR ANTIBIOTIC RESISTANCE GENES

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ABSTRACT

Throughout the years, there has been a vast evidence highlighting the benefits of probiotics in daily food products. Which confer potential health benefits by maintaining a proper balance in gut microbiota as well as treating specific pathological conditions. Numerous studies have shown the importance of manual and molecular methods for the identification of probiotics. Hereby, this study focuses on the identification of the probiotic, *Bifidobacterium* and the determining of its antibiotic resistance genes. Initially five yogurt drink samples were cultured in Bifidobacteria selective media. Phenotypic and Gram staining morphologies were observed, followed by DNA extraction using boiled cell and CTAB methods. Extracted DNA was quantified using spectrophotometer. Boiled cell method surpassed CTAB method in terms of yield and purity of the DNA obtained. The extracted DNA was then subjected to PCR where the products were observed for sample C and D from boiled cell method and not CTAB method.

Subsequently, the *Bifidobacterium* positive samples from boiled cell method were evaluated for antibiotic resistance genes through PCR based-detection respectively. The antibiotic resistant genes *tet*(M) and *erm*(B) were determined and tested, as they are prominently found in Bifidobacteria. Sample C displayed a band for *erm*(B) but not for *tet*(M). Nevertheless, Sample D displayed no bands for either *tet*(M) or *erm*(B) indicating susceptibility. The availability of these methods for identification of *Bifidobacterium* and their antibiotic resistance is therefore, vital in the fields of environmental and food microbiology.

Keywords: Daily food products, probiotic, yogurt drink, PCR, antibiotic resistance genes.

INTRODUCTION

Probiotics can be defined as living microorganisms administered in an adequate amount that continue to exist in the intestinal bionetwork, to perform a health positive effect on its host. Probiotics have been shown to be effective in preventing pediatric antibiotic-associated diarrhea, necrotizing enterocolitis in preterm infants, aid in lactose digestion and help to regulate digestion, immune system modulation,

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anti-allergy, hepatic encephalopathy and upper respiratory tract infections (Bourdichon *et al*., 2012; Azizpour *et al*., 2017). Lactic acid bacteria (LAB) such as The *Lactobacillus* (e.g., *L. bulgaricus, L. acidophilus, L. rhamnosus, L. casei, L. johnsonii, L. reuteri*, etc.), *Streptococcus* (e.g., *S. thermophilus*, etc.) and *Bifidobacterium* (e.g., *B. bifidum, B. longum, B. breve, B. infantis*) are few of the bacteria's to have achieved this status as probiotics (Amara and Shibl, 2015; Shigwedha and Ji, 2013).

Bifidobacterium and their significance

The genus *Bifidobacterium*, is a member of the *Bifidobacteriaceae* family, belonging to the *Actinobacteria* phylum. They are gram-positive, anaerobic microorganisms with a high G+C DNA content and they have the ability to survive passage through the gastrointestinal tract and eventually establish in the colon (gut microbiota) (Ventura, Turroni and van Sinderen, 2015). They are helpful in maintaining a proper balance in the gut microbiota by associating with health-promoting activities such as immune system stimulation and protection against pathogens (D'Aimmo, Modesto and Biavati, 2007). Though bifidobacteria are commonly found in the gut, they have been also found in: human blood (*Bifidobacterium scardovii*), sewage (e.g., *Bifidobacterium minimum*) and mainly food products (e.g., *Bifidobacterium animalis*). Furthermore, bifidobacteria is the predominant genus of bacteria in the normal intestinal flora of healthy breast-fed newborns which explains their low susceptibility to any enteric diseases (Tsuchida *et al*., 2017; Turroni *et al*., 2014). *Bifidobacterium* infections are extremely rare but in some cases *Bifidobacterium*-associated gastrointestinal and extra-intestinal infections have been found (Gueimonde *et al*., 2013).

