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IDENTIFICATION OF STREPTOCOCCUS IN TABLE OLIVE SAMPLES AND ANALYSIS OF THEIR ANTIBIOTIC RESISTANCE

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ABSTRACT

Fermented olives are a rich source of nutritional compounds and probiotics. Probiotics are defined as living bacteria that confer health benefits to the host when administered in adequate amounts. The purpose of this study was to identify the genus of Streptococcus in fermented olives samples and analyze their antibiotic resistance. The process was carried out by culturing 5 samples in MRS agar followed by biochemical tests to identify pure Streptococcus colonies and then sub culturing was performed. Afterwards DNA extraction was carried out by boil cell and kit based extractions method. The DNA yield and purity were compared between the two methods. PCR was performed using genus specific primers to identify Streptococcus. In addition to that, the resistance gene for tet(M) and erm(B) were detected by PCR and visualized by gel electrophoresis. Out of 5 samples, 4 samples had proper growth in MRS agar. After extraction when comparing DNA yield between the two methods were statistically significant (p-value < 0.05). The highest yield was observed by boil cell method. Out of 4 samples, 2 were positive for streptococci in PCR identification. Sample 2 was identified to contain erm(B) and tet(M) was not found. Some probiotics have been reported to harbour antibiotic resistance genes which can be transferred to pathogens in the gut. Therefore probiotic bacteria used in the food industry should be screened for resistance genes in order to ensure safety of the consumers.

Keywords: Streptococcus, Probiotics, Antibiotic Resistance

INTRODUCTION

Fermented olives, also known as table olives are consumed worldwide, especially in the Mediterranean countries. These olives has a great impact on the worldwide economy, since they have high nutritional content including vitamins, fibers, unsaturated fatty acids, amino acids and antioxidants (Moumita et al., 2018). These nutrients make olives a significant functional food in the meal. Moreover, the fermentation along with preservation eventually enhances the technological plus nutritional characteristics with the health benefits (Guantario et al., 2018).

The most frequently used and important functional food compounds are probiotics. These are various living bacteria that, when ingested in sufficient amounts, presents advantages on the host health. It is a part of the microbiota due to their beneficial health effects and hence it has been used for centuries (Xiao et al., 2017). Probiotic foods contain live microbes in an adequate amount to reach the intestine (Bron and Kleerebezem, 2018). They provide the balance of host intestinal microflora by the stimulation of the helpful microorganisms and the reduction of harmful bacteria (Varcamonti et al.,

2006). To express the health benefits, probiotic should contain at least 106–107 colony forming units/g in the food which is consumed.

There are number of requirements to select particular probiotic strain, which is safe for the host and it is important to assure safety, even in the middle of bacteria that are in generally recognized as safe (GRAS). Lactic acid bacteria (LAB) have a significant attention as probiotics over past few years. Mostly, LAB consists of rod-shaped microorganisms such as Lactobacilli, Bifidobacterium and cocci such as Streptococci, Lactococci, (Ben-Yahia et al., 2012) and Enterococci. These bacteria exist in the human gut flora and can also be taken orally as they are available in food products such as fermented vegetables and dairy products. The most important properties of probiotic include: gastric and bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria or fungi (Pu et al., 2017), bile salt hydrolase activity and the enhancing viability (Hummel et al.,2006). It should be stable during processing and storage and predominantly it should be safe to consume. Prevention of diarrhea, constipation, improvement of the anti-bacterial activity, changes in bile salt conjugation and anti-inflammation are several health benefits associated with probiotics (Schmitter et al., 2018)

Streptococcus is a gram-positive spherical bacteria that belongs to family Streptococcaceae, order Lactobacillus also known as Lactic acid bacteria, and belongs to phylum Firmicutes. Under microscopic identification of Streptococcus spp. can grow as pairs or chains that may appear bent or twisted since cell division in streptococcus arises along a single axis therefore the bacteria is able to form chains and pairs (Evivie et al., 2017). Most of the streptococci are oxidase-negative and catalase- negative and many are facultative anaerobes who are capable of growing under both aerobic and anaerobic conditions. Streptococcus thermophilus is one most important probiotic strains and also known as Streptococcus salivarius (Fontaine et al., 2009). Streptococcus thermophilus is a thermophilic LAB with key importance for the dairy production where strains of the species are generally used in manufacturing many cheeses varieties and yoghurt (Flórez and Mayo, 2017).

Antibiotic resistance (ABR) has become a most vital threat to the general communities' health (Devirgiliis, Barile and Perozzi, 2011). ABR bacteria can cause diseases which lead to dangerous infections and sometimes even death. It has become a difficult task to kill ABR bacteria and more it is costly to treat as well. There are few ways that bacteria can develop and acquire resistance to antibiotics; "neutralize" the antibiotic by altering it in a way that makes it harmless (De Vries et al., 2009), pumping antibiotics to the outer surface of the bacteria before it can do any harm (Ishihara et al., 2013), changing their outer structure by blocking binding sites and etc. After being exposed to antibiotics, sometimes even one bacteria can survive as it finds a way to counter attack the antibiotic and becomes resistant giving rise to a number of bacteria which can replace the dead bacteria (Gueimonde et al., 2013). After exposure by the selective pressure obtained from antibiotics the bacteria can become resistant or it can become resistant during a mutation of their genetic material (Villavicencio et al., 2018; Tsuyuki et al., 2017; Rizzotti et al., 2009) as shown in Figure 1.

