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Screening of Indigenous Lactic Acid Bacteria from Raw and Fermented Milk for Probiotic Potential

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ABSTRACT

Lactic acid bacteria are known to have probiotic attributes which are beneficial to human. This study was embarked upon to screen lactic acid bacteria (LAB) from raw milk and fermented milk (nono) samples for probiotic potential. Some of the assessed probiotic qualities include antimicrobial activities against food-borne pathogens, survival at acidic pH (2.5, 3.0) and bile salt concentrations (0.3%, 0.5%, 1.0%), safety test, and cell surface hydrophobicity assay indicative of epithelial adherence. Four LAB isolates namely Lactobacillus plantarumN17, L. plantarumN24, L. caseiN1 and L. brevisN10 had strong inhibition (10.00 to 15.15 mm) against selected food-borne pathogens, survived well at acidic pH and bile salt concentrations during 3 hours of incubation reaching viability of 10^5 -10^6 CFU/mL.In addition, they were DNase and Gelatinase negative, and had better(40.0 to 62.0 %) hydrophobicity indicative of epithelial adherence. Lactobacillus plantarumN24 and Lactobacillus caseiN1 were suitable probiotic candidates, and can be used as for food supplements.

***Key Words**: Lactic acid bacteria (LAB), Nono, Probiotic.*

1. INTRODUCTION

Lactic acid bacteria have been known in the food industries and still in existence till date. Moreover, they are also regarded as fastidious, acid tolerant, micro-aerophilic organisms, and have the ability to produce lactic acid (Rivera- Espinoza and Gallardo-Navaro, 2010; Brant and Todd, 2014).

Lactic acid bacteria (LAB)can be used as probiotics based on immense benefits. Most of the lactic acid bacteria in most work can be used as protective organisms which can be inoculated in yoghurt (Pérez-Chabela *et al*., 2008) or embedded with biopolymers (Pérez-Chabela *et al*., 2012; Pérez-Chabela *et al*., 2013; Mohammed *et al*., 2016). Probiotics refer to living microorganisms that has the tendency to exert some biological changes in the gut or gastrointestinal compartment when ingested by man (Masci *et al*., 2013; Vasiee *et al*., 2017). These probiotic organisms have the tendency to inhibit pathogens, but should also exhibit some important probiotic potentials. Moreover, some criteria used for regarding an organisms as probiotic bacteria includes tolerance to bile salts, survival at acidic pH, adherence to intestinal mucosa or epithelial cells, and should also be safe (Mohammed *et al*., 2016). However, survival of LAB in yoghurt is a challenge to quality product delivery because most LAB cannot withstand the low pH during yoghurt production and harsh conditions of the gut, hence limiting their probiotic potentials. Therefore, this study was designed to screen and select probiotic LAB starters

2. MATERIALS AND METHODS

2.1 Collection of samples

Raw milk from cow, goat and traditional fermented milk product (*nono* samples) were randomly purchased purposively from 'kara' at Bodija in Ibadan, Nigeria. They were brought into the laboratory in sterile bottles for microbiological analysis.

2.2 Collection of Indicator organisms

Indicator organisms were obtained from the culture collection unit of Federal Institute of Industrial Research, Oshodi (FIIRO).The organisms are *Escherichia coli* ATCC 8739, *Salmonella tyhimurium* ATCC 13311, *Bacillus subtilis* ATCC 6633, *Proteus* spp, *Shigella flexneri ATCC* 29833, *Bacillus cereus* CMGB 215.

2.3 Isolation and characterisation of isolates

Isolation of lactic acid bacteria was done using pour plate technique. One millilitre of each raw milk samples from goat, cow, and *nono* were taken aseptically and transferred into separate bottles containing 9.0 mL of sterile distilled water, and serial dilutions of the milk samples were made. One millilitre of 10^{-6} dilutions of the samples was plated into sterile Petri dishes containing MRS agar, and incubated in anaerobic jars at 37ºC for 48 hr. Isolates were sub-cultured and repeated streaking was done to obtain pure cultures (Nikolic *et al*., 2008). The isolates were characterized using conventional procedures by employing macroscopic, microscopic, physiological and biochemical tests.

2.4 Screening of isolated LAB for probiotic potential

The isolated LAB were assessed for probiotic potential which includes**:**

2.5 Antimicrobial activities of LAB against food-borne pathogens

Antimicrobial effects of metabolites of presumptive species of LAB isolates against *Escherichia coli* ATCC 8739, *Salmonella tyhimurium* ATCC 13311, *Bacillus subtilis* ATCC 6633, *Proteus* spp, *Shigella flexneri ATCC* 29833, *Bacillus cereus* CMGB 215earlier stated were determined by Agar diffusion method (Nikolic *et al*., 2008). The indicator organisms were incubated in nutrient broth at 37ºC for 24 hr, and 100 µL (approximately inoculum size of 10^7 CFU/mL) of standardized over night cultures was used. A 50 µL of cell free supernatants of cultured MRS broth obtained by centrifugation (4000 g for 15 minutes), was filled in 8 mm diammeter sealed wells cut on Mueller Hinton agar containing the test organisms. It was stored in the refrigerator for 2 hr, and the inoculated plates were incubated at 37ºC for 24 hr. The diammeter of the inhibition zone was measured with a transparent ruler to the nearest milliliters from the point of inhibition to the end.

2.6 Tolerance (growth) of LAB to different concentration of NaCl

One milliliter of the LAB isolates were inoculated into 10 mL freshly prepared MRS containing 4.0%, 6.0%, 8.0% and 10.0% NaCl and their growth were assessed for 48 hr. The growth was confirmed on MRS broth by visual reading due to its turbidity (Hoque *et al.*, 2010).

