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Probiotic Potentials and Antibiotic Susceptibility of a Yoghurt Analogue from a Mixture of 3-Plants Water Extracts.

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Abstract Yoghurt is one of the best sources of probiotics and its importance to the human gastrointestinal system provides a perfect food matrix for transporting probiotics to the body. Unfortunately, animal milk dominates the typical commercial yoghurt; hence, the need to produce probiotic yoghurt from plant milk. This paper has outlined a new yoghurt analogue, made from a mixture of plant-based materials and analyzed their probiotic potentials, microbial assay and Antibiotic susceptibility. The yoghurt analogue was made from tigernuts, coconut and dates water extracts inoculated with strains of Lactobacillus delbruekii subsp. bulgaricus and Streptococcus thermophillus isolated from commercial dairy yoghurts. Probiotics analysis of Yoghurt from vegetable milk showed that the acid tolerance test on the fermentation organisms- Lactobacillus bulgaricus (Strain 3) and Streptococcus thermophillus (Strain 1) had a survival rate at pH. 2.8; tolerance for Bile salt was positive (0.272 and 0.462) for up to 2% bile concentration. Results from the Salt tolerance test showed values of 0.636 at 1% concentration and 0.017 at 9% concentration for Lactobacillus bulgaricus (strain 3), in Strain 1, values reduced from 0.739 to 0.032. Positive results showed survival on known salt concentrations. The full growth of the yoghurt analogue on the antibiotics disc showed resistance to the antibiotics. Antibiotic drugs did not inhibit the growth of the probiotics in the newly developed yoghurt. There was greater zone of inhibition measuring 21.5 mm and 16.0 mm around disk with Escherichia coli; 15.0 mm and 11.0 mm for Shigella dysentriae and no significant inhibition for Staphylococcus aerus as it proved resistant to the plant yoghurt. The strains alone showed similar trend, 20.5mm and 10.0 mm for strains 01 and 03 on E. coli and 7.0 mm and 11.0 mm on Shigella dysentriae, while Staphylococcus aerus showed resistance. The finding of this study affirms that the plant yoghurt is a probiotic yoghurt analogue that can inhibit the growth of some pathogens and resist antibiotic effects when consumed.

Keywords: *Antibiotic sensitivity, pathogenic bacteria, yoghurt analogue*

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1. Introduction

Yoghurt is a fermented product usually obtained by starter culture activities (*Streptococcus thermophilus and Lactobacillus delbrueckii, L. bulgaricus spp.*) in milk. It produces a characteristic and desirable flavor and aroma [1]. The fermentation process of yoghurt usually improves the milk's keeping quality due to lowered pH and lactic acid formation. It improves the nutritive, therapeutic, and prophylactic effects on humans. Milk-like extracts from plants and their products like yoghurt are becoming a preferred choice to consumers as a thirst-quenching beverage, lifestyle change, or functional food due to the presence of beneficial health ingredients [2]. There have been studies on the possibility of yoghurt production using plant milk like coconut [3,4], soybean [5], and tiger nut [6].

'Kunnu aya' is a typical refreshing beverage in Nigeria made from a mixture of tiger nuts, coconuts, and dates. It has a milky appearance, and pleasant flavor, usually has no alcoholic content and a liquid with low viscosity and sweet taste [7,8]. Nigerian open markets hawk with no standard ratio tiger nuts, coconuts, and dates blends, hence consumers get varied tastes with each purchase and individual vendor. The low keeping quality of 'kunnu aya' aroused the interest in yoghurt production since fermentation provides an acidic environment that can prevent spoilage. All *Lactobacillus* genus are also known to have an inhibitory role on pathogenic bacteria [9].

Yoghurt is one of the best sources of probiotics. The importance of probiotics in the human gastrointestinal

system cannot be over-emphasized as yoghurt is a perfect food matrix for transporting probiotics to the body. Unfortunately, animal milk dominates the typical commercial yoghurt; hence, the need to fill a probiotic yoghurt gap from plant milk.

Lactic acid bacteria consisting of *Lactobacillus* species utilized in the fermentation process can serve a dual function by acting as agents for food fermentation and possibly impacting health benefits [9]. As there has been an increasing number of infections arising from excessive antibiotics consumption [10], the need to create antibiotics alternatives becomes important. Antibiotic-associated diarrhea (AAD) prevention and antibiotic healing has been reported from the ingestion of live probiotic strains of *Saccharomyces boulardii* or *Lactobercillus bulgarecus* GG) from Yoghurts [11]. Probiotics have also been reported to play a role in reducing the occurrence, duration, and severity of flu [12,13].