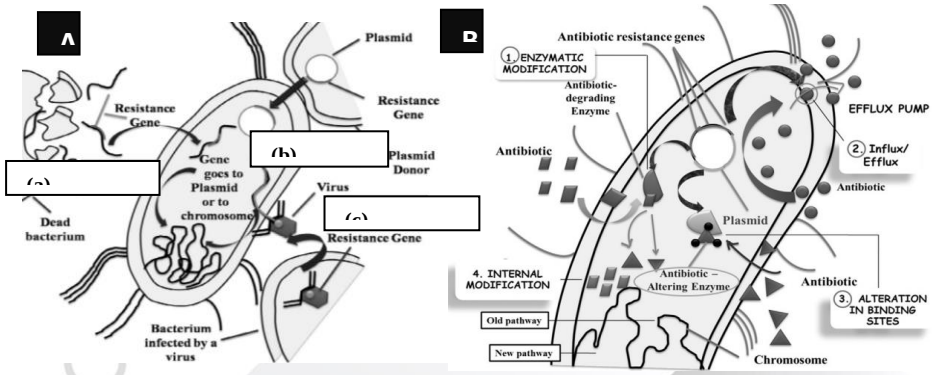

Figure 1. Mechanisms of antibiotic resistance in probiotics. (A) Intrinsic antibiotic resistance 1) efflux pumps, 2) antibiotic degrading enzyme, 3) antibiotic altering enzyme and 4) Inner change (B) Acquired antibiotic resistance (a) transformation, (b) conjugation, and (c) transduction (Sharma et al., 2014).

Antibiotic resistance of streptococcus can be determined by minimal inhibitory concentration (MIC) and disk diffusion assay (Brenciani et al., 2103). In addition, the presence of resistance gene(s) can be evaluated by molecular methods identification of streptococcus spp. using PCR amplification which is considered as a gold standard method and to identify antibiotic resistance the most frequent used resistance genes are tet(M) and erm(B). Molecular methods are conventional, specific and efficient over culturing methods, other than that the consumed time is lesser than the culturing methods. Antibiotic resistance has become an urgent threat to public health and despite the health benefits some probiotics have reported to harbor resistance and these can be transferred to other bacteria. The Purpose of this study was to identify probiotic genus of Streptococcus in fermented olives samples and analyze their antibiotic resistance. Therefore, it can be prevented and probiotics can be safely engineered to commercialize and to be used for next generation.

METHODOLOGY

Sample preparation

Five international brand of fermented olives were purchased from local market. Five separate beakers were taken and labeled from 1 to 5. Followed by transferring of the different samples (one olive and 5 ml of brine samples) into their respective beakers in equal amounts.

Culturing of the Streptococcus sample in MRS agar

Aseptic conditions were followed throughout the procedure. Using a sterile inoculation loop, the sample (crashed olive and brine mixture) was picked. It was streaked on MRS media using the four quadrant streak plate method. After streaking, all five petri plates were parafilmed and placed in the incubator. It was incubated in 37°Cfor 2 days.

Biochemical Tests to identify streptococcus

Gram staining of the cultivated colonies from the MRS agar

Initially, the bacterial smear was prepared with the specific colony picked and placed on a clean glass slide and was allowed to air dry and fixed by flaming. Then a drop of crystal violet was added and was allowed to dry for 30 - 60 seconds. The excess, was washed out. Thereafter, a drop of Grams iodine was added to the slide and it was allowed to air dry for 1 – 2 minutes. The excess amount

of iodine was washed out. Grams decolourizer (10 – 30 secs) was added to wash off excess stain followed by the addition of safranin (10 – 30 secs). The slide was washed with water and allowed to dry. A cover slip was placed and observed under the microscope (10x, 40x and but most importantly 100x (oil immersion)).

Catalase test to identify anaerobic bacteria

Aseptic conditions were followed throughout the procedure. Using a sterile inoculation loop, the colony sample from the previously cultured plate was picked and mixed with a water drop which was on the slide and a drop of H2O2 was added.

Sub culture of the Streptococcus pure colonies in MRS agar

Aseptic conditions were followed throughout the procedure. Using a sterile inoculation loop, the colony sample from the previously cultured plate was picked. It was streaked on MRS media using the four quadrant streak plate method. After streaking, all four petri plates were parafilmed and placed in the incubator. It was incubated in 37°C and 45oC for 24 hours.

DNA extraction of the cultivated colonies from the MRS agar

Heat shock DNA extraction method

Aseptic conditions were followed throughout the procedure. A 2 ml of autoclaved distilled water was added to each labelled falcon tube (1-4) followed by an inoculation of sufficient amount of the selected colonies into tube. The falcon tubes were then centrifuged at 4000rpm for 20 minutes. 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.

