POTENTIAL PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM MALTED AND SPONTANEOUSLY FERMENTED ACHA (*Digitaria exilis*) FLOUR.

ABSTRACT

Aim: To isolate, characterise and identify Lactic Acid Bacteria (LAB) with potential probiotic properties from malted and fermented Acha (*Digitaria exilis*).
Place and Duration of Study: The study was carried out at the Department of Microbiology, University of Ibadan, between May 2017 and October, 2017.
Methodology: Collected acha grains was sorted, malted for 48 hours, dried milled and sieved. The flour was reconstituted, fermented spontaneously for 72 hours and sampled every 24 hours for isolation of LAB.
Results: The probiotic properties of 40 LAB strains isolated were evaluated *in vitro*. Based on their antimicrobial activity against some common foodborne pathogen and antibiotic susceptibility pattern to standard antibiotics, 14 LAB strains were selected for further screening. They all exhibited strong antimicrobial activity against *Staphylococcus aureus, Salmonella sp., Escherichia coli, Bacillus sp.* and *Pseudomonas aeruginosa.* All selected strains were investigated for acidic pH and bile salt tolerance, tolerance to NaCl and simulated gastric juice, cell surface characteristics which includes hydrophobicity and auto-aggregation assay. Production of DNAase, gelatinase, Exopolysaccharide and haemolytic ability were investigated for safety assessment. Five strains (L12, L15, L118, L213, L214) exhibited tolerance to 10% NaCl concentration and pH 2. All the selected 14 LAB strains resisted the bile salt up to 1.0% except L15 and tolerated simulated gastric juice with a sharp decrease by 90min followed by an increase in count after 180min except for isolates L12 and L17. Auto-aggregation value ranged from 0.88% to 93.71% with the highest value recorded for L17 (93.71%). LAB117 had highest (43.39%) microbial adherence to hydrocarbon (MATH) value and least in L19 (2.08%). All the 14 LAB strains were negative to safety tests but all produced exopolysaccharide except LAB L13 and L19. Based on the morphological, biochemical and physiological characteristics, the 14 LAB isolates were identified as *Lactobacillus plantarum* (L15, L17, L117, L214), *Lactobacillus casei* (L113, L116, L213), *Lactobacillus sp*. (L13, L19, L22, L211), *Enterococcus sp*. (L12, L115) and *Pediococcus* sp. (L118).
Conclusion: The selected 14 isolates have the probiotic properties required for use as potential probiotic in weaning food supplements with the best probiotic properties recorded with *Lactobacillus plantarum* L117 strain.

KEYWORDS: Malted acha, Probiotic properties, Foodborne pathogen, Antimicrobial activity, Safety assessment**.**

1.0 INTRODUCTION

Locally fermented foods have been produced through the activity of microorganism such as Lactic acid bacteria (LAB), yeasts etc. via the process of fermentation [1, 2, 3]. This could be achieved either by spontaneous or controlled fermentation of cereals. Acha (*Digitaria exilis* Stapf) is a traditional African cereal in the family Gramineae. Grown in areas with low rainfall especially in the plateau and savannah. In Nigeria, it is widely grown in the cool region of Plateau State, part of Bauchi, Kebbi, Taraba, Kaduna and Niger States. It is either the staple food or a major part of the diet. The crop supplies food to 3-4 million people [4].

Fermentation in food processing is the conversion of carbohydrates to alcohol and carbon dioxide or organic acids using yeast and/or bacteria, under anaerobic conditions [5]. The production of these paste-like fermented foods which also serves as weaning food sometimes become a course for concern as the paste is prone to the risk of contamination in the production and handling [6] and exposes the infant to the risk of diarrhoea. The handlers of traditional fermented foods need to be conscious of food hygiene, as there are many instances where food is contaminated by bad handling after cooking. LAB fermentation fits into primary care initiatives and can reduce child mortality by supplying the minimum required nutrients [1, 7]. Lactic acid bacteria (LAB) isolated from these foods displayed probiotic properties such as hypolipidemic, hepatoproctective and antibacterial and had been found to be effective in treating gastroenteritis in man and animals [2].

Probiotics by FAO/WHO are live microorganisms which when administered in adequate amounts; confer a health benefit on the host by improving the intestinal microbial balance [8, 9, 10]. Probiotic bacteria are able to change the population of the gut micro biota by influencing the metabolic and nutritional functions of commensal bacteria.

The use of probiotic requires that the microorganism must be screened and selected strains must meet safety, technological, functional and physiological requirements [9, 10, 11, 12]. This study is therefore aimed at the isolation and screening of Lactic Acid Bacteria with good probiotic potentials from spontaneously fermented *Digitaria exilis.*

2.0 MATERIALS AND METHODS

2.1 Collection of sample

Acha (*Digitaria exilis*; unprocessed (hulled) was bought from a major market in Jos, Plateau State, Nigeria and brought to the Postgraduate laboratory of Department of Microbiology, University of Ibadan, and kept in air tight containers at 4^0C until use.