2.7 Survival of LAB at acidic pH under various incubation time

The LAB isolates were tested for their tolerance to acidic conditions similar to those of the stomach. The acid resistance was examined in MRS broth adjusted with Conc. HCl to pH 2.5, 3.0, and 4.0. Each LAB isolate was inoculated separately (10^7 CFU/mL) in 10 mL MRS broth at pH 2.5, 3.0, 4.0. After incubation for 45 minutes, 1 hr, 2hr, 3 hr and 24 hr, viable cells of the isolates were confirmed on MRS agar after anaerobic incubation at 37ºC for 48 hr following the protocol of Klingberg *et al.* (2005). A period of 45 minutes acclimatization time was used during the start of incubation in this study and was indicated as 0 hr.

2.8 Tolerance to bile salts under various incubation time

The LAB isolates were tested for their ability to survive different concentration of bile salts. Each LAB isolates were separately inoculated (10^7 CFU/mL) into 10 mL MRS broth containing varying concentration of bile salts (0.3%, 0.5%, 1.0%). After exposures to incubation for 45 minutes, 1 hr, 2hr, and 3 hr, viable cells were confirmed on MRS Agar after anaerobic incubation for 48 hr at 37ºC (Klingberg *et al*., 2005). A period of 45 minutes acclimatization time was used during the start of incubation in this study.

2.9 Adherence to intestinal mucosa using hydrophobicity assay

The protocol of Rosenberg *et al*. (2013) was used. The LAB isolates were first grown on MRS broth at 37^0C for 24 hr. They were centrifuged at 5000 x g for 15 minutes, pellets was washed twice with phosphate buffer saline (PBS) having pH 7.0, and the optical density was measured at 540 nm. Then, one milliliter of the bacterial suspension was added to 1 mL of different hydrocarbons (chloroform and xylene) and were vortexed for 30 seconds. After 30 minutes of phase separation, the optical density of aqueous separation was measured again at 540 nm and was compared with initial value. Hydrophobicity was calculated using the equation:

% hydrophobicity = (A_{540nm}initial value- A_{540nm}aqueous solution/A540nm) x100

2.10 Antibiotic susceptibility test of LAB

A total of eight antibiotics discs (Oxoid, England) were used to determine the sensitivity of LAB to antibiotics. They are Ceftazidime (10 µg), Cefuroxime (30 µg), Gentamycin (30 µg), Ceftriaxone (20 µg), Ofloxacin (10 µg), Erythromycin (10 µg), Augmentin (30 µg), and Cloxacin (10 µg). The bacterial cultures of 18 hr old were inoculated into 10 mL of normal saline which had being standardized to cell suspension (Mcfarland standard 0.5). The saline containing the cultures were flooded on Mueller-

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Hinton agar plates before introducing the disc with a sterile forceps. The plates was incubated for 48 hr at 37ºC, and the zone of inhibition was measured with a transparent ruler to the nearest milliliters, and compared with the values of susceptibility interpretation break points, and was expressed in terms of resistance and sensitive (Bauer *et al*., 1996).

2.11 Safety assessment of LAB based on gelatinase and Dnase production

Gelatinase production

Gelatinase production was determined by inoculating 18 hr old cultures of the LAB isolates on plate containing nutrient agar, supplemented with0.4% gelatin and was incubated at 37ºC for 48 hr. The incubated plate was flooded with saturated ammonium sulphate solution. The development of clear zones around the spots against the opaque background indicated a positive reaction while absence of clear zones indicated negative result (Gupta and Malik, 2007).

2.12 DNase production

DNase agar medium was used to check production of DNase enzyme. A streaked of the LAB cultures were made on the agar and incubated at 30ºC for 48 hr. After incubation, a clear pinkish zone around the colonies against dark-blue background was considered positive for DNase production (Gupta and Malik, 2007).

Statistical analysis

The experiments was carried out in duplicates, average was recorded, and Analysis of Variance (ANOVA) with Duncan Multiple Range Test for significance at P≤0.05 was also used.

3.0 RESULTS AND DISCUSSION

Fifty - five presumptive lactic acid bacteria were isolated from raw cow milk, raw goat milk and *nono* samples. On the basis of the cultural and morphological appearances, all the LAB isolates were small colonies, circular, whitish to creamy in colour, raised with entire edges. They were all Gram positive, short to long rods. On the basis of the identification tests, isolates were identified as *Pediococcus acidilactici, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus fermentum* and *Lactobacillus casei* according to Bergey's Manual of Determinative Bacteriology based on their similarities in characteristics with the organisms (Nikolic*et al*., 2008).The results was not shown.

3.1 Screening of LAB for probiotic potential

3.2 Antimicrobial activities of LAB against food –borne pathogens

The antagonistic activities exhibited by LAB isolated from raw milk and *nono* samples is presented in Table 1. Culture supernatants of the isolates obtained from *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus brevis, and Lactobacillus fermentum* exhibited varying degrees of inhibitory activity from 7 to 15 mm against strains of *Escherichia coli* ATCC8739, *Bacillus cereus* CMGB215, *Proteus* spp, *Bacillus sublitis*ATCC6633, *Shigella flexneri*ATCC29833 and *Salmonella typhimurium*ATCC13311. *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17 and *Lactobacillus brevis*N10isolated from *nono* samples had good inhibition against *Salmonella typhimurium*ATCC13311 with 15.00±0.00 mm, 15.15±2.76 mm, 15.10±1.27 mm, respectively and were not significantly different from each other at P≤0.05 but slightly different from *Lactobacillus casei*N1(14.00±0.57 mm). They also had inhibition against *Shigella flexneri*ATCC29833 and *E.coli*ATCC873. About 52.7 % of the LAB metabolites inhibited *Proteus* spp while 47.2% showed no inhibition. The results of some LAB are not shown in the table. In a study experimented by Yadesse *et al.* (2005), they suggested that the antimicrobial potential of LAB could be influenced by the medium they grew in, biochemical properties of the strains used including major factors such as physical and chemical conditions of growth.