Promega kit based extraction method

The DNA extraction procedure was carried out by promega kit method. In the initial step bacterial colonies were scraped and mixed with it 2 ml of autoclaved distilled water which was in eppendorf tube and centrifuge at 7000rpm for 4 minutes and water was removed.it was suspended in 480µl of 50mM EDTA and 120µl of lytic enzyme was added was added. Then incubated at 37oC for 30-60 Minutes, after that centrifuged the tubes in 7000rpm for 4 minutes add supernatant was removed. Then 600µl of nuclei lysis solution was added into the pallet contained eppendorf tube and gently mixed with pipette. Then the samples were incubated in the water bath at 800C for 5minutes. After that samples were let to cool down at room temperature. Here after 3µl of RNase solution was added and mixed. In the next step, samples were incubated again in the water bath at 370C for 45 minutes and kept in the room temperature to cool down. After this the samples were transferred into the labelled eppendorfs tubes using pipettes. 200µl of protein precipitation solution was added, and then samples were vortexed for 5-10 seconds. Then placed into ice and incubated for 5 minutes and then samples were centrifuged at 7,000 rpm for 6 minutes. Next the supernant of the samples were transferred in to the clean labelled eppendoff tubes which consisted with 600µl of isoproponal, and samples were mixed well with tapping and subsequently the samples were centrifuged at 7,000 rpm for 4 minutes. Here after the supernant of the samples were removed and 600µl of 70% ethanol was added into the samples at room temperature, consequently samples were mixed well by tapping. And then the centrifugation procedure at 7,000rpm for 4 minutes. Following that ethanol was aspirated via discarding form the samples, and afterwards the samples were placed to

air dry for 15-20 minutes. Once this procedure done, 100µl of DNA Rehydration solution was added into the samples to rehydrate the DNA pallet and subsequently the samples were stored at 40C temperature for further analysis.

Quantification of DNA from bacterial DNA isolated from Boiled cell and Kit based extraction methods using spectrophotometer

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 was calculated. These readings were taken for each of the 5 samples (Sample 1 – 5).initially Absorbance of TE was measured since it was been used as the blank.

Following the calculation of the DNA concentration and yield (Refer appendix B), the ratios (260/280) and (260/230) were calculated for both extraction methods to evaluate the purity of DNA. Equations used are mentioned below and these were adapted from a study conducted by Abdulamir et al., (2010):

Dilalution factor=(TE Used as Blank(3000µl)+Amount of DNA sample added(30µl))/(Amount of DNA sample added (30µl))

DNA concentration (n⟦gµl⟧^(-1))=(A260 in OD units)×⟦50µgml⟧^(-1)×Dialution Factor

DNA yield (µg)=DNA concentration(⟦µgµl⟧^(-1))×Amount of DNA kept as a stock

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

Statistical analysis was performed using one way ANOVA analysis in SPSS Statistics 21 software. This was done to find if there is any significance (p value <0.05) or there is no significance (p value >0.05) between the DNA yield when comparing it to the sample and the extraction method.

Identification of Streptococcus by Polymerase Chain Reaction (PCR)

Streptococcus was identified using genus specific primers (Table 1), Volumes and reagents used are included in Table 2. PCR cyclic conditions are given in Table 5 (Prabhu et al., 2012) PCR was performed using DNA extracts from both extraction methods.

Table 1. Streptococcus genus specific primer sequences and product size

Target Organism	Primer Set	Sequence (5' to 3')	Product size	Reference
Streptococcus genus	Forward primer	CAA CTT GAC GAA GGT CCT GCA	110	Prabhu *et al.*, (2012)
	Reverse primer	TGG GTT GAT TGA ACC TGG TTT A		

Table 2. Components of the PCR reaction mixture and their specific working concentrations

Reagents	Stock Conc.	Working Conc.	Volume for one reaction (µl)	Volume for 6 reactions (µl)
PCR buffer	5X	1X	5	30
dNTP	10Mm	0.2 Mm	0.5	3.0
Forward Primer	2 µM	0.2 µM	2.5	15
Reverse Primer	2 µM	0.2 µM	2.5	15
MgCl2	25 mM	1.5 mM	1.5	9.0
Taq Polymerase	5 U/ µl	0.05 U/ µl	0.25	1.5
DNA	-	-	100 ng/µl	-
Autoclaved distilled H20	-	-	-	-
Total volume			25	73.5

The amount of DNA and autoclaved distilled water added for each sample was given in Table 3 and Table 4 since the DNA amount added vary from sample to sample.

