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Potentials of Lactic Acid Bacteria isolated from Traditional Fermented Foods as Starter Cultures in Yoghurt Production

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ABSTRACT

The demand for functional foods has increased the interest in finding and developing more functional starter cultures that will not only serve as good starters but will also be used as human probiotics. The aim of this study was to isolate, characterize and identify Lactic Acid Bacteria (LAB) that can serve as good starters for yoghurt production and can also be used to complement existing yoghurt starters. A total of 120 samples of traditional fermented foods (*Akamu*, *Ugba*, *Akpu*, *Nunu* and *Kunu*) were screened. Out of 28 isolates recovered, 21 were considered presumptively after gram staining. Five LAB namely *Lactobacillus plantarum, L. lactis, L. fermentum, Leuconostoc mesenteroides* and *L. pentosus* had the highest percentage occurrence ranging from 56% - 28% with *Akamu* having the highest LAB occurrence. Isolates were Gram positive rod/cocci, mesophilic, acidiophilic, catalase negative and showed good growth at 2.5-6-5% NaCl concentration and diverse sugar fermentation abilities. Isolates had high tolerance to low pH and bile indicating their ability to withstand the acidic nature of the stomach. Similarly, they showed antibacterial activity against *S. aureus, E. coli, P. aeruginosa* and *Bacillus*. *E. coli* was inhibited most (14.33mm-16.67mm) while *Bacillus* had the least (9.67mm - 10.33mm). Antibacterial activity was affected by different temperature and pH ranges. Evaluation for use as starters, confirmed them to be safe as there was no haemolysis, gelatinase and DNase activity recorded. They also hydrolysed lactose to glucose and produced Exopolysaccharides (2.0 mm - 3.0mm). They demonstrated a strong performance as starters for yogurt production when compared with commercial starter cultures of *S. thermophilus, L. bulgaricus* and *L. acidiophilus.* Sensory evaluation and acceptability tests revealed that, yoghurt of *L. fermentum* was the most preferred in terms of colour, taste and general acceptability. While in terms of aroma and mouth feel, the yoghurt of *L. lactis* was the most preferred (6.82±0.10 and 6.49±0.01 respectively). In conclusion, this study has shown that *L. plantarum, L. fermentum* and *L. lactis* can be used as good starter cultures for yoghurt production.

Keywords: Fermented Foods, LAB, *L. fermentum*, Starter Culture, Yoghurt, Exopolysaccharides

Introduction

Presently, there is a surge in the desire to generally improve health through dietary/natural means. In the food and dairy industry, Lactic acid bacteria (LAB) has proven to be such organisms suitable for the production of functional foods (Pangiota *et al.*, 2013). They not only improve the nutritional value of foods but also improve the general well-being of individuals. Over the decades, investigation on lactic acid bacteria has tremendously increased. This is as a result of their usefulness and health promoting abilities. Lactic Acid Bacteria (LAB) are widely spread organism which can be found in any environment rich mainly in carbohydrate such as plants, fermented food and the mucosal surfaces of humans, terrestrial and marine animals (Pangiota *et al.*, 2013). They are gram positive, aero tolerant anaerobes and catalase negative bacteria that produce lactic acid as a major metabolic product. They are among the important and useful groups of microorganisms used in the food industry. They include members of the genera *Streptococcus, Enterococcus, Lactococcus, Lactobacillus* and *Leuconostoc* (Rijkers *et al.*, 2011).

LAB has been researched on by mankind for thousands of years for the production of fermented food because of their usefulness (Egbe *et al.*, 2017). These include ability to produce desirable changes in taste, flavour, aroma, and texture of food.