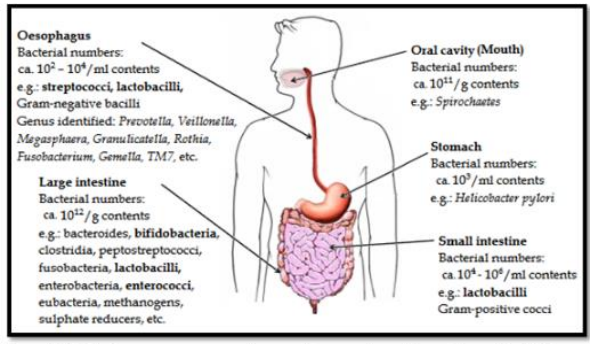

Figure 1.1.1. *The human gastrointestinal tract and its microbiota (Shigwedha and Ji, 2013).*

The morphological and physiological features of these microorganisms (Figure 1.1.2) are similar to those of *Lactobacillus* and they were classified as members of the genus *Lactobacillus* for most of the 20th century and only starting from the year 1974, they have been recognized as a separate genus (Turroni *et al*., 2014). Most of the species belonging to the *Bifidobacterium* genus display long and short club-shaped rods, most of which are long and thin with blunted ends. Additionally, with conventional "V" or "Y" shaped cells (Shigwedha and Ji, 2013).

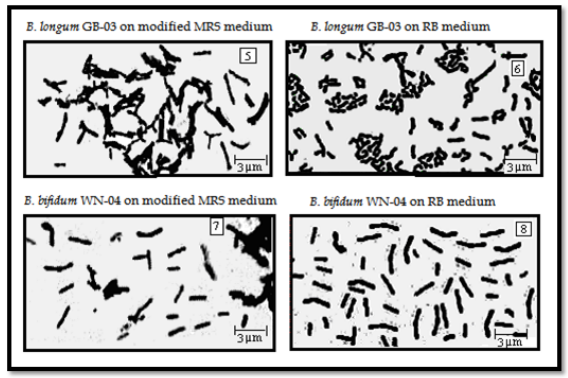

Figure 1.1.2. *Micrographs of the a few strains of Bifidobacterium taken at 100× magnification by oil immersion (Shigwedha and Ji, 2013).*

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These various *Bifidobacterium* strains have been incorporated into a wide variety of food products including baby foods, livestock feed supplements, pharmaceutical preparations and fermented dairy products (Yogurt, Yogurt Drink, Curd, Sauerkraut etc.) (Salmerón *et al*., 2013). Thus, there is great interest in the development of novel probiotic foods containing these lactic acid strains to obtain its beneficial statuses. Due to the bacterium's anaerobic nature, Shigwedha and Ji, (2013) speculated that its survival rate in most daily products is short due to low pH and/or exposure to oxygen. However, their oxygen sensitivity differs among the bacterium's species, and these species have become tolerant to these low pH's ranges and exposure to oxygen thereby elevating its growth. One example of this oxygen and acid tolerant strain when compared to other strains is *Bifidobacterium lactis* (Yerlikaya, 2014).

Antibiotic resistance

The spread of antibiotic resistance among pathogenic bacteria represents a major concern and increasing medical problem for decades. Since most probiotics are common members of the human intestinal tract they are ingested in large amounts for their beneficial traits, and the presence of antibiotic resistance determinants in their genome poses a major risk. Because these probiotics can constitute a reservoir from which these determinants could be transmitted to non-pathogenic or pathogenic bacteria present in the gut. (Onyibe *et al*., 2013; Hummel *et al*., 2006). Using horizontal gene transfer mechanisms, antibiotic resistance genes may not only be exchanged among members of the gut microbiota, but may also be transferred to other bacteria that are just passing through the gastrointestinal tract, including several diet-associated bacteria which can result in infections (Duranti *et al*., 2016).

Types of antibiotic resistance

The known mechanisms of antibiotic resistance can be broadly classified into two types: Intrinsic (natural) and acquired resistance which also includes mutations. Certain mechanisms, such as lack of cell wall permeability or the modification of target site etc, are more likely to be inherited to a bacterial species or genus; therefore, this type of resistance is classified as intrinsic or natural. However, strains belonging to a group naturally susceptible to an antibiotic can acquire resistance through gain of exogenous DNA through mobile genetic elements, such as plasmids and transposons or by mutation of indigenous genes in their chromosome. This is known as acquired resistance (Martínez *et al*., 2018; Patel, Shah and Prajapati, 2012). Towards the antibiotic resistance of the *Bifidobacterium* genus, the resistance of tetracycline deserves attention as the proteins that protect the ribosome from the action of tetracyclines, the *tet* genes (Table 1.2.1.1.) have been detected in several *Bifidobacterium* species (Wang *et al*., 2017).