Initially the PCR mastermix was prepared consisting of PCR buffer, dNTP, Forward primer, Reverse primer, MgCl2 and Taq polymerase. The mastermix was prepared for 7 reactions (5 samples + negative control + extra reaction). Followed by the aliquotion of the PCR master mix (12.25 µl) to each tube. Amount of DNA was calculated by below mentioned equations:

Amount of DNA = DNA working conc. (100ng/ µl) / DNA concentration

Amount of Water = Total volume (25 µl) – (12.25 + Amount of DNA)

Table 3. Amount of DNA and Water need for each sample for the PCR reaction for boiled cell method

Sample	Amount of PCR Mater mix added(µl)	Amount of DNA added(µl)	Amount of water added(µl)
Negative control	12.25	-	12.75
Sample 1	12.25	0.7	12.05
Sample 2	12.25	2.0	10.75
Sample 3	12.25	0.3	12.45
Sample 4	12.25	0.3	12.45

Table 4. Amount of DNA and Water need for each sample for the PCR reaction for Kit Based extraction method

Sample	Amount of PCR Mater mix added(µl)	Amount of DNA added(µl)	Amount of water added(µl)
Negative control	12.25	-	12.75
Sample 1	12.25	2.5	10.25
Sample 2	12.25	2.1	10.65
Sample 3	12.25	4.3	8.45
Sample 4	12.25	8.5	4.25

PCR cyclic parameters for Heat Shock and Kit based extraction methods

Table 5. PCR cyclic parameters for Heat Shock and kit based extraction PCR procedure

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		

Step	Temperature (°C)	Time/min	Cycles
Initial denaturation	95	5	
Denaturation	95	1	35
Annealing	49.5	1	
Extension	72	2	
Final Extension	72	12	
Final hold	4		

PCR for the identification of resistance for tetracycline and erythromycin towards Streptococcus isolates

PCR was performed for the Streptococcus positive samples using both tet(M) (tetracycline) and erm(B) (erythromycin) resistant genes specific primers separately according to Table 6. The same PCR components (Table 2.) were used in both of these PCR procedures but the number of reactions for each including the amount of DNA and water added were calculate and according to Table 7 for tet(M) and erm(B) respectively. But the primers used are different including the cyclic parameters (Gad, Abdel-Hamid and Farag, 2014).

Table 6. 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

Table 7. Amount of DNA and Water need for each sample for the PCR reaction for Kit Based extraction method.

Methods	Sample	Amount of PCR Mater mix added(µl)	Amount of DNA added(µl)	Amount of water added(µl)
Boil cell method	Negative control	12.25	-	12.75
	Sample 2	12.25	2.0	10.75
	Sample 4	12.25	0.3	12.45
Kit Based extraction	Negative control	12.25	-	12.75
	Sample 2	12.25	2.1	10.65
	Sample 4	12.25	8.5	4.25

The separate cyclic parameters published by Gad, Abdel-Hamid and Farag, 2014 are given below in Table 8 and Table 9.

Table 8. 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 9. 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		

Detection by agarose gel electrophoresis

With aseptic conditions, 2% agarose gel was prepared and 8µl of each samples and negative control was loaded onto the wells followed by 2µl of the DNA ladder (100bp). Then the gel was run at 45V for 15 minutes and 55V for 45 minutes. This protocol was followed for all PCR related methods in this stud

RESULTS

Morphological identification of Streptococcus growth on MRS media

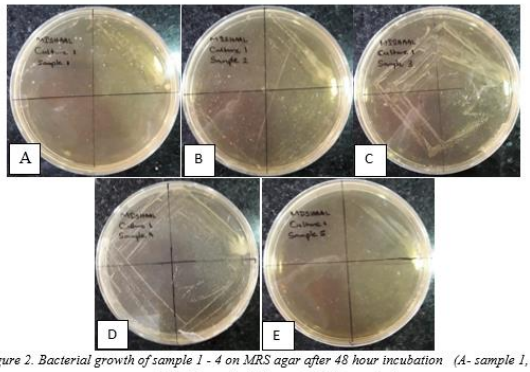

Figure 2. Bacterial growth of sample 1 - 4 on MRS agar after 48 hour incubation (A- sample 1, B-sample 2, C-sample 3, D-sample 4, E-sample 5)

As shown in Figure 2 Creamy colour, circular, relatively small colonies were observed in MRS agar. Less growth were observed in A and B and in C and D the growth was higher. There were no colonies observed in sample 5 and there were no contaminations present in any agar media.

Gram staining results of Streptococcus culture

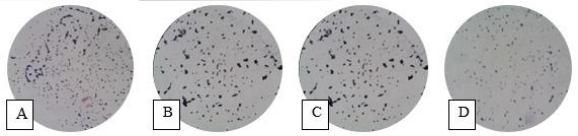

Figure 3. Bacterial identification by Gram staining images of sample 1 - 4 under 100X magnification (A- sample 1, B-sample 2, C-sample 3, D-sample 4)

The isolated bacteria shown in Figure 3 were purple in colour (Gram positive) cocci bacteria, there were cocci chains, diplo cocci and mostly single cocci in the smear

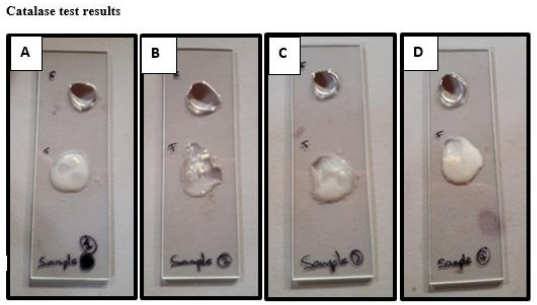

Figure 4. Catalase test results of all the sample A- sample 1, B-sample 2, C-sample 3, and D-sample 4. All the samples showed negative results for catalase test.