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Other usefulness include their use as probiotics to improve the general health of humans, their antimicrobial activity against pathogens and spoilage organisms, extends shelf life and promotes safety of food products (Mahantesh *et al.,* 2009). LAB are generally recognized as safe (GRAS) which gives them an advantage over other microorganisms for suitable use in biotechnology (Zamfir *et al.,* 2014). They have shown also to improve shelf-life of fermented food due to wide range production of metabolites which has an antagonistic effect and as such can serve as a bio-preservative (Fan *et al.,* 2012). These metabolites include organic acids, carbondioxide, ethanol, hydrogen peroxide, bacteriocins, *etc*. However bacteriocin have attracted serious attention and interest due to their potential use as safe and natural preservative (Egbe *et al.,* 2017).

Yoghurt is a fermented dairy product that can be produced through the fermentation of lactose by microorganisms especially Lactic acid bacteria. It can be used as a vehicle (medium) for probiotics. Yoghurt is of great significance as it provides and preserves vast quantities of nutrients in foods with wide diversity of flavours, aromas and textures which enrich the human diet and also are consumed throughout the world (Oyedeji *et al.,* 2013).

In this study, the potentials of Lactic Acid Bacteria isolated from traditional fermented foods were investigated as starter cultures in yoghurt production.

Materials and Methods

Sample collection and processing

Twenty samples each of traditional fermented foods: *Akamu* (from maize and sorghum), *Ugba* (from fermented oil bean seeds), *Akpu* (from fermented cassava tuber), *Nunu* (from fermented milk) and *Kunu* (from fermented sorghum) were purchased from the retailers in Umuahia market in Abia State. They were packaged in universal sterile bottles and placed inside a cooler with ice and quickly transported to the laboratory for analysis.

Isolation, identification and characterization of Lactic Acid Bacterial (LAB)

One gram of each sample was homogenized in 0.1% peptone water (9 ml)) and serially diluted in 9 ml of Normal Saline Solution (0.85% NaCl/M/V) and 0.1ml of aliquots of the appropriate dilution was inoculated by spread plate method on De Man Rogosa and Sharpe (MRS) media supplemented with 1% calcium carbonate $CaCO_3$ to distinguish the acid producing bacteria and incubated anaerobically at 37^{o}C for 48 hours (Harrigan and McCane, 1976). Colonies formed were purified by sub-culturing on MRS agar by streaking. Pure colonies were then selected and preserved in 40% glycerol and stored for further analysis. The pure isolates were characterized based on morphological, biochemical properties. Gram staining was carried out followed by sugar fermentation (Harrigan and McCane, 1976).

Molecular identification of LAB

Extraction of DNA was carried out using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba, South Africa. The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Standard methods were adopted.

Screening of LAB for probiotic properties

Acid tolerance was determined based on the method by Dowarah *et al.* (2018). The pH of MRS broth was adjusted to pH 2.0 to 8-0 using 1m HCl. 100µL of overnight grown LAB was added into 5ml MRS medium with different pH and incubated for 4hrs at 37^{o}C. Microbial growth was then observed.

Bile Salt tolerance in bile salt was measured according to the methods of Nami *et al.* (2019). The MRS medium was prepared with 0.3% bile and without bile salt. The one without bile salt served as control. The two media was inoculated with 1% of culture and incubated for 4hrs at 37^{o}C.The growth rate was determined by the viable plate count method. Growth rate was calculated as: follows:

$$\% \text{ Growth} = \frac{Growth\ in\ bile\ salt\ medium}{Growth\ in\ control\ medium} \times \frac{100}{1}$$

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Assessment of LAB isolates for use as starter culture

This was carried out by determining the Haemolytic activity of LAB (Monique *et al.*, 2020), Production of Gelatinase (Monique *et al.*, 2020), determination of DNase Activity (Shuhadha, *et al.*, 2017) and hydrolysis of lactose (Takahiro *et al.*, 1981). Production of Exopolysaccharides was carried out according to the method of (Guiraudi, 1998). Yoghurt production by milk fermentation using defined starter cultures was according to Dira (1993) with some modification.

Statistical analysis

Data were expressed as a Mean+ standard deviation of triplicate means. Mean separation was done using Duncan multiple range test using statistical package for social sciences (SPSS) version 22.0.