Table 1.2.1.1. *Antibiotic resistance genes identified and characterized in bifidobacteria. (Adapted from Gueimonde et al., 2013).*

Genes	Resistance	Mechanism	Location	Microorganism
erm(X)	Macrolides, lincosamides, streptogramines (MLS)	Ribosomal methylation	Transposon	*B. animalis* subsp. *lactis* and *B. thermophilum*.
tet(W), *tet*(M), *tet*(O), *tet*(W/32/O), *tet*(O/W	Tetracycline	Ribosomal protection	Chromosome	*B. longum* (subsp. *infantis* and subsp. *longum*), *B. breve*, *B. animalis* subsp. *lactis*, *B. bifidum*. *B. pseudocatenulatum*, and *B. thermophilum*
tet(L)	Tetracycline	Efflux	Chromosome	*B. longum*, *B. animalis*.

However, antibiotics with low concentrations of macrolides, vancomycin, chloramphenicol, beta – lactams, rifampicin, spectinomycin and

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erythromycin is said to cause resistance as well (van Hoek *et al*., 2008).

Identification and detection of antibiotic resistance

For the identification of *Bifidobacterium*, molecular methods such as PCR have proven to be the gold standard due to its advantageous properties of high sensitivity and specificity (Shigwedha and Ji, 2013). However, in contrast to susceptibility testing of these probiotics, methods such as use of bacterium specific growth medias, disk diffusion, broth microdilution and agar dilution have been used for the process of detection and evaluation of these bacteria (Wong *et al*., 2015). The purpose of this study is to identify *Bifidobacterium* in selected yogurt drink products and to detect their antibiotic resistance tetracycline and erythromycin by PCR. PCR detection is influenced by the purity and concentration of the extracted DNA. Therefore, two different extraction methods were carried out to find out which provides better yield with high purity.

MATERIALS AND METHODOLOGY

Materials

Reagents

Bifidobacterium selective media agar (HI media), Glacial acetic acid (CH3COOH), Sodium chloride (Nacl), Mueller Hinton Agar (HI media), 0.2% β-mercaptoethanol, Barium chloride (Bacl2), Hydrochloric acid (Hcl), Sulphuric acid (H2SO4), Crystal violet, Grams iodine, Grams decolourizer, Safranin, Ethanol (100%), isopropyl alcohol, CTAB powder (HI media), Tris base, EDTA, dNTP (Invitrogen), 5U/ul Taq polymerase (IDT), Ethidium bromide(EtBr), Agarose powder (Sigma Aldrich), 100bp Ladder (abm), 25mM Mgcl2 (Promega), 5X PCR buffer (Promega), Antibiotic disks (10 ug) for ampicillin, tetracycline, erythromycin and vancomycin (Liofilchem, Italy).

Sample preparation

Five commercially available yogurt drink products were purchased and 2ml from each was transferred to five beakers and labelled from A to E.

Culturing of the sample in Bifidobacterium selective media agar

Aseptic conditions were maintained throughout the procedure. Using a sterile inoculation loop, the sample (Yogurt Drink) was picked. It was streaked on Bifidobacterium selective media using the four quadrant streak plate method. After streaking, all five petri plates incubated in 37°C for 2 days.

Identification of the bacteria by Gram

Initially, the bacterial smear was prepared with the specific colony picked and gram staining procedure was followed (Modifed from: Coico, 2005). Gram positive bacteria with rod shape were selected for DNA extraction.

DNA Extraction of the Cultivated Colonies from the Selective Media Agar

Boiled cell DNA extraction method

Initially, 500µl of autoclaved distilled water was added to each labelled falcon tube (A-E) followed by the inoculation of sufficient amount of the selected colonies into tube. The falcon tubes were then centrifuged at 4000rpm for 20 minutes.

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The supernatant was discarded and to each tube 500µl of TE was added to each tube and vortexed briefly. Thereafter, the tubes were heated at 100°C for 20 minutes and immediately cooled at -20°C for 20 minutes. Then the tubes were centrifuged at 4000rpm for 10 minutes. Finally, the pellet formed was discarded and the supernatant was transferred to the Eppendorf tubes and stored at -5°C (Modified from: Abdulamir *et al*., 2010).