According to Figure 4. there were no bubbles present in all the samples in the presence of H2O2 and it confirms all the samples contain anaerobes. Furthermore, it confirms there is no Staphylococcus present in the samples.

Morphological identification of Streptococcus Sub culturing pure colonies in MRS agar

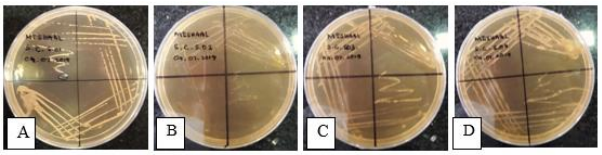

Figure 5. Streptococcus sub culture growth of sample 1-4 on MRS agar

As shown in ***Figure 5*** Creamy colour, relatively small and circular colonies in high numbers of growth were observed in MRS media.

Subculture growth under different temperatures

Table 10. Subculture growth under 37ºC and 45ºC for 24 hour incubation time

Table 10. Subculture growth under 37ºC and 45ºC for 24 hour incubation time

Sample	Growth at 37°C	Growth at 45°C
S1	✓✓	✓
S2	✓✓	✓✓
S3	✓✓✓	✓
S4	✓✓✓	(-)

No growth - (-), Less growth - ✓, Normal growth - ✓✓, High growth -✓✓✓

With the results mentioned in Table 10 at 37oC growth was observed in 4 samples and only 3 samples were grown in 45oC incubation specifically sample 4 didn't have any growth but streptococcus optimum temperature is between 37oC – 45oC. Sample 3 and sample 4 has maximum growth at 37oC.

Spectrophotometer results for DNA quantification

Table 11. Mean absorbance values for boiled cell method.

Sample	Mean Absorbance at 230nm	Mean Absorbance at 260nm	Mean Absorbance at 280nm
Sample 1	0.0173±0.0006	0.0287±0.0006	0.0137±0.0006
Sample 2	0.0070±0.0010	0.0100±0.0000	0.0053±0.0006
Sample 3	0.0403±0.0006	0.0767±0.0006	0.0420±0.0000
Sample 4	0.0437±0.0006	0.0763±0.0006	0.0390±0.0000

Table 12. Mean absorbance values for kit based extractions.

Sample	Mean Absorbance at 230nm	Mean Absorbance at 260nm	Mean Absorbance at 280nm
Sample 1	0.0047±0.0006	0.0080±0.0000	0.0043±0.0006
Sample 2	0.0053±0.0006	0.0093±0.0006	0.0050±0.0000
Sample 3	0.0030±0.0000	0.0047±0.0012	0.0020±0.0010
Sample 4	0.0013±0.0006	0.0023±0.0006	0.0010±0.0000

According to Table 11 and Table 12 Mean absorbance at 230nm, 260nm, 280nm sample 1-4 in Boil cell method is higher and Kit based method shows less values compared to boil cell method. Highest values are from sample1, 3 and 4 in boil cell method.

Table 13. Mean concentration values for both boil cell and kit based extractions methods.

Sample	Mean DNA Concentrations At 260nm ($ngul^{-1}$) Boil cell method	Mean DNA Concentrations At 260nm ($ngul^{-1}$) Kit based extractions
Sample 1	144.77±2.915619	40.40±0
Sample 2	50.50±0	47.13±2.915619
Sample 3	387.17±2.915619	23.57±5.831238
Sample 4	385.48±2.915619	11.78±2.915619

According to Table 13 Mean concentration values are higher in sample 1, 3 and 4 boil cell method and in kit based method comparatively sample 2 has the higher value. When comparing both methods all the DNA Concentrations values are higher in boil cell method which are higher than kit based method.

Table 14. Table depicting the DNA purity ratios for Boiled cell and kit based extraction methods.

Boil cell extraction method			Kit based extraction method		
Sample	260/230 Mean Absorbance ratio	260/280 Mean Absorbance ratio	Sample	260/230 Mean Absorbance ratio	260/280 Mean Absorbance ratio
Sample 1	1.65±0.048	2.10±0.118	Sample 1	1.71±0.231	1.85±0.231
Sample 2	1.43±0.209	1.88±0.192	Sample 2	1.75±0.252	1.87±0.115
Sample 3	1.90±0.023	1.83±0.014	Sample 3	1.56±0.385	2.33±1.155
Sample 4	1.75±0.020	1.96±0.015	Sample 4	1.75±1.000	2.33±0.577
Standard values	2.0-2.2	1.8 – 2.0	Standard values	2.0-2.2	1.8

If the 260/280 ratio value is ≥ 1.8 it is consider as pure DNA if its < 1.8 it can be concluded that there is a protein present in the DNA sample, if the ratio is ≥ 2.0 it confirms the presence of RNA in the sample. If the 260/230 ratio is ≥ 2.0-2.2 its considered as pure if its less the value its due to the presence of a organic solvent in the sample. With regard to these information and according to Table 14 all the 260/280 ratio value in boil cell and kit based extraction are above the standard therefore it is considered as pure. But in

boil cell sample 1 and kit based extractions sample 3 and 4 ratio values are > 2.0, this confirms the presence of RNA in the sample. All the 260/230 ratio values are lower than the standard value therefore it confirms the presence of organic solvents.