Yoghurt from plants can be successfully employed to tackle hidden hunger, protein-calorie malnutrition, and some other health benefits. Manufacturers can improve the sensory properties and consumer acceptability of the water extracts used in making yoghurt analogue by combining two or more plant materials to leverage on their different physicochemical and sensory properties [14]. In this study, the combination of tiger nuts, coconuts, and date fruits will increase the yoghurt's aesthetic and sensory value [15]. Thus improving the storage life of "kunnu aya" will provide consumers a better opportunity to appreciate the aphrodisiac claim on the product; as some consumers are unable to patronize the street vendors who sell kunnu aya due to lack of standardized production formula as well as food safety concerns.

Materials and method

2. Culture Preparation

Bacterial strains (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) were isolated and further confirmed using biochemical methods at the Sheda Science and Technology complex, Abuja, Nigeria. The bacterial strains were streaked severally after which a distinct colony was sub-cultured into a sterile MRS agar slant, incubated at 40^{o}C anaerobically for 18 hours. They were stored under sterile glycerol at 4^{o}C in the refrigerator for further use.

3. Preparation of Tiger Nut for Extraction

The method of Badau, *et al*., [5] was used with some slight modifications in the extraction of the tigernut milk. Tigernuts (200 g) were sorted and surface sterilized using 70% ethanol. The seeds were submerged in ethanol and placed in a stirrer for 15 minutes, subsequently rinsed using sterile water 4 to 5 times and drained with sterile cheese cloth, soaked in a stainless bowl with 400 ml sterile water inside a refrigerator at 4°C for 12 h. This treatment was done to prevent growth of fermentative organisms while softening the nuts for effective milling and extraction of the milk.

4. Preparation of Coconut for Extraction

The method of Edem and Elijah, [16] was used to prepare coconut for milk extraction with some modifications. To produce coconut milk, coconut heads were cracked by hitting the nut hard on the floor till opened and the coconut meat removed with a sharp knife. It was cut into smaller pieces to achieve size (4 mm by 2mm) reduction and easier blending, then the ratio in experimental design (see Table 3.1) was weighed out. Surface sterilization of the weighed coconut meat was done with 70% ethanol (at the ratio of 1:3 coconuts: ethanol) for 60 seconds and rinsed off with sterile water. This was to ensure that the coconut meat was sterile and does not absorb the ethanol.

5. Preparation of Dates Seed for extraction

The method of Nizar, *et al*., [17] was used with some modifications to produce date extract. To produce the date extract, various ratios shown in the experimental design of dried wholesome seeds were weighed, pitted and surface sterilized with 70% ethanol for 5 minutes after it was rinsed 3 to 4 times and soaked in 200ml of sterile water for 3h. They were stored in the refrigerator for further use.

6. Experimental Design for Production of the Plant Milk Blends from Tigernuts, Coconts and Dates.

The experiment was a mixture design that was carried out using a statistical software (Design Expert, version 8.7.0.1). The design type was Simplex Lattice. It generated 14 treatments. Each run (sample) contained the blend of the three components: coconut milk (A), tiger nut milk (B) and date extract (C). The mixture total for every sample was equal to 1.0.

$$\sum \text{coconut} + \text{tiger nut} + \text{dates} = 1.0 \quad \textit{(Eqn. 1)}$$

7. Production of Milk Blend from Tigernuts, Coconts and Dates

Production of the plant milk blend was according to the method of Odoom [18] with some modifications. The weighed tiger nut, coconut and date extracts (200g) were mixed in 14 bowls at different ratios following the experimental (mixture) design . Each mix was wet milled using exactly 200 ml of sterile water in a Stephan universal machine industrial blender (Type VCM 12, Hameln, Germany) to obtain the slurry, and then filtered with the use of a 1.19mm mesh size muslin cloth. Pressure was applied to the content during filtration so as to achieve maximum milk extraction. The filtrate was collected into close glassware and pasteurized at 70^{o}C for 30 min in a water bath. Pasteurized blend from the mix (tigernuts, coconuts and dates) was allowed to cool, then

poured into 14 sterilized bottles and labelled samples and kept separately for further analysis.