CTAB DNA extraction method

Following any aseptic conditions, 500µl of autoclaved CTAB buffer (Refer Appendix B for preparation method) were added to each falcon tubes. Then the selected colony was picked with a sterilized loop and was inoculated in the CTAB buffer. Thereafter it was centrifuged at 4000rpm for 20 minutes followed by incubation at 65°C for 30 min. Then the supernatant was transferred to a fresh falcon tube and equal amounts of Isopropanol was added and tube was inverted to mix. Thereafter the tubes was centrifuged until the precipitate was observed. After receiving the pellet, the supernatant was discarded without disturbing the pellet. After removing the supernatant, 500µl of 70% ethanol was added to wash the pellet and it was centrifuged at 4000rpm for 1 minute. Then the excess ethanol was pipetted out and the pellet was dried for 24 hours to evaporate any remaining ethanol. Finally 500 µl of TE was added to the pellet and the tubes were stored at -18°C (Modified from: Abdulamir et al., 2010).

Spectrophotometric quantification of bacterial DNA isolated from Boiled cell and CTAB extraction methods

DNA quantification for the two extraction methods was performed in a spectrophotometer to calculate the DNA concentration and DNA yield for each sample.

Readings were taken in triplicates at 230, 260 and 280nm wavelengths and the mean calculated. These readings were taken for each sample (Sample A – E).

Following the calculation of the DNA concentration and yield, the ratios (260/280) and (260/230) was calculated for both extraction methods to evaluate the purity of DNA.

Statistical analysis of the DNA yield and its comparison with the sample and extraction method

Statistical analysis was performed on SPSS Statistics 21 by One way-Anova analysis. This was done to find if there is any significance (p value <0.05) or not (p value >0.05) between the DNA yields when comparing it to the sample and the extraction method.

Identification of Bifidobacterium by Polymerase Chain Reaction (PCR)

Bifidobacterium was identified using genus specific primers (Table 2.8.1). PCR was performed according to the cyclic parameters given in table Table 2.8.1.1

(Modified from: Abdulamir *et al*., 2010).

Table 2.8.1. *Bifidobacterium* genus specific primer sequences and product size

Target Organism	Primer Set	Sequence (5' to 3')	Product size	Reference
Bifidobacterium genus	g-Bifid-F	CTCCTGGAAACGGGTGG	562 (549-563)	(Matsuki *et al*., 2004)
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA		

The following PCR procedure was followed for extracted bacterial DNA from both boiled cell and CTAB DNA extraction methods. 25 µl of PCR mastermix contained 5x PCR buffer, 10 Mm dNTP, 2 µM forward primer, 2 µM reverse primer, 25mM MgCl2 and 5U µl^{-} Taq polymerase, autoclaved distilled water and 100 ng bacterial DNA. The

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PCR products were detected by agarose gel electrophoresis using 2% agarose gel.

2.8.1. PCR cyclic parameters for Heat Shock and CTAB DNA extraction methods

Table 2.8.1.1. *PCR cyclic parameters for Heat Shock and CTAB PCR procedure*

Step	Temperature (°C)	Time/min	Cycles
Initial denaturation	94	5	
Denaturation	94	1	35
Annealing	55	1	
Extension	72	2	
Final Extension	72	12	
Final hold	4		

Identification of resistance to tetracycline and erythromycin

PCR was performed for the Bifidobacterium positive samples using both tet(M) (tetracycline) and erm(B) (erythromycin) resistant genes specific primers separately. The same PCR components were used in this PCR procedures as well. Primers and the cyclic parameters used are mentioned in table 2.9.1.1, 2.9.1.2 and 2.9.1.3 respectively

Table 2.9.1.1. *tet(M) (tetracycline) and erm(B) (erythromycin) specific primer sequences and product size.*

Primer Set	Sequence (5' to 3')	Product size	Reference
tet(M) forward	GGTGAACATCATAGACACGC	401	Gad, Abdel-Hamid and Farag, 2014).
tet(M) reverse	CTTGTTCGAGTTCCAATGC		
erm(B) forward	CATTTAACGACGAAACTGGC	405	
erm(B) reverse	GGAACATCTGTGGTATGGCG		

The separate cyclic parameters published by Gad, Abdel-Hamid and Farag, 2014 is given below:

Table 2.9.1.2. *PCR cyclic parameters for erm(B)*

Step	Temperature (°C)	Time/min	Cycles
Initial denaturation	94	5	
Denaturation	94	1	35
Annealing	52	1	
Extension	72	2	
Final Extension	72	12	
Final hold	4		

Table 2.9.1.3. *PCR cyclic parameters for tet(M)*

Step	Temperature (°C)	Time/min	Cycle times
Initial denaturation	94	5	
Denaturation	94	1	35
Annealing	55	1	
Extension	72	2	
Final Extension	72	12	
Final hold	4		

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RESULTS

Phenotypic identification of samples in *Bifidobacterium* specific agar

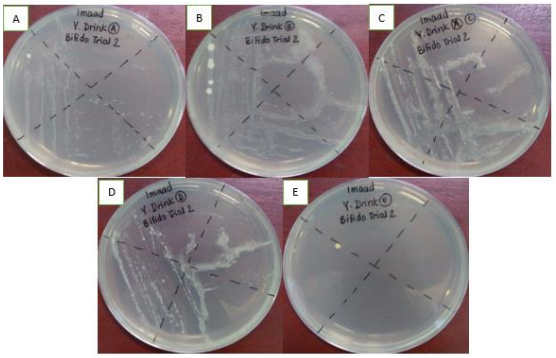

Figure 3.1.1. *Visualization of Yogurt Drink Sample A-E cultured in Bifidobacterium specific agar after 72 hours of incubation*

On phenotypic identification of Sample A – D, a white transparent and opaque circular raised colonies was observed. In sample E, a single opaque white circular colony was observed.

Gram staining performed on selected colonies cultured in Bifidobacterium selective agar

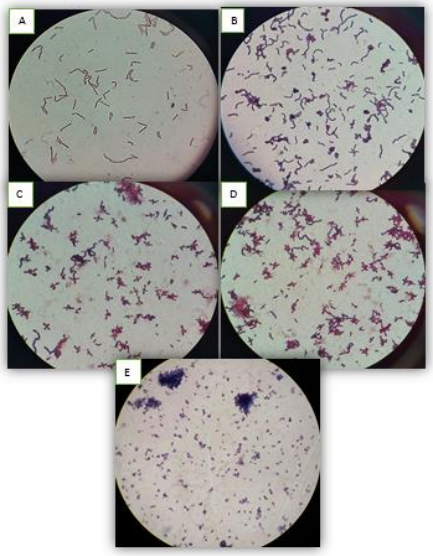

Figure 3.2.1. *Photomicrograph of Yogurt Drink sample A-E under 100x magnification.*

During the observation, long slender rods arranged in chains or palisades was observed in sample A which are very prominent features of lactobacillus. Where the cells appeared to be light red in colour. Sample B showed long, short and thin cells with bends and protuberances at the end of the cells. Where the cells appeared to be in dark purple in colour. Both sample C and D showed very similar short and thin cells with bends and protuberances at the end of the cells disposed in ''V'' and ''Y'' shaped arrangements. Which included a very few circular cocci shaped cells, appearing to be in reddish purple colour. Thus, on observation of sample E, short and thin cells with bends were present. Where the cells were observed in dark blue colour.

Spectrophotometric quantification for Boiled cell and CTAB DNA extraction methods

Table 3.3.1. *Calculated mean DNA concentrations for each sample under boiled cell method*

Sample	DNA Concentration (ng/µl)
A	75.75 ± 10.51
B	80.8 ± 10.51
C	80.8 ± 2.92
D	106.05 ± 5.83
E	80.8 ± 11.51

Table 3.3.2. *Calculated mean DNA concentrations for each sample under CTAB method*

Sample	DNA Concentration (ng/µl)
A	60.6 ± 5.05
B	45.45 ± 16.23
C	65.65 ± 10.10
D	70.7 ± 13.36
E	60.6 ± 5.83

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Comparison of DNA yield between Boiled cell and CTAB DNA extraction method

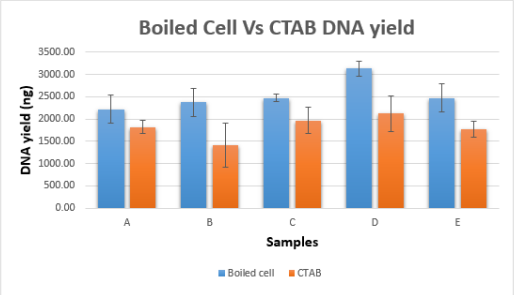

Figure 3.4.1. *Bar chart depicting the differences in the DNA yield between boiled cell and CTAB method including error bars representing standard error of the mean*

Referring to Figure 3.4.1. When comparing the DNA yield between the two DNA extraction methods, it was indicated that boiled cell method gave the highest DNA yield compared to CTAB method.