Statistical analysis using one-way **ANOVA**

Table 15. Comparison of DNA yield between methods and brand(sample)

Tests of Between-Subjects Effects

Dependent Variable: DNA yield

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	476227628.156[a]	7	68032518.308	6467.104	.000
Intercept	407793582.094	1	407793582.094	38764.455	.000
Method	236225513.344	1	236225513.344	22455.364	.000
Brand	94481980.781	3	31493993.594	2993.788	.000
Method * Brand	145520134.031	3	48506711.344	4611.000	.000
Error	168316.500	16	10519.781		
Total	884189526.750	24			
Corrected Total	476395944.656	23			

a. R Squared = 1.000 (Adjusted R Squared = .999)

With respect to the Table 15 when comparing DNA yield between method and brand (sample) individually and both method and brand (sample) together all has a p – value less than 0.05(0.000 for all) respectively indicating there is a statistically significant difference between the DNA yields.

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
S1	S2	1186.7500*	59.21650	.000	1017.3304	1356.1696
	S3	-3383.5000*	59.21650	.000	-3552.9196	-3214.0804
	S4	-3181.5000*	59.21650	.000	-3350.9196	-3012.0804
S2	S1	-1186.7500*	59.21650	.000	-1356.1696	-1017.3304
	S3	-4570.2500*	59.21650	.000	-4739.6696	-4400.8304
	S4	-4368.2500*	59.21650	.000	-4537.6696	-4198.8304
S3	S1	3383.5000*	59.21650	.000	3214.0804	3552.9196
	S2	4570.2500*	59.21650	.000	4400.8304	4739.6696
	S4	202.0000*	59.21650	.017	32.5804	371.4196
S4	S1	3181.5000*	59.21650	.000	3012.0804	3350.9196
	S2	4368.2500*	59.21650	.000	4198.8304	4537.6696
	S3	-202.0000*	59.21650	.017	-371.4196	-32.5804

Based on observed means.

The error term is Mean Square(Error) = 10519.781.

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

According to Table 16 when comparing DNA yield between brands (samples) together all has a p – value less than 0.05 respectively indicating there is a significant difference between the DNA yields obtained from different samples used.

Comparison of DNA yields

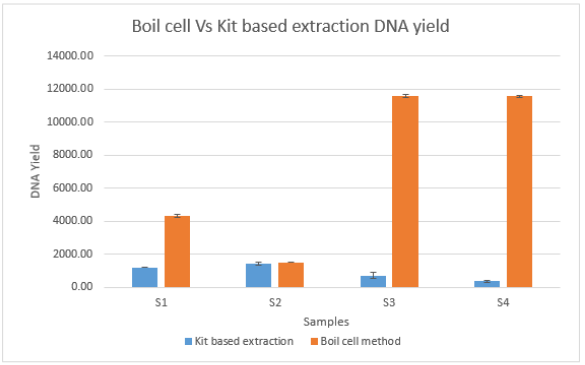

Figure 6. Comparison between two extraction methods using DNA yield

As shown in Figure 6 Boil cell methods show high DNA yield in sample 3 & 4. Generally, boil cell method gave highest DNA yield compared to kit based method.

Identification of Streptococcus with polymerase chain reaction using DNA extracts from Boil cell method and kit based extraction methods

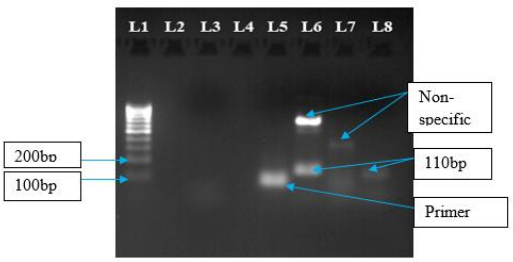

Figure 7. Amplified PCR products of Sample 1-4 of boil cell method to identify Streptococcus in 2% agarose gel. In Lane 1-100bp ladder, Lane 3- negative control, Lane 5- sample 1, Lane 6- sample 2, Lane 7- sample 3 and Lane 8- shows sample 4.

According to Figure 7 110bp pair length Products were observed in sample 2 and 4. Therefore, sample 2 and 4 contains Streptococcus. Negative control was not contaminated. Non-specific bands were observed on sample 3 and 4, primer dimers were observed on sample 1.

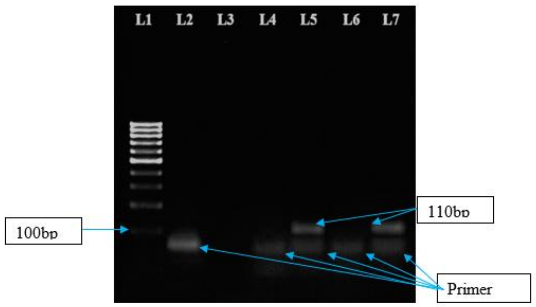

Figure 8. Amplified PCR products of Sample 1-4 of kit based extraction method to identify Streptococcus in 2% agarose gel. In Lane 1-100bp ladder, Lane 2- negative control, Lane 4- sample 1, Lane 5- sample 2, Lane 6- sample 3 and Lane 7- shows sample 4.