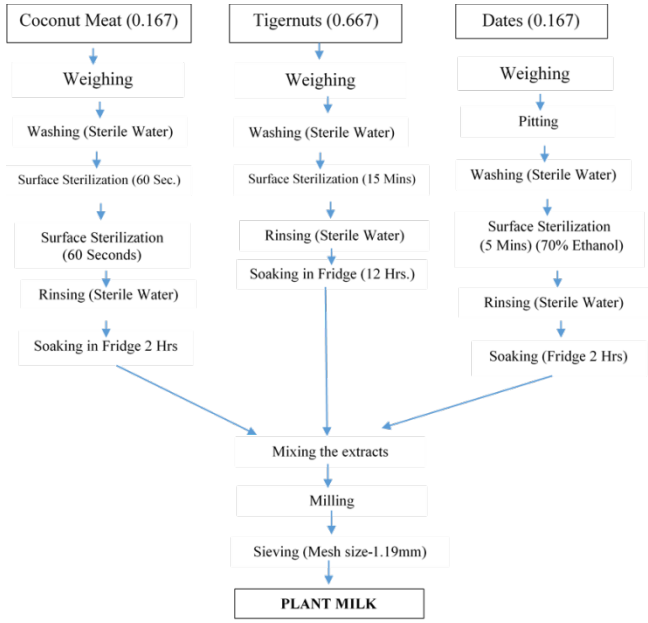

Figure 1. Production of Milk Blend from Tigernuts, Coconts and Dates

8. Production of the Probiotic Yoghurt

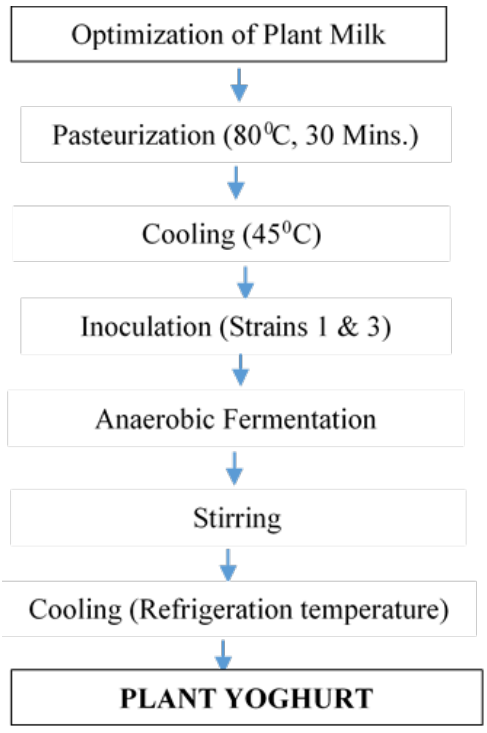

Figure 2. Production of the Probiotic Yoghurt from Plant based milk blend

The production of the yoghurt as shown in Figure 2 was carried out according to the procedure of Tamime and Robinson The plant based milk blend with the ratio 0.167 (Coconut), 0.667 (Tigernut), 0.167 (Date) was used in yoghurt production. The milk was freshly prepared and pasteurized at 70°C for 30 min, it was thereafter transferred into the biosafety cabinet and allowed to cool to 45°C before inoculating it with the isolated strains (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) confirmed during the preliminary investigation of this study at the chosen concentration, then transferred into the fermentation jar and incubated anaerobically at the corresponding temperature and time (Figure 2). The yoghurt produced was stored in a refrigerator at 4°C for sensory evaluation and further analyses.

9. Probiotics Analysis of Yoghurt from Plant Base Milk

Acid Tolerance Test

Adopting the methods of Tsai, *et al*. [19], 1 ml of Man, Rogosa and Sharpe (MRS) broth containing approximately 10^9 CFU/ml of LAB (*Lactobacillus bulgaricus:* Strain 3 and *Streptococcus thermophillus*: Strain 1) was transferred into 9 ml phosphate-buffered saline (PBS) (pH 2.0) and incubated at 37°C for 3 h. Viable bacteria were counted by plating serial dilutions on MRS agar. Bacterial acid tolerance was assessed by calculating the ratio of viable LAB cultured on MRS agar to surviving cells after incubation at pH 2.0 for 3h. Simultaneously, spectophotometer readings were also taken at 560 nm according to the methods outlined by Tsai, *et al*. [19].

Bile salt Tolerance Test

The method of Graciela and Maruia [20] was used to determine the growth rate of bacterial cultures. MRS broth containing different levels (0.05, 0.1, 0.15, 0.3 and 0.5%) of bile salts were used to determine this growth rate of the cultures. Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h under anaerobic condition. The optical density of each sample was measured using a spectrophotometer at 560nm.