Comparison of DNA purity ratios between Boiled cell and CTAB DNA extraction methods

Table 3.5.1. *Table depicting the DNA purity ratios for Boiled cell and CTAB DNA extraction methods*

Boiled Cell			CTAB		
Sample	**260/280**	**260/230**	**Sample**	**260/280**	**260/230**
A	1.15 ± 0.29	1.15 ± 0.26	A	1.00 ± 0.05	0.52 ± 0.03
B	1.00 ± 0.15	0.89 ± 0.11	B	0.64 ± 0.33	0.64 ± 0.16
C	1.00 ± 0.08	1.23 ± 0.16	C	0.76 ± 0.11	0.87 ± 0.17
D	0.84 ± 0.14	1.00 ± 0.21	D	1.00 ± 0.25	0.88 ± 0.15
E	0.89 ± 0.07	1.07 ± 0.15	E	0.86 ± 0.05	0.86 ± 0.07
Reference ranges	**1.8**	**2.0 - 2.2**	Reference ranges	**1.8**	**2.0 - 2.2**

Statistical analysis using One-way ANOVA

Tests of Between-Subjects Effects

Dependent Variable: DNA yield

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6158088.68[a]	9	684232.075	7.777	.000
Intercept	142121097.1	1	142121097.1	1615.313	.000
Method	3856743.075	1	3856743.075	43.835	.000
Brand	1869843.300	4	467460.825	5.313	.004
Method * Brand	431502.300	4	107875.575	1.226	.331
Error	1759672.500	20	87983.625		
Total	150038858.3	30			
Corrected Total	7917761.175	29			

a. R Squared = .778 (Adjusted R Squared = .678)

Figure 3.6.1. *Table showing comparisons of DNA yield between method and brand (sample)*

When comparing the DNA yield between the method and brand (sample), Method and brand separately has a p – value less than 0.05 (0.000 & 0.004) respectively indicating there is a significant difference between the DNA yield. Nevertheless, both Method and brand had e p value of 0.331 (> 0.05) indicating that method and brand collectively have no significant contribution towards the DNA yields obtained.

Multiple Comparisons

Dependent Variable: DNA yield

Tukey HSD

(I) Brand	(J) Brand	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
a	b	126.2500	171.25383	.945	-386.2056	638.7056
	c	-202.0000	171.25383	.763	-714.4556	310.4556
	d	-606.0000*	171.25383	.016	-1118.4556	-93.5444
	e	-101.0000	171.25383	.975	-613.4556	411.4556
b	a	-126.2500	171.25383	.945	-638.7056	386.2056
	c	-328.2500	171.25383	.341	-840.7056	184.2056
	d	-732.2500*	171.25383	.003	-1244.7056	-219.7944
	e	-227.2500	171.25383	.678	-739.7056	285.2056
c	a	202.0000	171.25383	.763	-310.4556	714.4556
	b	328.2500	171.25383	.341	-184.2056	840.7056
	d	-404.0000	171.25383	.168	-916.4556	108.4556
	e	101.0000	171.25383	.975	-411.4556	613.4556
d	a	606.0000	171.25383	.016	93.5444	1118.4556
	b	732.2500*	171.25383	.003	219.7944	1244.7056
	c	404.0000	171.25383	.168	-108.4556	916.4556
	e	505.0000	171.25383	.055	-7.4556	1017.4556
e	a	101.0000	171.25383	.975	-411.4556	613.4556
	b	227.2500	171.25383	.678	-285.2056	739.7056
	c	-101.0000	171.25383	.975	-613.4556	411.4556
	d	-505.0000	171.25383	.055	-1017.4556	7.4556

Based on observed means.
The error term is Mean Square(Error) = 87983.625.
*. The mean difference is significant at the 0.05 level.