According to Figure 8 110bp pair length Products were observed in sample 2 and 4. Therefore, sample 2 and 4 contains Streptococcus. Negative control was not contaminated. Only primer dimers were also observed on all the samples.

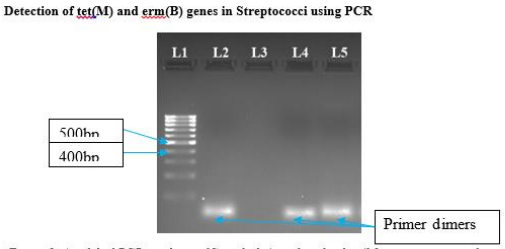

Figure 9. Amplified PCR products of Sample 1-4 to identify of tet(M) gene using specific primer in 2% agarose gel. In Lane 1-100bp ladder, Lane 2- negative control, Lane 4- sample 2 and Lane 5- shows sample 4

According to Figure 9. 401bp pair length Products were not observed in sample 2 and 4. Therefore, sample 2 and 4 does not contains tet(M). Negative control was not contaminated. Only primer dimers were observed in both the samples.

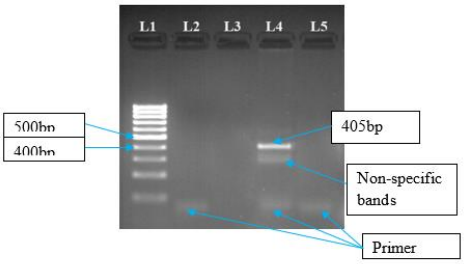

Figure 10. Amplified PCR products of Sample 1-4 to identify of erm(B) gene using specific primer in 2% agarose gel. In Lane 1-100bp ladder, Lane 2- negative control, Lane 4- sample 2 and Lane 5- shows sample 4

According to Figure 10 405bp pair length Products were observed in sample 2. Therefore, sample 2 contains erm(B) and sample 4 did not have a band and its negative for erm(B). Negative control was not contaminated. A non-specific band was observed in sample 2 and primer dimers were observed.

DISCUSSION

Lactobacillus spp. and Bifidobacterium spp. are the two main types of probiotis, isolated mainly from dairy products. The present study was focused to identify Streptococcus spp. by its probiotic characteristics and to observe its antibiotic resistance. Table olives are a natural source of probiotic bacteria and are well known for their health promoting useful products (Bonatsou et al., 2017). In the previous studies, streptococcus was grown in M17 agar (Yang et al., 2018; Guevarra and Barraquio, 2015; Collado and Hernández 2006; Mater et al., 2005), but in this study De Man Rogosa and Sharpe (MRS Agar) was used instead and colony growth was observed. The purpose of using MRS agar was to identify the presence of other probiotic bacteria in fermented olive and to find Streptococcus to subculture, and 4 out of 5 samples had a growth. Sample 5 did not show a growth, suggesting there is no lactic acid bacteria

present in the sample. Bacterial growth of the samples were creamy and opaque in colour, the colonies were relatively small and circular in appearance as shown in (Figure 2). The colonies obtained had the same characteristic for Streptococcus colony appearances in study conducted by Guevarra and Barraquio in 2015; hence further tests were carried out in 3 different stages to confirm the presence of Streptococcus. Gram staining followed by the catalase test and growth under different temperatures was tested to identify Streptococcus spp. in the samples. Since sample 5did not have any colony growth, further tests were not performed on the particular sample. Gram staining of all the samples (Figure 3) showed gram positive coccus only and mainly chains, pairs and single cocci and fewer clusters were also observed, in dark purple colour.

The intention of the Catalase test is to find the action of catalase enzyme that transforms hydrogen peroxide into H2O and oxygen. Usually aerobic or facultative anaerobic bacteria contain this enzyme, thus the Catalase test was carried out with the intention of finding the presence of catalase enzyme in bacteria (Iwase et al., 2013). The samples were found to be catalase negative and it confirms that all the bacteria present are anaerobes as shown in Figure 4 and there is no Staphylococcus growth in the samples. With this relevant information sub culturing was carried out in MRS agar plates and the agar plates had good yield of bacteria. Pure colonies were sub cultured in MRS agar and incubated under two temperatures respectively 37oC and 45oC. As shown in Table 10, only 3 samples had growth in 45oC which had only cream colour colonies while the sample 4 did not grow under 45°C, and all the 4 samples had growth under 37oC where the sample 1 had creamy and opaque colour colonies while the rest were all cream colour. Similar results were shown in a study carried out by Gad,Humaid and Farag (2014).Streptococcus will grow in 37oC and 45oC but the study by Radke-Mitchell and Sandine, (1986) have shown the growth between 30oC and 42oC and maximum growth at 43oC-46oC. Lactococcus have growth in 15oC and 37oC with related to this sample 4 can be Streptococcus or no growth can be due to a growth inhibition.