Salt Tolerance Test

Using the method as outlined by Graciela and Maruia [20], isolated probiotic strains (*Lactobacillus bulgaricus and Streptococcus thermophiles*) used for the production of yoghurt were grown in MRS broth which were adjusted with different concentrations of NaCl (1-9%). Tubes were then autoclaved after which they inoculated with 10 µl of overnight with culture of lactobacilli and incubated anaerobically at 37°C for 24 h. After 24 h incubation, the bacterial growth was measured using a spectrophotometer at 560 nm.

Antibiotic Susceptibility Test

To assay the antibiotic susceptibility pattern, the disc diffusion method as reported by Okafor and Umeh, [21] was used with some modifications. In place of the Muller Hinton agar, MRS agar was used. Plates were swabbed with suspension of selected isolates using sterile cotton buds. After which antibiotic discs (ampicillin, tertracycline, gentamycine, azithromycin, chrolamphenicol and ciprofloxacin) were placed on the surface of the plates at equidistance. The plates were then incubated at 4°C for 1 to 2 hours for proper disc diffusion of antibiotics. In order to check for zones of inhibition, plates where then incubated for 18 to 24 hours at 37°C. After which the zone

of inhibition was observed for antibiotic sensitivity or resistance and zone diameter was measured.

10. Antimicrobial Sensitivity Test of the Yoghurt against Pathogenic Bacteria

Bacterial isolates were screened for their antimicrobial activity against pathogenic bacteria, *Enterobacteriacea*. Sterile Muller Hinton agar and broth media were prepared and a drop of an overnight growth of the pathogenic cell suspension was seeded onto the agar plates and was evenly distributed using a sterile wire loop, 100 µl of the test isolate was placed in a 6 mm well made with a sterile cork borer and was temporarily kept in the refrigerator at 4^{o}C for 15 minutes to allow diffusion to take place and then incubated at 37°C. Growth inhibition was evaluated after incubation for 24 hours, zone of inhibition around the spots with the test organism was measured using a graduated ruler.

11. Determination of Total Viable Count of Probiotic Bacterial Strain

Serial Dilution

The optimized yoghurt sample was then subjected to serial dilution as described by Cheesbrough, [22]. A serial dilution involving series of sequential dilution was carried out to reduce the dense culture of cell to a more usable concentration. 1ml aliquot of fermented yoghurt homogenized with sterile peptone water was serially diluted into eights (10^{-8}) folds after which the 10^{-8} diluent of the yoghurt (plant blend) was used to prepare pour plate in Nutrient agar and MRS agar [23].

The total viable count was carried out as outlined by the American Public Health Association [24]. For Microbiological Analysis, Nutrient agar and Muller Hinton agar were prepared according to manufacturer's (Titan Biotech Ltd) instruction. The spread plate method of inoculation after serial dilution of the sample up to 10^{9} dilution factor was applied as described by Oyeleke and Manga [25]. A drop of the diluted sample was placed on the surface of sterilized and solidified agar and bent glass rod was used to carefully spread the sample onto the agar. Colonies of bacteria were counted using a colony counter the colony were further sub-cultured from the mixed cultures on sterile nutrient agar and MRS agar plate, incubated at 37^{o}C for 18 hours under aerobic and anaerobic conditions, respectively.

Isolation and identification of the microorganisms responsible for spoilage were carried out by pour plate method on various selective media (PDA, EMB, MacConkey, Nutrient agar, MRS), the bacterial isolates were incubated according to their selective conditions and counted using a magnifying lens.

Data Analysis

The analysis was done in triplicate; results were expressed as Mean ± Standard error of mean. All microbial counts were converted to the base -10 logarithm of the number of colony forming units per ml of tiger nut blended yoghurt samples (log10cfu/ml). Data were subjected to one-way Analysis of Variance (ANOVA) and Dunnet compared all versus control was used to test for the level of significance between mean. Statistical significance was accepted at $p < 0.05$. The results obtained from microbial screening was compared with Codex standard of microbial limit for fermented food.

12. Results and Discussions Probiotic tests on the strains and yogurt

Acid Tolerance Test

Table 1 shows the spectrophotometer readings Acid Tolerance of the organisms- *Lactobacillus bulgaricus* (Strain 3) and Streptococcus *thermophilus* (Strain 1) subjected to very low acid concentrations used for fermentation. Negative spectrophotometer readings shows no survival of organisms while positive readings show there was survival. Results showed survival at ph. 2.8 (Table 1).