However, these compounds produced by LAB lowered the pH of the medium which could result to strong antagonistic effect against the studied food -borne pathogens or indicator organisms (Krishnendra e*t al.*, 2013).

3.3 Tolerance (growth) of LAB to various NaCl concentration

The result showed that all the studied LAB isolates were able to tolerate 4-10% NaCl concentration with different growth rate as shown in Table 2. At 4.0% NaCl concentration, normal growth was observed by *Lactobacillus plantarum*N17, *Lactobacillus plantarum*N24, *Lactobacillus brevis*N10 and *Lactobacillus casei* N1,of which profuse growth was observed by them at 6.0% and 8.0% NaCl concentration. However, 70.37% (38) of the LAB tolerated 4.0% NaCl concentrations while 29.6% (16) were not able to grow. *Pediococococcus acidilactici* did not grow at 4% but grew at other concentration of NaCl. Moreover, 100% of the LAB were able to grow at 6%, 8% and 10% salt concentrations.The presence of this salt will have no adverse effects on the organisms.

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Hoque *et al*. (2010) reported the tolerance of some *Lactobacillus* species isolated from fermented milk products to 1- 9.0 % NaCl concentrations as indicated in their experiments. However, results of this experimental studies are similar to the work done by Adebayo-tayo and Onilude (2008), on the tolerance of LAB to 1- 6.5% NaCl.

3.4 Survival of LAB at acidic pH under various incubation time

The results of survival at different acidic pH are shown in Figures 1 and 2. The LAB isolates were able to grow at pH 2.5 for 45 minutes but *Lactobacillus plantarum*N24 exhibited the best survival (3.6 x 10^6 CFU/mL). However, all the isolates lost their viability except for *Lactobacillus plantarum*N17, *Lactobacillus plantarum*N24, *Lactobacillus casei*N1and *Lactobacillus brevis*N10 showing viability of 10^6 CFU/mL during 2 hr of incubation (1.0 x 10^6, 1.8 x 10^6, 2.8 x 10^6 and 1.5 x 10^6 CFU/mL, respectively). At pH 3.0 as shown in Figure 2, *Lactobacillus plantarum*N24 and *Lactobacillus plantarum*N17 survived better reaching viability of (5.5 x 10^6 CFU/mL) and (5.6 x 10^6 CFU/mL), respectively at 45 mins but *Lactobacillus brevis*N10 survived best after 3 hr of incubation.

Moreover, this work reveals that four LAB isolated from fermented milk product (*nono* samples) were able to survived pH 2.5 and 3.0 for 3 hr.. Kabore *et al*. (2012) also isolated LAB from a fermented milk product, and they were able to survive well at pH 2.5 and above, suggesting it could be intrinsical. Furthermore, LAB strains were shown such as *Lactobacillus casei* can resist pH 3.0 for 3 hr. This could be attributed to the medium they grew in or transfer of acidic genes that can resist low pH (Mishra and Prasad, 2005; Kumar and Kumar, 2015).

Table 1: Antimicrobial activities of LAB isolates against food-borne pathogens (diameter of zones of inhibition (mm))

LAB isolates	Zones of Inhibition (mm)					
	Bacillus cereus CMGB 215	*Proteus spp*	*E coli* ATCC 8739	*Salmonella typhimurium* ATCC 13311	*Bacillus subtilis* ATCC 6633	*Shigella flexneri*ATC C29833
*P. acidilactici*G1						
*P. acidilactici*G2						
*P. acidilactici*G3						
*P. acidilactici*G4						
*P. acidilactici*G5			7.00±0.00^{j}			
*P. acidilactici*G6						
*L. plantarum*G7	11.00±0.00^{def}	10.00±0.00^{fgh}	12.15±0.1^{b}	12.00±0.00^{cd}		
*P. acidilactici*G8						
*P. acidilactici*G9						
*P. acidilactici*G10						
*P. acidilactici*G11						
*L. plantarum*G12	8.00±0.00^{i}		10.70±0.5^{ef}	11.00±0.00^{def}		
*P. acidilactici*C1						
*P. acidilactici*C2						

DOI: 10.31695/IJASRE.2019.33268

*L. plantarum*C3	8.00±1.41^{i}	11.00±0.35^{ef}			10.00±2.62^{f}
*L. plantarum*C4	10.00±0.00^{fgh}	10.00±1.41^{fgh}			
*L. plantarum*C5		12.00±0.00^{b-f}	10.25±0.3^{f}	11.15±0.07^{de}	
*L. plantarum*C6				11.00±.0.00^{def}	

*Means of duplicates with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) for separation of statistically significant. 8-15mm = Good Inhibition, - = No inhibition, low inhibition= 7mm , G=Isolates from goat milk, C=Isolates from cow milk, N= Isolates from *nono* samples, LAB isolates = Lactic acid bacterial isolates

Table 1 (Cont'd):