Differences in significance were considered using one way ANOVA (Analysis of Variance at $P<0.05$).

Results

Isolation and characterization of LAB isolates

A total of 21 LAB isolates were recovered from various fermented foods and were found to be members of *Lactobacillus plantarum* (7 out of 21), *Lactococcus lactis* (4 out of 21), *Lactobacillus fermentum* (4 out of 21), *Leuconostoc mesenteroides* (2 of 21) and *Lactobacillus pentosus* (4 out of 21).

Table 1: Morphological and Biochemical Identification of Lactic Acid Bacteria Isolate from Traditional Fermented Foods

Number of isolate /code	Colony Morphology	Gram reaction	Cell shape	Spore	CO_2	Motility	Catalase	Indole	Oxidase	Methyl-red	Voges-Proskauer	Glucose	Sucrose	Lactose	Mallose	Mamitol	Fructose	Xylose	Suspected LAB Isolate
07AM	Circular, Creamy, convex	+	Rod	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	*Lactobacillus plantarum*
04K	Creamy almost flat	+	Cocci	-	+	-	-	-	-	+	-	+	-	+	+	-	+	+	*Lactococcus lactis*
04A	Circular white glistening convex	+	Rod	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	*Lactobacillus fermentum*
02U	Circular, milky, slimy	+	Cocci	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	*Leuconostoc mesenteroides*
04K	Circular, milky glistening	+	Rod	-	+	-	-	-	-	+	-	+	-	+	+	+	+	+	*Lactobacillus pentosus*

Key: AM = *Akamu*, K = *Kunu*, A = *Akpu*, U = *Ugba*; + = Positive, - = Negative

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The Agarose gel electrophoresis showing the amplified 16srRNA. Lanes 1-3 represent the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder is presented in Plate 1.

The phylogenetic tree showing the evolutionary relationship between the Lactic acid bacterial isolates is presented in Figure 1.

Table 2 shows the growth rate of the isolates at various growth parameters. Viable growth was observed at pH 4, 5, 6 while at pH 7, *Lactococcus lactis* and *Lactobacillus pentosus* had no growth. At pH 8, no growth was observed from all the isolates. No growth was observed at temperatures 45^{o}C and 60^{o}C. Again, no growth was recorded at 10% salt concentration.