Figure 3.6.2. *Table showing post HOC tests showing multiple comparisons between brands*

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When comparing the DNA yield between brands, significance difference between the yields obtained from sample A and D as well as sample B and D is evident (Figure 3.6.2.)

Agarose gel electrophoresis for boiled cell and CTAB DNA extraction methods

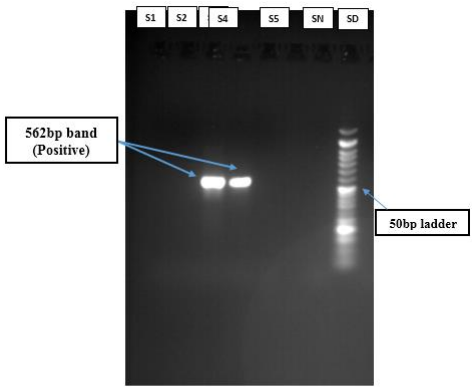

Figure 3.7.1. *2% agarose gel image of PCR products of the DNA extracted from Boiled cell method*

'S1' = Yogurt drink sample A
'S2' = Yogurt drink sample B
'S3' = Yogurt drink sample C (Positive)
'S4' = Yogurt drink sample D (Positive)
'S5' = Yogurt drink sample E
'SN' = Negative control
'SD' = 50bp ladder

Yogurt Drink sample C and D gave bands **(562bp)** for boiled cell method.

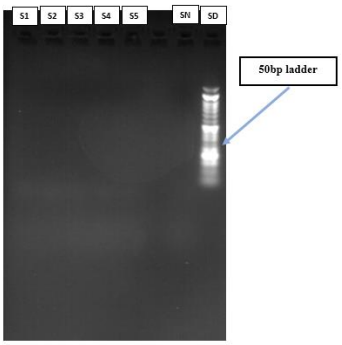

Figure 3.7.2. *2% agarose gel image of PCR products of the DNA extracted from CTAB method*

'S1' = Yogurt drink sample A
'S2' = Yogurt drink sample B
'S3' = Yogurt drink sample C
'S4' = Yogurt drink sample D
'S5' = Yogurt drink sample E
'SN' = Negative control
'SD' = 50bp ladder

No bands were observed for CTAB method.

Agarose gel electrophoresis for antibiotic resistance

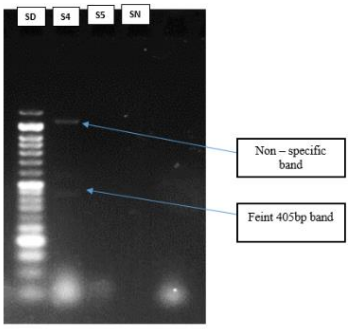

Figure 3.8.1. *2% agarose gel image of PCR products of the positive DNA samples tested for erm(B) gene*

'S4' = Yogurt drink sample C (Feint 405bp positive band)
'S5' = Yogurt drink sample D
'SN' = Negative control
'SD' = 50bp Ladder

Yogurt drink sample C gave a feint band of **405bp** including a nonspecific band. No bands were observed for sample D.

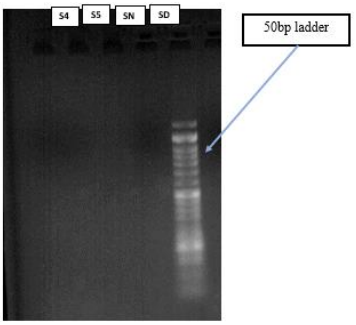

Figure 3.8.2. *Agarose gel image of PCR products of the positive DNA samples tested for tet(M) gene*

'S4' = Yogurt drink sample C
'S5' = Yogurt drink sample D
'SN' = Negative control
'SD' = 100bp Ladder

No bands were observed for both samples tested for tetracycline resistance.