LAB isolates	Zones of Inhibition (mm)					
	*Bacillus cereus*CMGB215	*Proteus spp*	*E coli* ATCC 8739	*Salmonella typhimurium* ATCC 13311	*Bacillus subtilis* ATCC 6633	*Shigella flexneriATCC 29833*
*L. plantarum*C7	11.00±0.35^{efg}	11.00±0.14^{def}	8.00±0.00^{g}			
*L. plantarum*C8	11.00±0.00^{def}					
*L. plantarum*C9		12.00±0.57^{c-f}			10.00±0.28^{ef}	
*L. plantarum*C10	10.00±1.41^{fgh}		10.00±0.28^{f}		11.00±2.55^{def}	
*P. acidilactici*C11						
*L. plantarum*C12	10.00±0.28^{fgh}	12.00±1.34^{b-f}		8.00±0.42^{g}	10.00±2.26^{ef}	
*L. plantarum*C13		13.00±1.41^{a-d}		10.20±0.28^{ef}	10.00±1.48^{f}	
*L. plantarum*C14	12.00±0.42^{cd} -	12.00±0.00^{b-f}				
*L. plantarum*C15	12.00±0.21^{cde}	10.00±2.83^{fgh}	11.00±0.28^{ef}	12.00±1.41^{cd}	12.00±1.41^{cd}	
*P. acidilactici*C16						
*P. acidilactici*C17						
*L. casei*N1	14.00±0.28^{ab}	13.00±2.83^{a-d}	13.00±0.21^{ab}	14.00±0.57^{ab}	14.00±0.14^{ab}	14.00±1.41^{b}
*L. plantarum*N2	12.00±0.21^{cde}	11.00±1.41^{def}	11.00±0.00^{ef}	13.00±0.00^{bc}	13.00±0.00^{bc}	
*L. plantarum*N3	11.00±2.83^{def}	12.00±0.28^{b-f}	11.35±0.49^{c-}			
*L. plantarum*N4		11.00±0.00^{def}				
*L. fermentum*N5	10.10±0.14^{fgh}	10.35±0.07^{ef}	14.25±0.35^{a}	14.00±0.00^{ab}	13.00±0.00^{bc}	

DOI: 10.31695/IJASRE.2019.33268

*L. plantarum*N6	15.00±0.00[a]	15.00±0.00[a]	14.00±0.00[a]	14.00±0.00[ab]	14.00±0.35[ab]
*L. plantarum*N7	10.00±0.00[fgh]	10.20±0.28[efg]		12.00±0.28[cd]	

*Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) separation of statistically significant means. Data collected were represented as "Means

Table 1(contd).

LAB isolates	Zones of Inhibition (mm) *Bacillus cereus* CMGB 215	*Proteus* spp	*E.coli*ATCC 8739	*Salmonella typhimurium* ATCC 13311	*Bacillus subtilis*ATCC 6633	*Shigella flexneriATCC C29833*
*L. brevis*N8	11.00±0.14[def]		14.30±0.42[a]	10.40±0.85[ef]		
*L. casei*N9		12.00±0.71[b-f]			12.00±0.00[cd]	
*L. brevis*N10	14.00±2.83[ab]	14.00±1.41[a-c]	10.20±0.28[f]	15.10±1.27[a]	15.00±0.00[a]	8.00±0.28[b]
*L.plantarum*N1	12.00±0.07[cde]		10.75±0.35[e-f]			
*Labrevis*N12	8.00±0.21[i]		12.20±1.13[b-e]	11.20±0.28[de]		
*L.fermentum*N13		11.00±0.49[def]	13.00±4.17[abc]			
*L. plantarum*N14	9.00±0.99[ghi]	12.00±1.41[b-f]		11.00±0.00[def]		
*L. casei*N15			11.00±0.42[def]	8.15±0.21[g]		
*L. plantarum*N16	10.00±0.21[fgh]	10.00±0.21[e-f]		11.00±1.41[def]		
*L. plantarum*N17	13.00±0.35[bc]	14.00±0.35[a-c]	12.00±2.69[b-e]	15.15±2.76[a]	14.00±1.56[ab]	15.00±1.41[a]
*L. fermentum*N18						
*L. plantarum*N19		12.00±0.21[b-f]	12.00±2.69[b-e]			
*L. plantarum*N20	13.00±1.34[bc]		13.00±1.41[a-d]			
*L. brevis*N21				11.05±2.89[def]	12.00±0.92[cd]	
*L. casei*N22	11.00±0.28[def]	11.00±0.71[def]		11.20±0.28[de]		
*L. plantarum*N23	9.00±2.61[hi]	10.00±2.12[e-h]		-		
*L. plantarum*N24	14.00±1.20[ab]	12.00±0.21[b-f]	14.00±0.28[ab]	15.00±0.00[a]	12.00±0.49[cd]	12.00±0.00[c]
*L. plantarum*N25		8.00±0.14[gh]	8.00±1.56[g]	9.80±0.57[f]		
L. plantarumN26		8.00±0.99[h]		10.05±0.07[ef]	10.00±1.41[f]	

Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range T (DMRT) for separation of statistically significant means. Data collected were represented as "Means of duplicates ± Stand

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Deviation (SD)" only. 8-15 mm = Good Inhibition, - = No inhibition, 7mm= low inhibition, LAB isolates= Lactic acid bacte
isolates