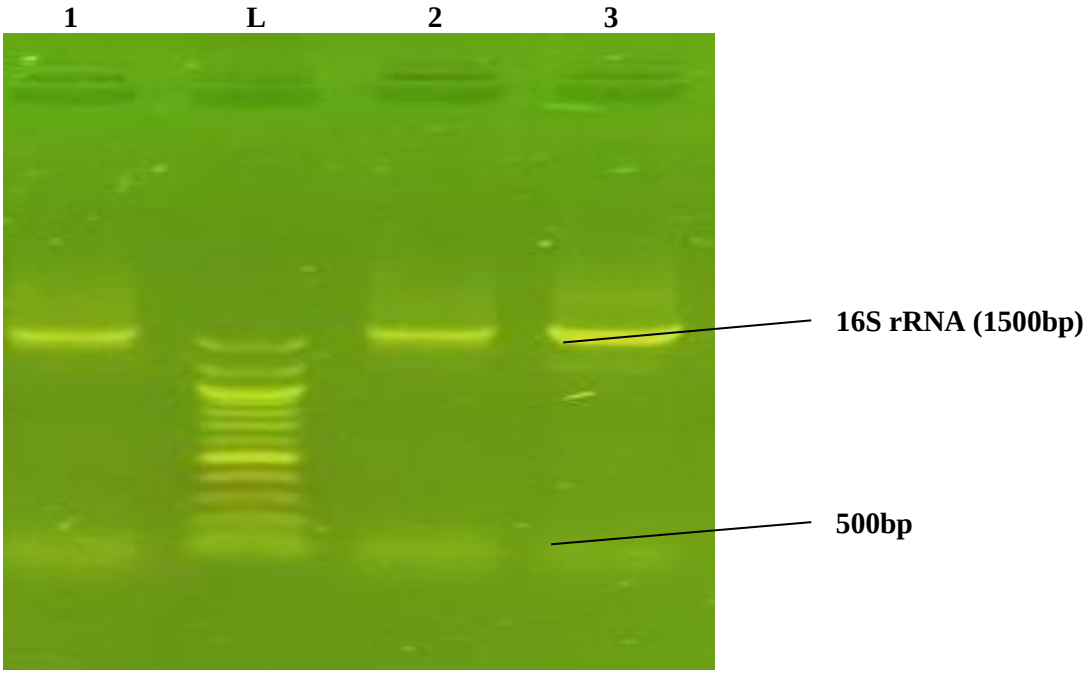

Fig. 1: Agarose gel electrophoresis showing the amplified 16srRNA

Lanes 1-3 represent the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder. L1 = *Lactobacillus fermentum.* L2 = *Lactococcus lacti*, L3 = *Leuconostoc mesenteroides*

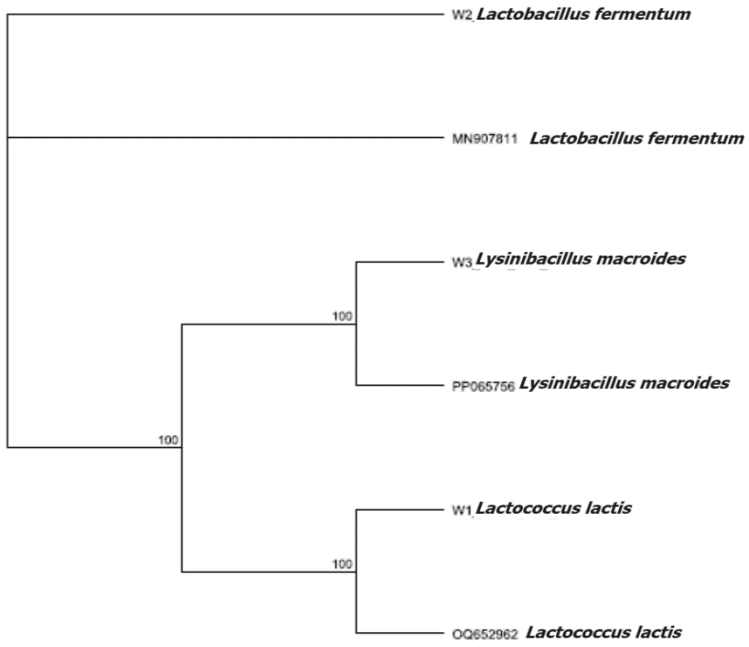

Fig. 1: Phylogenetic tree showing the evolutionary relationship between the bacterial isolates

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Table 2: Physiological Characterization of LAB Isolated from Fermented Foods

LAB Organisms	Growth Rate												
	pH					Temp (ºC)				NaCl %			
	4	5	6	7	8	30	37	45	60	2.5	4.5	6.5	10
L. plantarum	+	+	+	+	-	+	+	-	-	+	+	+	-
L. lactis	+	+	+	-	-	+	+	-	-	+	+	+	-
L. fermentum	+	+	+	+	-	+	+	-	-	+	+	+	-
L. mesenteroides	+	+	+	+	-	+	+	-	-	+	+	+	-
L. pentosus	+	+	+	-	-	+	+	-	-	+	+	+	-

Key: Positive (+) = Growth : Negative (-) = No Growth

Probiotic evaluation of LAB isolates

Acid tolerance

Table 3 shows the result of acid tolerance by the LAB isolates. It shows they survive pH 2 and 3 upon exposure for 3 hours. There was profuse growth at pH 3, 4 and 5 but non at pH 8.