Table 1. Acid Tolerance Test Result for probiotic tests on the bacterial strains

Lactobacillus bulgaricus **Strain 3**		Streptococcus thermophillus **Strain 1**	
Conc.	000	Conc.	000
1.2	-0.605	1.2	-0.390
1.5	-0.088	1.5	-0.321
2.8	0.166	2.8	0.175

Bile Tolerance Test

The results of the bile test for organisms- *Lactobacillus bulgaricus* (Strain 3) and *Streptococcus thermophillus* (Strain 1) used for fermentation is reported in table XXX. Results shows survival of up to 2.0% bile concentration. Spectrophotometer readings were positive at 0.272 and 0.462 for strains 3 and 1 respectively.

Table 2. Bile Tolerance Test

Conc. of Bile (%)	*Lactobacillus bulgaricus* **Strain 3**	*Streptococcus thermophillus* **Strain 1**
0.3	4.275	2.922
0.5	1.974	2.656
1.0	1.026	1.395
1.5	0.300	0.564
2.0	0.272	0.462

Salt Tolerance Test

Table 3. Salt Tolerance Test Result

Lactobacillus bulgaricus **Strain 3**		Streptococcus thermophillus **Strain 1**	
Conc. of salt	**Spec reading**	**Conc. of salt**	**Spec reading**
1%	0.636	1%	0.739
2%	0.491	2%	0.499
3%	0.422	3%	0.473
4%	0.339	4%	0.445
5%	0.359	5%	0.372
6%	0.161	6%	0.343
7%	0.114	7%	0.165
8%	0.044	8%	0.033
9%	0.017	9%	0.032

Table 3 shows the salt tolerance test result using two strains of organisms *Lactobacillus bulgaricus* (Strain 3)

and *Streptococcus thermophillus* (Strain 1) used for fermentation of the product. Result showed that concentration increased from 1% to 9% with a corresponding decrease in spectrophotometer readings from 0.636 at 1% concentration to 0.017 at 9% concentration for strain 3, while strain 1 spectrophotometer reading reduced from 0.739 to 0.032. Positive results showed survival on known salt concentrations.

Antibiotics Susceptibility Test

Figure 3 and 4 shows the resistance to the effect of drugs on the organisms. *Lactobacillus bulgaricus* (Strain 3) and *Streptococcus thermophillus* (Strain 1). The full growth of the plant and dairy yoghurt on the antibiotics disc showed that the yoghurt had resistance to the antibiotics used for analysis. The 2 strains of bacteria used in fermentation, showed resistance to the effect of drugs on the organisms. Antibiotic drugs did not inhibit their growth as probiotics.

Figure 3. Antibiogram of isolated *Lactobacillus bulgaricus*

Figure 4. Antibiogram of isolated Streptococcus thermophile

Key: OFX – TARIVID, PEF – REFLACINE, CPX – CIPROFLOX, AU – AUGUMENTIN, CN – GENTAMYCIN, S – STREPTOMYCIN, CEP – CEPOREX, NA – NALIDIXIC ACID SXT SEPTRIN, PN – AMPLICIN

Probiotic Sensitivity Test to Pathogens (Zone of Inhibition)

Probiotic sensitivity test shows the ability of the Yoghurt analogue and cow milk to inhibit the growth of pathogenic organisms are reported in Tables 4 and 5. Results shows that the Yoghurt analogue and cow yoghurt created a greater zone of inhibition measuring 21.5 mm and 16.0mm around the hole filled with *Echericha coli;* 15.0 mm and 11.0 mm for *Shigella Dysentriae* and No significant inhibition for *Staphylococcus aerus* as it proved resistant to the strains. The strains alone showed similar trend, 20.5mm and 10.0 mm for strains 01 and 03 on *E. coli* and 7.0 mm and 11.0 mm on *Shigella Dysentriae*, while *Staphylococcus aerus* showed resistance.

Table 4. Probiotic Sensitivity Test (Zone of Inhibition) of the yoghurt analogue and Dairy yoghurt.