Table 2: Tolerance of LAB to various concentrations of NaCl

Isolates	NaCl concentrations (%)			
	4.0	6.0	8.0	10.0
*P. acidilactici*G1	-	+++	+++	++
*P. acidilactici*G2	-	+++	+++	++
*P. acidilactici*G3	-	+++	+++	++
*P. acidilactici*G4	-	+++	++	++
*P. acidilactici*G5	-	++	++	++
*P. acidilactici*G6	-	++	++	++
*L. plantarum*G7	-	++	++	+
*P. acidilactici*G8	-	++	++	+
*P. acidilactici*G9	-	++	++	+
*P. acidilactici*G10	-	++	++	++
*P. acidilactici*G11	-	+++	+++	+++
*L. plantarum*G12	++	++	++	++
*P. acidilactici*C1	-	++	+++	++
*P. acidilactici*C2	_	++	++	++
*L. plantarum*C3	++	++	++	++
*L. plantarum*C4	++	+++	++	++
*L. plantarum*C5	++	++	++	++
*L. plantarum*C6	++	++	++	++
*L. plantarum*C7	++	+++	++	++
*L. plantarum*C8	++	+++	++	++
*L. plantarum*C9	++	++	++	++
*L. plantarum*C10	++	++	++	++
*P. acidilactici*C11	-	++	++	++
*L. plantarum*C12	++	++	++	++
*L. plantarum*C13	++	++	+++	++
*L. plantarum*C14	++	++	++	+
*L. plantarum*C15	++	++	++	++
*P. acidilactici*C16	-	++	++	++
*P. acidilactici*C17	-	++	++	++
*L. casei*N1	++	+++	+++	+++
*L. plantarum*N2	++	++	++	+
*L. plantarum*N3	++	++	+	+

'+++ Profuse growth, ++ normal , + less growth, - no growth

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Table 2 contd.

Isolates	NaCl concentration (%)			
	4.0	6.0	8.0	10.0
*L. plantarum*N4	++	+++	+	+
*L. fermentum*N5	+	+++	+++	++
*L. plantarum*N6	+	++	++	++
*L. plantarum*N7	+	++	+	+
*L. brevis*N8	++	++	++	+
*L. casei*N9	+	++	++	++
*L.brevis*N10	++	+++	+++	+++
*L. plantarum*N11	+	++	++	++
*L. brevis*N12	++	++	++	+
*L. fermentum*N13	+	++	++	+
*L. plantarum*N14	+	++	++	+
*L. casei*N15	+	++	++	++
*L. plantarum*N16	++	++	++	++
*L. plantarum*N17	++	+++	+++	+++
*L. fermentum*N18	++	++	++	++
*L. plantarum*N19	++	++	++	++
L.plantarum 20	+	++	++	++
*L. brevis*N21	++	++	++	++
*L. casei*N22	++	++	++	+
*L. plantarum*N23	++	++	++	++
*L. plantarum*N24	++	+++	+++	++
*L. plantarum*N25	++	++	++	+
L. plantarumN26	++	++	+	+

'+++ Profuse growth, ++ normal growth, + less growth

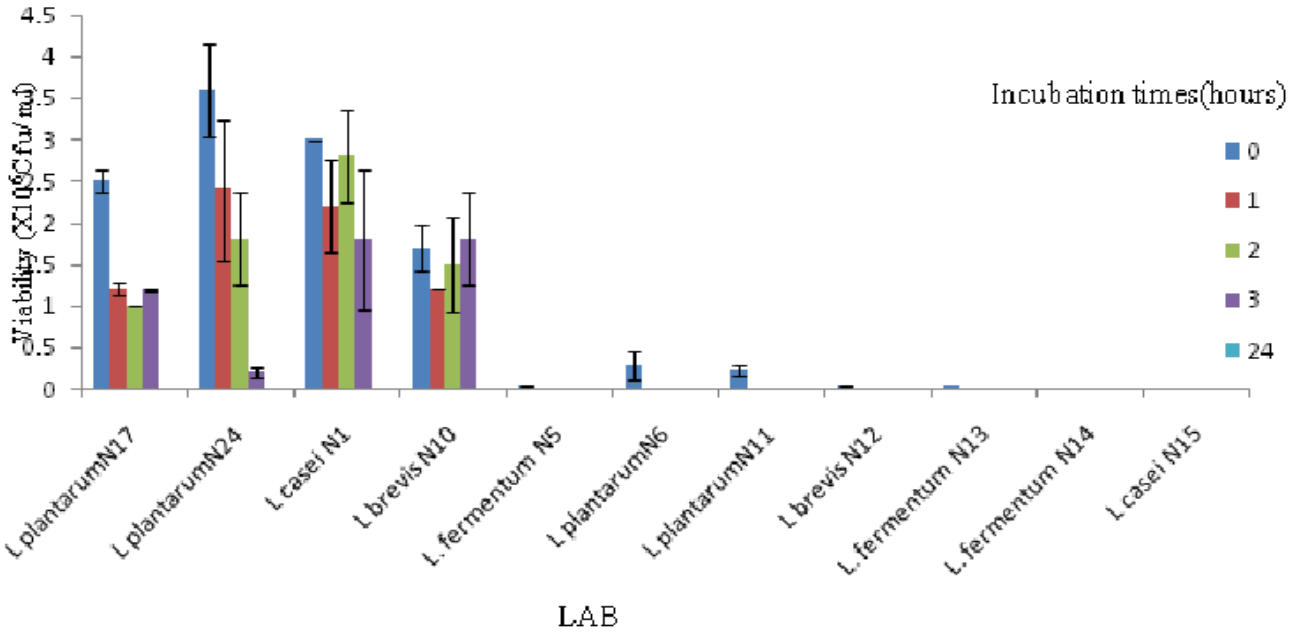

Figure 1: Survival of LAB at acidic pH(pH 2.5) under various incubation times (X10^6 CFU/mL). Error bars indicate standard deviations. 0 hr indicates the first(start) incubation time in this study = 45 minutes, inoculum size= 10^7 CFU/mL