Bile tolerance

The survivability of the LAB isolates in bile salt is shown in Table 4. The result shows some significant level of growth reduction in comparison with the control without bile.

Assessment of LAB isolates for use as starter culture

Table 5 shows the results of haemolytic activity of the isolates.

The results show that both the Beta haemolytic test and the alpha haemolytic tests were all negative in all the isolates.

However in contrast, the Gamma haemolysis test showed positive results indicating the absence of haemolysis in all the LAB. Similarly all organisms reacted negatively to gelatinase and DNase activities which were confirmed by the absence of clear halo zones in the test plates inoculated with all the studied Isolates.

Lactose hydrolysis DNase Activity

The result of hydrolysis test is shown in Table 6. The different LAB isolates demonstrated their ability to hydrolyse Lactose which is the sugar in Milk. This was evident by the positive results of the glucose test with strip. This indicates that lactose was hydrolysed.

Table 3: pH Tolerance for 3hrs by the LAB isolated from fermented foods

LAB Isolate	pH tolerance for 3hrs by the LAB						
	2	3	4	5	6	7	8
L. plantarum	+	++	++	++	+	+	-
L. lactis	+	++	++	++	+	+	-
L. fermentum	+	++	++	++	+	-	-
L. mesenteroides	+	++	++	++	+	+	-
L. pentosus	+	++	++	++	+	-	-

Key: + = minimal Growth. - = No Growth. ++ = Profuse Growth

Table 4: Bile tolerance (0.3%) of the LAB isolated from fermented foods for 3hrs

LAB Isolate	TVC (CFU/ml) control (WB)	TVC (CFU/ml) bile medium	Growth rate % tolerance
Lactobacillus plantarum	$2.29x10^7$	$1.19x10^7$	51.96%
Lactococcus lactis	$2.38x10^7$	$1.43x10^7$	60.80%
Lactobacillus fermentum	$2.41x10^7$	$1.39x10^7$	57.68%
Leuconostoc mesenteroides	$2.35x10^7$	$1.34x10^7$	57.02%
Lactobacillus pentosus	$2.44x10^7$	$1.47x10^7$	60.25%

Key: WB = without bile

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Table 5: Safety Evaluation of LAB isolated from fermented foods

Organism	β-haemolysis (clear Halos)	α-Haemolysis (green Halos)	γ-haemolysis (None)	Gelatinase (No zone)	DNase Activity
Lactobacillus plantarum	-	-	+	-	-
Lactococcus lactis	-	-	+	-	-
Lactobacillus fermentum	-	-	+	-	-
Leuconostoc mesenteroides	-	-	+	-	-
Lactobacillus pentosus	-	-	+	-	-

Key: + = positive; - = negative

Table 6: Hydrolysis result (after 24 hrs at 37ºC)

Isolates	Glucose/Testing with Strip
Lactobacillus plantarum	+
Lactococcus lactis	+
Lactobacillus fermentum	+

Key + = Positive; - = Negative

Exopolysaccharide (EPS) production and quantification

Table 7 shows the ability of LAB to produce exopolysaccharide with *Lactococcus lactis* giving the highest result (3.0) while *Leuconostoc mesenteroides* had the lowest result (2.0).

Table 7: Exopolysaccharide (EPS) Production and Quantification - slime length (mm)

LAB Isolate	Preliminary Test	Confirmatory (Colour test)
L. plantarum	2.5	+
L. lactis	3.0	+
L. fermentum	2.5	+
L. mesenteroides	2.0	+
L. pentosus	2.5	+

Key: = + = Positive; - = Negative

Sensory evaluation of yoghurts

Table 8 shows the results of comparative assessment of yogurts product with commercial starter culture through sensory evaluation. The result which was based on a 9-point Hedonic scale showed an acceptability score ranging from 6.52 to 6.66 as against 6.80 recorded for the commercial starter culture yogurt. This translates to acceptability levels of 72.44% to 44.00% of the LAB isolates as against 75.56% of the commercial starter culture yogurt. This implies a relative acceptance of 95.87%.