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DISCUSSION

Following the culturing of the sample in the *Bifidobacterium* selective media, phenotypic identification showed a variety of transparent and opaque white circular raised colonies in the samples (Figure. 3.1.1.). This may indicate the growth of different species of *Bifidobacterium*. With reference to Gram staining, Shigwedha and Ji, (2013) explains the morphology of *Bifidobacterium* as: short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances. Additionally, single or chains of various arrangements (Eg: star-like aggregates or disposed in "V" or "Y" shaped arrangements). Sample A depicted short as well as long slender rods arranged in chains or palisades *(*Figure 3.2.1.), this gives clear indication of the growth of lactobacillus bacteria (Sharma *et al*., 2015). But in samples B, C, D and E comparatively similar morphologies were observed as explained by (Shigwedha and Ji, 2013). Furthermore, according to (Sarkar and Mandal, 2016) *bifidobacterium* are gram positive, anaerobic microorganisms which are coloured blue or dark purple after gram staining. The A260/280 ratio can be used to assess the DNA purity concerning the protein and other contaminations. Additionally, assessing the A260/230 ratio depicts contamination of organic solvents. Furthermore, determination of DNA concentration and purity is important for downstream applications, such as PCR for the detection of *Bifidobacterium.* PCR has a specific target or amount of DNA concentration that is required in order to perform optimally. This helps to prevent unnecessary consumption, enhance reproducibility and amplification of the sample bands (Boesenberg-Smith, Pessarakli and Wolk, 2012). When comparing the ratios of both extraction methods, the ratio A260/A280 was found to be **< 1.8** for both boiled cell and CTAB extraction methods. Ratios less than 1.8 indicate the contamination of proteins (Sigma-Aldrich, 2018). Boiled cell had the least amount of protein contamination compared to CTAB when analyzing the ratio (A260/A280) except for sample D. In conclusion, the DNA yields obtained from boiled cell method is far greater than the CTAB method. *(*Figure 3.4.1.)

One-way ANOVA analysis (Figure 3.6.1) showed initially the p-value for method and brand was less than 0.05, indicating there is a significant contribution of brand and method individually towards the DNA yield obtained but the p-value for method and brand collectively was more than 0.05, indicating that there is no significant contribution of brand and method collectively toward the yield (Figure 3.6.2.). In addition, it has been discussed in several studies that CTAB enzymatic extraction method has been accredited with producing the highest amount of DNA compared to boiled cell method. As through the use of chemicals such as Tris and EDTA solutions, it has the ability to efficiently damage the cell membrane, the cell wall of the Gram-positive bacterium (*Bifidobacterium*) and release its cytoplasmic components (Esfahani *et al*., 2017; Kamel, Helmy and Hafez, 2014). But in boiled cell method, only due to its high temperatures it has the ability to disrupt peptidoglycan cell walls which is sufficient in some DNA extraction studies (Ribeiro Junior *et al*., 2016), Nevertheless, despite low values for (A260/A280) ratio, positive bands were still observed for Sample C and D of boiled cell method and no bands were observed for CTAB method (Figure 3.7.1). This observation of the bands are possible as studies have shown that even at very low concentrations of DNA, the

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sensitivity of PCR is high enough to produce a reasonable amount of amplicons for visualization (Ribeiro Junior *et al*., 2016; Kamel, Helmy and Hafez, 2014; Oliveira *et al*., 2014).

Based on the highest DNA yield obtained and the comparisons done, the positive samples observed for boiled cell method was taken identification of antibiotic resistance for tetracycline and erythromycin for the positive samples (C and D) was performed using PCR. In support of erythromycin resistance, a study done by (Sato and Iino, 2010) revealed erythromycin resistance in *Bifidobacterium bifidum* and *Bifidobacterium breve* species which acquired resistance through gain of mutation of indigenous genes in their chromosome (Additional studies include: Martínez *et al*., 2018). Erythromycin resistance for sample C was confirmed by PCR where a feint band of 405bp was observed for the presence of the erm(B) gene (Figure 3.8.1). Non-specific band formation can be avoided by optimizing the PCR conditions. *Bifidobacterium* in sample D showed no bands either for *erm*(B) nor *tet*(M) gene.

CONCLUSION

In conclusion, *Bifidobacterium* were detected in two yogurt drink samples (C and D) and boiled extraction method found to be the best method to obtain DNA with high yield and purity. The *Bifidobacterium* isolated from sample C showed resistance to erythromycin due to presence of *erm*(B) and was may be susceptible to tetracycline due to absence of *tet*(M). Nevertheless, *Bifidobacterium* isolated from sample D may be susceptible to both tetracycline and erythromycin due to absence of the genes *tet*(M) and erm(B). The erm(B) and *tet*(M) are not the only genes which are responsible for tetracycline and erythromycin resistance. Therefore, other resistance genes should also be tested to confirm these results.

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