DOI: 10.31695/IJASRE.2019.33268

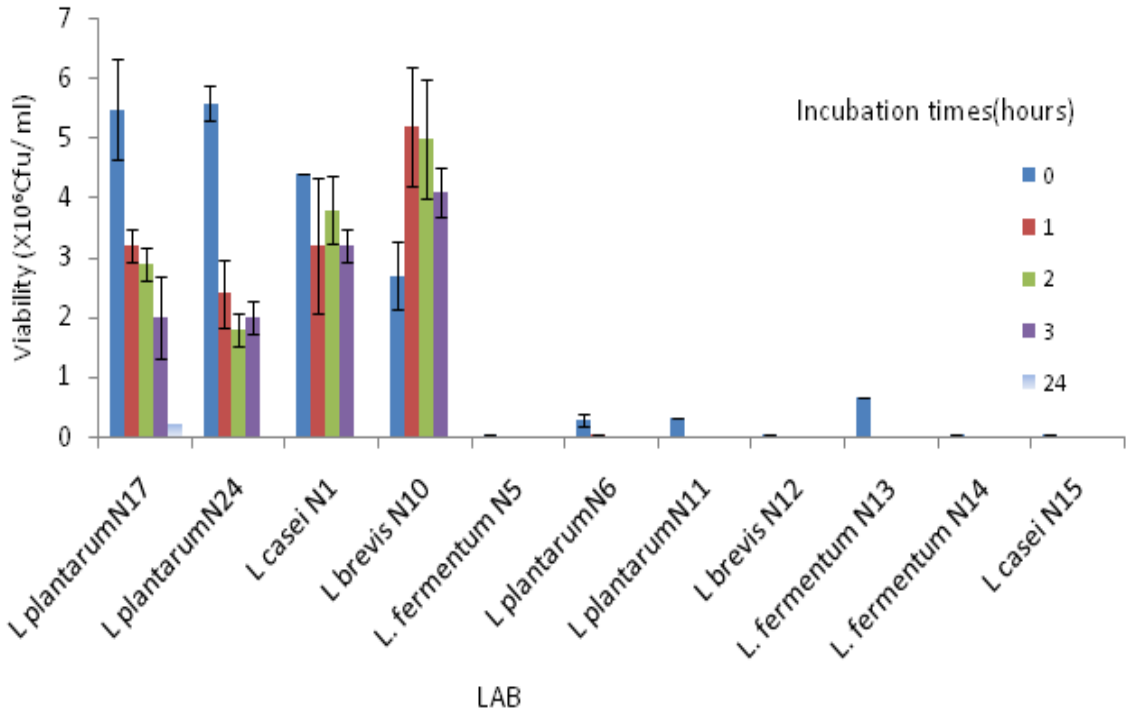

Figure 2: Survival of LAB at acidic pH(pH 3.0) under various incubation time (X10^6 CFU/ml). Error bars indicate standard deviations. 0 hr indicates the first(start) incubation time in this study =45 minutes, inoculum size = 10^7 CFU/mL

3.6 Tolerance of LAB to bile salts under various incubation time (CFU/mL).

Of all the studied LAB, four survived effectively at all the three tested bile salt concentrations (0.3%, 0.5%, 1.0%) as shown in Tables 3, 4 and 5 in which the initial inoculum size was 10^7 CFU/mL. At 0.3% bile concentration as shown in Table 3, the studied LAB showed good survival rate but the level of significance varied and ranged between 0.10 - 4.0 x 10^6 CFU/mL. *Lactobacillus plantarum*N17, *Lactobacillus brevis*N10, *Lactobacillus plantarum*N24, and *Lactobacillus casei*N1were seen as the best during (45 minutes) reaching viability of 10^6 CFU/mL (3.5±1.55, 3.0±1.55, 4.0±0.14, 3.00±1.13 CFU/mL), respectively but were not significantly different from each other at P≤0.05. They also survived 2 hrs of incubation. At 0.5% and 1.0% bile concentration as shown in Tables 4 and 5, only the four above isolates reached viability of 10^6 CFU/mL at 3 hrs of incubation. Generally, there was a slight decrease in the survival rate of the studied LAB as the bile concentration increased but four were able to grow effectively reaching viability of 10^5-10^6 CFU/mL except for the bile sensitive LAB which lost viability during one hour of incubation at 0.5% and 1.0% bile concentration. However, similar work were reported in an *in vitro* study of *Lactobacillus* strains which deconjugated the conjugated bile salts due to presence of bile salt hydrolases in the systems. This is attributed to presence of some enzymes that can convert the salts to less toxic substance. Furthermore, most probiotic bacteria were reported to survive and remain viable in MRS medium having above 0.5% conjugated bile salts (Noruga *et al*., 2006). Therefore, tolerance to bile salts had being used as a criteria or prerequisite for colonization and metabolic activity of bacteria in the intestine of most host. This present study also revealed that the organisms were able to tolerate at least 0.3% bile salts which is regarded as the concentration in human.

3.7 Adherence of LAB to intestinal mucosa using Microbial adherence to hydrocarbons (hydrophobicity assay)

Table 6 shows the adherence of LAB to hydrocarbons and there was a significant different (P<0.05) of hydrophobicity of the LAB to hydrocarbons. The percentage hydrophobicity for the tested LAB ranged between 20.8±0.56 to 62.0±0.99%. *Lactobacillus casei*N1 had the highest value of 62.0±0.99% towards chloroform which was significantly different (P<0.05) from that of *Lactobacillus plantarum*N17 (45.8±4.80 %) and *Lactobacillus brevis*N10 (40.3±0.00%). The least value (20.8±0.26%) was observed by *Lactobacillus fermentum*N13. *Lactobacillus plantarum*N24, *Lactobacillus brevis*N10, *Lactobacillus plantarum*N17 and *Lactobacillus casei*N1 showed above 40% towards the two solvents (chloroform and xylene) used in this study.