For further confirmations, Polymerase Chain Reaction (PCR) was used. DNA extraction was carried out in 2 different methods to obtain DNA for the process, namely boil cell extraction and Promega kit based extraction method out using the study Abdulamir et al., (2010).PCR requires a specific amount of DNA concentration for ideal performance. This helps to prevent unnecessary consumption, enhance reproducibility and amplification of the samples (Boesenberg-Smith, Pessarakli and Wolk, 2012). A study was done to find the accuracy of DNA concentration with DNA samples using spectrophotometric DNA quantification, and compared it with fluorometric quantification method. Spectrophotometric DNA quantification was the most precise method (Haque et al., 2003) and this was supported by a study carried out by Shokere, Holden and Jenkinsin (2008).

Absorbance at 230nm, 260nm and 280nm were obtained in triplet values using spectrophotometer and mean was calculated. All the samples, especially sample 3 and 4 had high values in Boil cell extraction absorbance values confirming the presence of high DNA yield. After thatA260/A230 ratio and A260/A280 ratio was calculated with the DNA absorbance mean and compared with the standard values of the ratios A260/A280 standard was 1.8 -2.0 (Desjardins and Conklin, 2010) or above values if it's less than that it confirms the presence of protein but all the values from both extraction methods had values passing standard values

showing higher values and but values obtained by boil cell method had purity. Sample 1 in boil cell method and sample 3 and 4 in kit based method had above A260/A280 ratio above 2.0 and it confirms the presence of RNA. A260/A230 ratio standard is above 2.0 -2.2 (Aphale and Kulkarni, 2018) whereas no sample had that value, thus confirms the presence of organic solvents. According to the graph shown in (Figure 6) boil cell method has a higher DNA yield compared to Promega kit based extraction method.

the study carried out by Abdulamir et al., in (2010) shows higher purity and DNA yield was obtained from Promege kit method compared to boil cell method but in another study done by Peng et al., (2013) confirms boil cells method has similar DNA yield like other kits methods.

According to SPSS data suing one way ANOVA results in the Table 15 comparing DNA yield between method and brand (sample) and both method and brand (sample) together all has a p – value less than 0.05(0.000 for all) respectively indicating there is a significant difference between the DNA yields of brand and methods because the yield obtained from boil cell method is higher than Promege kit based method. With relevant to the data in Table 16 DNA yield between brands (samples) together all has a p – value less than 0.05 respectively indicating there is a significant difference between the DNA yields and samples used since the bacteria concentration available in each sample is different from one other.

In order to further confirm the presence of Streptococcus spp. and to identify, molecular based techniques were used along with extracted quantified DNA and genus specific primers for streptococcus. According to the study carried out by Prabhu et al., (2012) expected amplicon size is 110bp.Nevertheless, with the lower values of A260/A230 ratios and proper A260/A280ratios positive bands were observed between 100bp and 200bp in sample 2 and 4 in both boil cell and Promega kit based extraction methods. Nonspecific binding was observed in boil cell method and not in kit based method. The annealing temperature of the primers should be optimized to prevent the formation of primer dimers. In addition there were no bands observed in negative control and can be concluded there is no contaminations (Ali et al., 2014). A study was carried out by Preethirani et al., (2015) using same primer sequence (tuf) to identify Streptococcus and out of 5 samples all gave positives and there were no primer dimers present, the only difference was annealing temperature they used which was 50oC.

PCR was performed to detect the presence of tet(M) and erm(B) in sample 2 and 4.Molecular methods are more sensitive and reliable compared to other conventional methods and it is less time consuming as well, and that is why molecular methods were utilized in the present study to confirm the results (Wong et al., 2015). As shown in (Figure 9) no band was observed at 400bp level and it confirms all the samples are negative for tet(M) gene and since negative control did not show a band it can be concluded that the reagents and samples are not contaminated. Since there was a band which is 400bp in sample 2 (Figure 10) that is the only sample which is positive for erm(B) while sample 4 was negative for erm(B) gene and the negative control wasn't contaminated. With these results it can be concluded that Streptococcus can have antibiotic resistance for Erythromycin (Zheng et al., 2017).

This study demonstrated that presence of Streptococcus as a probiotic in olive samples and its antibiotic resistance. Out of all the 5 samples, sample 2 and 4 were streptococcus positive and sample 2 was resistant for erythromycin and susceptible for tetracycline. Antibiotic resistance has become urgent threat to public health and despite the health benefits some probiotics

have reported to harbour resistance and these can be transferred to other bacteria. In order to prevent these it should be safely engineered to commercialize and to be used for next generation.

CONCLUSION

This study aimed to isolate Streptococcus from table olive samples. The boil-cell method was proved to be a better extraction procedure since it produced a greater DNA yield compared to kit-based method. PCR amplification indicated the presence of Streptococci bacteria, further confirming the gram staining results. The detection of tet(M) and erm(B) genes using PCR showed positive results for the sample 2, confirming the presence of erm(B) gene. However, none of the samples possessed the tet(M) gene. Further analysis of other resistant genes are required as there is a possibility of resistant genes to transfer and pose a threat to the human health.

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