Organisms	Zone of inhabitation of Yoghurt analogue in (mm)			Zone of Inhabitation of Dairy Yoghurt in (mm)		
	a	b	C	a	b	C
Escherichia Coli	22.0	21.0	21.5	16.0	16.0	16.0
Shigella dysenteriae	15.0	15.0	15.0	10.0	12.0	11.0
Staphylococcus aureus	NSI	NSI	NSI	NSI	NSI	NSI

Table 5. Zone of inhabitation of strains 01 and 03

Organisms	Zone of inhabitation of 01 (mm)			Zone of Inhabitation of 03 mm)		
	A	B	c	a	b	C
Escherichia Coil	20.0	20.0	20.5	10.0	10.0	10.0
Shigella Dysenteriae	6.00	8.00	7.00	12.0	10.0	11.0
Staphylococcus aureus	NSI	NSI	NSI	NSI	NSI	NSI

Key:* Diameter of cork borer (4mm) included (a.)First reading(b).Second reading.(c). a+b/2(Average) NSI Nosignificant inhibition

Results on Figure 5, Figure 6, and Figure 7 show that for the 2 strains of bacteria used in fermentation were able to inhibit the growth of some pathogenic organism. The yoghurt and milk samples filled inside a cork bored hole was able to inhibit the growth of *E. coli* and *Shigella dysenteriae*, however there was no inhibition of *Staphylococcus aureus.*

Figure 5. Probiotic sensitivity test on Shigella for the plant and dairy yoghurt showing zones of inhibition

Figure 6. Probiotic sensitivity test on Staphylococcus for plant and dairy yoghurt showing zones of inhibition.

KEY: A - Plant yoghurt, M - Dairy yoghurt

Figure 7. Probiotic sensitivity test on *E. coli* for the plant and dairy yoghurt showing zones of inhibition

13. Discussions

The damages caused by extreme consumption of antibiotics was reported by Butler, *et al.*, [26], and the need for alternatives required to fight the increasing number of infections equally emphasized. The significance of antibiotic resistance's by the probiotics used in this study is targeted towards aid in the prevention of Antibiotic-Associated Diarrhoea (AAD). It shows that antibiotics consumption cannot kill the probiotics in the yoghurt when consumed during treatment. Control trials have been used to prove the advantage of these probiotics in preventing Antibiotic-Associated Diarrhoea (AAD). Agamennone, *et al.* [11] reported the effectiveness of *Saccharomyces boulardii* or *Lactobercillus bulgarecus* GG in adults or children undergoing antibiotic healing. The most common disruption of the flora is produced by antibiotics that can cause diarrhea (AAD). The pattern of resistance or sensitivities of a given microorganism towards a range of antibiotics is called an antibiogram. This study was able to ascertain if the organisms used would survive when antibiotics are ingested alongside. An antibiotic is a substance produced by a microorganism and, at low concentrations, inhibits or kills other microorganisms [27,28]. In the present study, an antibiotic susceptibility test was performed for the two strains present in the new yoghurt and labelled 03 and 01 (*Lactobacillus bulgaricus* (ANOL 2) and *Streptococcus thermophilus* (ANOL 5) respectively, and both strains thrived well in the presence of the antibiotic disc.

The yoghurt samples filled inside a cork bored hole were able to inhibit the growth of *E. coli* and *Shigella dysenteriae*; however, there was no inhibition of *Staphylococcus aureus.* This finding affirms the Yoghurt analogue as probiotic that can inhibit the growth of pathogens when consumed. *E. coli* is responsible for producing toxins when consumed that causes stomach cramps, diarrhea, and vomiting. The gastrointestinal micro-flora helps maintain a microbial barrier against the colonization and proliferation of pathogens in the digestive tract [29], however, antibiotics ingestion disrupts the bacteria in the gut.

The yoghurt and milk samples filled inside a cork bored hole were able to inhibit the growth of *E. coil* and *Shigeila dysenteriae*; however, there was no inhibition of *Staphylococcus aureus.* This finding affirms the plant yoghurt as a probiotic. *E. coli* is responsible for producing toxins when consumed that causes stomach cramps, diarrhea, and vomiting. The gastrointestinal micro- flora helps maintain a microbial barrier against the colonization and proliferation of pathogens in the digestive tract [29].

14. Conclusions

Yoghurts produced by using starter cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* have higher therapeutic and/or antimicrobial properties than organoleptic characteristics. Certain lactic acid-producing bacteria may reduce cholesterol by breaking down bile in the gut. The two strains employed in this study were able to tolerate high percentages of bile, low acid concentration, and high salt concentration. This shows they can survive in the intestine when consumed. This survival is essential as it enables the product to exert its probiotics benefits to the consumer.

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