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In xylene, the percentage hydrophobicity ranged between 23.0-53.2%. The highest adherence (53.2±0.99%) was also observed by *Lactobacillus casei*N1 which was significantly different from *Lactobacillus plantarum*N24 (40.7±2.68%) and *Lactobacillus plantarum*N17 (40.7±2.68%). The least adherence was observed by *Lactobacillus fermentum*N5 showing 23.0±0.42%.

Microbial adhesion to hydrocarbon has been widely used to determine the cell surface hydrophobicity as adherence to intestinal mucosa or epithelial cells. Orlowaski and Bieleck (2006) suggested that such protocol could determined cell surface hydrophobicity. *Lactobacillus species* had above 40 % cell surface hydrophobicity towards chloroform and xylene indicating they are strong electron donors with good potential. Kos *et al*. (2003) reported that *Lactobacillus plantanum* had maximum cell surface hydrophobicity towards solvents like chloroform and xylene. Other studied LAB had lower percentage (≤ 40%) indicating that they are not good probiotics or cannot adhere to intestinal mucosa due to lack of electrostatic interactions. Martin *et al.* (2005) also reported that some LAB could have low affinity towards chloroform and xylene due to the absence of electrostatic interactions. However, surface hydrophobicity was determined in this study to test for possible correlation between the physiochemical property of LAB and the ability to adhere to the intestinal muscosa. This could also depends on the strain of the microorganisms, surface charge of the bacteria cell and ability of the LAB to express the some associated proteins (Nwanyanwu *et al*., 2012).

3.8 Safety assessment ofLAB based on Gelatinase and DNase production.

Th studied LAB were negative to both gelatinase and DNaseproduction at incubation period of 48 hrs suggesting that they are safe and lack the enzymes indicating. Therefore, they are good probiotic candidates. This is similar to the work of Hasegawa *et al*. (2010) who reported that probiotic organisms should not produce these enzymes because it can serve as substrates for pathogens and evade the immune systems.

3.9 Antibiotic susceptibility of LAB

Table 8 shows the antibiotic susceptibility of the LAB strains. LAB were sensitive to Ceftazidime, Cefuroxime, Gentamycin, Geftriaxone, Ofloxacin, Erythromycin, Augmentin, Cloxacin except *Lactobacillus fermentum*N5 which was resistance to the some of the antibiotics. *Lactobacillus plantarum*N6 and *Lactobacillus plantarum*N11 were also resistant to Augmentin and Cloxacin, respectively but sensitive to other antibiotics. This work reveals that most of the lactic acid bacteria including *Lactobacilluscasei*N1 and *Lactobacillus plantarum*N17were sensitive to all the antibiotics which could be intrinsical. This also shows that they lack resistant traits which prevent them from transferring resistant genes to pathogenic organisms. Handa and Sharma (2016) reported that *Lactobacillus plantarum* were sensitive to majority of the studied antibiotics suggesting such LAB are of advantage, especiallyin the case of transfer of resistance genes to pathogenic organisms**.**

Table 3: Tolerance of LAB to bile salts (0.3%) under various incubation time (X10^6 CFU/mL)

	Incubation time (hr)			
Isolates	0(45 minutes)	1	2	3
*L. plantarum*N17	3.5±0.84^{a**}	2.8±0.84^{a}	2.5±0.70^{a}	1.70±0.42^{a}
*L. plantarum*N24	4.0±0.14^{a}	3.1±0.70^{a}	1.8±0.42^{a}	1.00±0.00^{a}
*L. casei*N1	3.00±1.13^{a}	2.4±0.99^{a}	1.6±0.56^{a}	1.2±0.28^{a}
*L. fermentum*N5	0.35±0.04^{b}	0.10±0.00^{b}	-	-
*L. plantarum*N6	0.30±0.05^{b}	-	-	-
*L. plantarum*N11	0.28±0.05^{b}	-	-	-
*L. brevis*N12	0.18±0.02^{b}	0.13±0.00^{b}		
*L. fermentum*N13	0.18±0.08^{b}	-	-	-
*L. plantarum*N14	0.20±0.11^{b}	-	-	-
*L. casei*N15	0.27±0.00^{b}	-	-	-
*L. brevis*N10	3.0±1.55^{a}	2.5±0.14^{a}	2.8±0.84^{a}	0.20±0.04^{b}

**Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) for separation of statistically significant means. Data collected were represented as "Means of duplicates ±

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Standard Deviation (SD)"Values are in CFU/mL, - = not viable, inoculum size = 10^7 CFU/mL, 45 minutes was used as the first incubation time (start) in this study. Zero point (0.) indicates 10^5 CFU/mL

Table 4: Tolerance of LAB to bile salts (0.5%) under various incubation time (X10^6 CFU/mL)

	Incubation time (hr)			
Isolates	0(45 minutes)	1	2	3
*L. plantarum*N17	3.0±1.27^{a**}	2.4±1.13^{a}	1.8±0.42^{a}	1.4±0.14^{a}
*L. plantarum*N24	3.1±1.13^{a}	2.5±0.42^{a}	2.4±0.42^{a}	1.0±0.00^{b}
*L. casei*N1	2.5±0.56^{a}	2.0±0.70^{a}	1.4±0.42^{a}	1.4±0.00^{b}
*L. fermentum*N5	0.30±0.00^{b}	-	-	-
*L. plantarum*N6	0.28±0.02^{b}	-	-	-
*L. plantarum*N11	0.16±0.00^{b}	-	-	-
*L. brevis*N12	0.13±0.02^{b}	-	-	-
*L. fermentum*N13	0.17±0.07^{b}	-	-	-
*L. plantarum*N14	0.13±0.02^{b}	-	-	-
*L. casei*N15	0.20±0.00^{b}	-	-	-
*L. brevis*N10	2.60±1.13^{a}	2.4±0.14^{a}	0.2±0.07^{b}	0.14±0.05^{c}

**Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) for separation of statistically significant means. Data collected were represented as "Means of duplicates ± Standard Deviation .Values are in CFU/mL, - = not viable, inoculum size = 10^7CFU/mL, 45 minutes was used as the first incubation time (start) in this study.