However, variations of significant differences ($P< 0.05$) were recorded between the LAB starter culture yogurt and the commercial starter as it affects the sensory attributes of taste, aroma, mouth feel and consistency. Yoghurt produced by *L. fermentum* was the most liked in terms colour, taste and consistency with preferential score over and above those of the commercial starter culture yogurt. No synergic improvement was recorded by the combination of the different LAB isolates when used as starter culture. But the mixture of *L. plantarum* and *L. fermentum* scored highest in colour, aroma and mouth feel (texture).

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Table 8: Sensory evaluation results of yoghurts produced with the different Lactic Acid Bacteria isolated from traditional fermented foods

Food Sample	Colour	Aroma	Taste	Mouth feel (Texture)	Consistency	Acceptability
YG 1 Control	7.26± 0.01^{b}	6.72± 0.05^{bc}	7.25± 0.71^{ab}	6.29±0.05^{c}	6.40± 0.31^{bcd}	6.80±0.01^{a}
YG 2	7.04± 0.06^{C}	6.59± 0.13^{c}	6.70± 0.26^{ab}	6.19± 0.09^{c}	6.48±0.04^{bc}	6.52±0.04^{c}
YG 3	7.13 ± 0.02^{c}	6.82± 0.10^{b}	6.80± 0.06^{ab}	6.49± 0.01^{b}	6.27±0.02^{d}	6.64±0.02^{b}
YG 4	7.49± 0.04^{a}	6.74± 0.01^{bc}	4.28± 0.75^{a}	6.28±0.04^{c}	6.73±0.03^{a}	6.66± 0.01^{b}
YG 5	7.56± 0.01^{a}	7.01± 0.01^{a}	6.29± 0.06^{b}	6.72±0.05^{a}	6.51±0.01^{b}	6.66±0.02^{b}
YG 6	6.81± 0.01^{d}	6.70± 0.02^{bc}	6.74± 0.03^{ab}	6.40±0.01^{b}	6.30±0.02^{cd}	6.60±0.05^{bc}
YG 7	7.31± 0.10^{b}	6.67± 0.02^{c}	6.74± 0.028^{ab}	6.30±0.02^{c}	6.28±0.04^{cd}	6.52 ± 0.09^{c}
YG 8	7.31± 0.02^{b}	6.62± 0.01^{c}	6.73± 0.02^{ab}	6.25±0.02^{a}	6.37±0.08^{bcd}	6.62±0.06^{bc}

Values show means of fifteen responses to standard deviation

Key: YG 1= Yoghurt with commercial mixed starter. YG 2 = Yoghurt with *L.plantarum*. YG3 = Yoghurt with *L. lactis*. YG 4 = Yoghurt with *L. fermentum*. YG 5 = Yoghurt with *L. Plantarum, L. lactis and L. fermentum*. YG 6 = Yoghurt with *L.plantarum and L. lactis*. YG 7 = Yoghurt with *L. plantarum* and *L. fermentum*. YG 8 = Yoghurt with *L. lactis* and *L. fermentum*

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Discussion

In this study, Lactic Acid Bacteria isolated from various fermented food sources (*Akamu* made from maize and Sorghum), *Ugba, Akpu, Nunu* and *Kunu*) are reported. They were identified molecularly as *Lactococcus lactis, Lactobacillus fermentum* while the suspected *Lactobacillus plantarum* turned out to be *Lysinibacillus macroides*. They were gram-positive, catalase negative, rod/cocci shaped and mesophilic organisms. They showed good growth at 2.5-6.5% NaCl concentration and showed diversity in their ability to ferment different sugars. Some were also confirmed to be hetero-fermenters due to their ability to produce CO_2.