Table 5: Tolerance of LAB to bile salts (1.0%) under various incubation time(X10^6 CFU/mL)

	Incubation time (hr)			
Isolates	0(45minutes)	1	2	3
*L. plantarum*N17	2.8±0.99^{a**}	2.0±0.42^{a}	1.2±0.28^{a}	1.2±0.00^{a}
*L. plantarum*N24	2.2±0.56^{a}	1.4±0.42^{b}	1.0±0.00^{a}	1.0±0.00^{a}
*L. casei*N1	2.0±0.70^{a}	1.6±0.28^{ab}	1.2±0.28^{a}	1.8±0.56^{a}
*L. fermentum*N5	0.28±0.01^{b}	-	-	-
*L. plantarum*N6	0.18±0.01^{b}	-	-	-
*L. plantarum*N11	0.12±0.02^{b}	-	-	-
*L. brevis*N12	0.13±0.00^{b}	-	-	-
*L. fermentum*N13	0.12±0.02^{b}	-	-	-
*L. plantarum*N14	0.14±0.01^{b}	-	-	-
*L. casei*N15	0.10±0.00^{b}	-	-	-
*L. brevis*N10	2.0±0.99^{a}	1.4±0.14^{b}	0.12±0.02^{b}	0.12±0.02^{b}

**Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) for separation of statistically significant means. Data collected were represented as "Means of duplicates ± Standard Deviation (SD)". Values are in CFU/mL, - = not viable, inoculum size = 10^7CFU/mL, 45 minutes was used as the first incubation time (start) in this study. Zero point (0.) indicates 10^5 CFU/mL

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Table 6: Adherence of LAB to intestinal mucosa using hydrophobicity assay

	Hydrophobicity (%)	
Isolates	Chloroform (mL)	Xylene (mL)
*L. plantarum*N17	45.8±4.80^{b*}	40.7±2.68^{bc}
*L. plantarum*N24	40.3±0.00^{bc}	45.8±2.12^{b}
*L. casei*N1	62.0±0.99^{a}	53.2±0.99^{a}
*L. fermentum*N5	32.5±1.41^{de}	23.0±0.42^{f}
*L. plantarum*N6	28.5±1.13^{ef}	30.7±2.54^{e}
*L. plantarum*N11	35.0±1.98^{cde}	38.5±0.99^{cd}
*L. brevis*N12	38.7±0.14^{cd}	28.5±1.27^{ef}
*L. fermentum*N13	20.8±0.56^{g}	35±2.26^{cde}
*L. plantarum*N14	25.0±2.54^{fg}	32.5±2.40^{de}
*L. casei*N15	22.0±0.00^{fg}	31.0±7.77^{e}
*L.brevis*N10	40.0±7.49^{bc}	41.0±0.14^{bc}

*Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT) for separation of statistically significant means. Data collected were represented as "Means of duplicates ± Standard Deviation (SD)"

≥ 40%=hydrophobic or adherence, less than 40% = not adhering

Table 7: Safety assessment of LAB based on Gelatinase and DNase production.

	Safety parameters	
Isolates	Gelatinase production	DNase production
*L. plantarum*N17	–	–
*L. plantarum*N24	–	–
*L. casei*N1	–	–
*L. fermentum*N5	–	–
*L. plantarum*N6	–	–
*L. plantarum*N11	–	–
*L. brevis*N12	–	–
*L. fermentum*N13	–	–
*L. plantarum*N14	–	–
*L. casei*N15	–	–
*L. brevis*N10	–	–

- = negative

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Table 8: Antibiotics susceptibility of LAB

LAB isolates	Ceftazidime	Cefuroxime	Gentamycin	Ceftriaxone	Ofloxacin	Erythromycin	Augmentin	Cloxacin
*L. plantarum*N17	S	S	S	S	S	S	S	S
*L. plantarum*N24	S	S	S	S	S	S	S	S
*L. casei*N1	S	S	S	S	S	S	S	S
*L. fermentum*N5	R	S	R	S	S	R	R	R
*L. plantarum*N6	S	S	S	S	S	S	R	S
*L. plantarum*N11	S	S	S	S	S	S	S	R
*L. brevis*N12	S	S	S	S	S	S	S	S
*L. fermentum*N13	S	S	S	S	S	S	S	S
*L. plantarum*N14	S	S	S	S	S	S	S	S
*L. casei*N15	S	S	S	S	S	S	S	S
*L. brevis*N10	S	S	S	S	S	S	S	S

Keys: The range according to CSLI, 2000., R= Resistance, S= Sensitive

5.0 CONCLUSIONS

The results showed that LAB strains such as *Lactobacillus casei*N1, *L.brevis*N10, *L. plantarum*N24,and *L. plantarum*N17from fermented milk product (*nono)* had maximum antimicrobial activity, survived acidic pH and bile, sensitive to various antibiotics, and safe indicating they are suitable probiotic candidates which can be used as food supplements.

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