The molecular identification showed that there is 100% similarity/ relationship with previously studied species. Out of 21 LAB recovered, 5 LAB *Lactobacillus Plantarum, Lactococcus Lactis, Lactobacillus ferementum, Leuconostoc Mesenteroides* and *Lactobacillus pentosus* occurred most. They showed morphological, physiological and biochemical properties that qualified them as Lactic acid bacteria taxonomically (Cowan and Steel, 1993; Holzapel and Stiles, 1997). *Akamu* from maize had the highest LAB occurrence and this dominance can be linked to the nature of the substrate which are fermentable carbohydrates. This clearly shows that fermented foods present good sources of Lactic Acid Bacteria. This is in agreement with the work of Liu *et al.* (2014).

For organisms to be used as probiotics, they must overcome the unfavourable conditions of the human gastrointestinal tract. So, they should be resistant or tolerate the acid and bile conditions. This is important as it will allow them withstand gastric stresses and also allows them to survive longer in carrier food such as yogurt without reduction in number (Wang, *et al*., 2010). In this study, the isolates showed profuse growth at pH 3-4 while at pH 2 showed minimal growth. At pH 7 (Neutral pH) only 3 recorded minimal growth occurred while at pH 8, no growth was recorded. This finding agrees with the work of Guo *et al*, (2010). Due to the toxic nature of bile salt to living cells, its tolerance is considered as one of the criteria in selecting probiotic strains (Succi, *et al*, 2005). In this study, all the LAB isolates tolerated bile salt at 0.3%, though at varying extent. This shows a major functional probiotic property as also seen in numerous studies by various scholars (Guo *et al.*, 2010).

In order to produce a functional food, development of good starter culture is needed. However, for an organism to be used as a starter culture, it must be certified safe. Lactic acid bacteria are generally recognized as safe (GRAS) which gives them an advantage over others for use in biotechnology (Holzapfel and stiles, 1997). In this study, the safety evaluation of the studied LAB was evaluated. Results showed no sign of haemolysis, DNase and gelatinase activity. This indicates the safety of the isolates to be used as starters and their suitability for biotechnological and industrial applications. This agrees with the findings of Monique *et al*, (2020) and Shuhdha *et al*, (2017). Again, utilization of lactose is the primary function of LAB used in dairy fermentation. The activities of B-galactosidase were evident through the hydrolysis result. This indicates that it can be used in production of lactose-free dairy drinks and can be beneficial to lactose intolerant individuals. Similar was reported by Takahiro *et al*. (1981) and Tamara *et al.*, (2005).

In the screening for Exopolysaccharide (EPS) production, we observed that all studied strain showed slimy and ropy colonies with slight variations among the isolates. This agrees with the findings of Almalki (2020). EPS plays a role in forming the desired mouth feel, thickness, texture and flavour property of yogurt. The sensory analysis revealed a favourable report in using the studied LAB as starter cultures in the fermentation of milk to produce yogurt, when compared with yoghurt produced using the commercial starter. The acceptability and sensory evaluation by the evaluators showed that *L. lactis* and L. *fermentum* individually and as mixed cultures had good performances. Again, among the studied starters, *L. fermentum* was the most liked in terms of colour, taste, consistency and general acceptability but in terms of aroma. *L. lactis* had the highest acceptability and this confirms its contribution to the yoghurt flavor.

Conclusion

This present study demonstrated that *L. Plantarum, L. fermentum* and *L. lactis* can be isolated from traditional fermented food products and have proven to be abundant in environments rich in carbohydrate. Results showed too that they proved to be probiotics and have antibacterial qualities with a broad spectrum, thus can serve as bio preservatives.

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From their safety result, they were confirmed to be safe for use as starter culture. As starters, even though the *S. thermophilus* and *L. bulgaricus* mixed starters has been the most accepted and widely used starters, *L. fermentum* and *L. lactis* mixture showed great potentials and may be recommended as potential starter culture for production of yogurt. This study has proved that yoghurt produced with these two isolates of Lactic Acid Bacteria (*L. fermentum* and *L. lactis* mixture) produced good flavor to the yoghurts they produced.

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