2.2 Enumeration and Isolation of Microorganisms

A 1:10 dilution of the fermenting slurry was made up to 10^{-10} with sterile distilled water, 0.5ml of higher dilutions were pour-plated out onto De Mann Rogosa and Sharpe (MRS) medium for lactic acid bacteria (LAB) isolation. The plates were incubated at 35±2^{o}C anaerobically for 48 hours. Pure cultures of the

isolates were obtained by sub-culturing unto fresh MRS agar plates twice and young pure cultures were used for further screening.

2.3 Evaluation of Probiotic Potential of the LAB Isolates

2.3.1 Determination of antagonistic activity of lactic acid bacteria isolates against some selected pathogens

The antagonistic activities of the isolated LAB against selected pathogenic indicator organisms such as *Pseudomonas aeruginosa, Escherichia coli, Bacillus* spp.*, Salmonella* spp. and *Staphylococcus aureus* was determined using agar well diffusion method of Mahnaz *et al*. [13]. The test LAB isolates were inoculated into MRS broth, incubated at 35 ± 2^{o}C for 48 hours and cell- free culture supernatant was obtained by centrifuging the MRS broth culture at 6000 rpm for 20 minutes. 60 microlitres (µl) of the supernatant was dispensed into each well in the plates and tested against approximately 1.5 × 10^{8} cfu/ml of indicator organisms on Mueller Hinton agar and incubated aerobically at 37˚C for 24 hours. Zones of inhibition around each well indicated the antagonistic activity of the LAB isolate against the indicator organisms.

2.3.2 Antibiotic Susceptibility Test

The antibiotic disc diffusion method was used for the LAB isolates as described by Pundir *et al*.[14]. The diameter of zones of inhibition around each disc was noted and recorded.

2.3.3 Assay for Sodium Chloride Tolerance.

The determination of tolerance for the LAB isolates was carried out using MRS broth adjusted to different concentration of sodium chloride (NaCl) (2, 4, 6, 8 and 10% w/v). Observation for growth was qualitatively determined by checking for turbidity compared with the control. Maximum growth were indicated (++), normal growth (+) and no growth (-) [15].

2.3.4 Growth at different pH

One millilitre of overnight cultures of LAB cultures adjusted to 0.5 Mac Farland's standard were inoculated into MRS broth with varying pH values of 2, 3, 4, 5 and 6. The inoculated broth was incubated at 35±2^{o}C for 24 hours. Growth was determined spectrometrically at optical density 560nm against the unadjusted, uninoculated broth as blank or control [15].

2.3.5 Bile Salt Tolerance

A modified method of Oluwajoba *et al.* [16] and Agaliya and Jeevaratnan, [17] were employed. All cultures were evaluated for growth in MRS broth containing bile salt No.3 (Oxoid, England) in the following concentrations 0.05, 0.1, 0.3, 0.6, and 1% (w/v). An aliquot of 0.5ml of freshly prepared cell suspension adjusted to 0.5 Mac Farland's standard was inoculated into bile containing medium, the experiment was allowed to stand for four hours. During the four hours, viable cells or bacterial growth were monitored at the first and the fourth hour spectrophotometrically at 620nm (JENWAY 6850uv/vis), followed by pour plating of 0.1millilitre of each concentration and incubated at 37°C. Bacterial growth was enumerated by plate counts after 24 hours of incubation, while tube without bile salt served as control.

2.3.6 Microbial Adherence to Hydrocarbon (MATH) Test.

Adhesion to hydrocarbons was carried out using a modified method of Rosenberg *et al*.[18] and Agaliya and Jeevartnam [17]. The isolates were grown in MRS broth at 37^{o} C for 24 to 48 hours. LAB were harvested at 4000 rpm for 15 minutes and washed twice in PBS (phosphate buffer saline, pH 7.0) and re-suspended in PBS and the Optical Density (OD) determined at 600nm. Then 3mls of the bacterial suspension was mixed with 1ml of hydrocarbon (xylene) and vortex (using "mrc" VORTEX MIXER) at speed 10 for 120 seconds. For the separation or partitioning of the aqueous and organic phase, it was then incubated at 37^{o}C for 30 minutes. 1ml of the lower aqueous phase was removed carefully and the optical density (OD at 600nm) was determined.

% Hydrophobicity = $[OD_{initial} - OD_{final}]/ OD_{initial} \times 100$

Where OD= optical density

$OD_{initial}$ and OD_{final} are the absorbance before and after extraction with hydrocarbon.

2.3.7 Autoaggregation assay

Autoaggregation assay was performed as described by Sourabh *et al.,* [19] and Syal and Vohra [20] with minor modifications. The cells were harvested by centrifugation (4000×g) for 15mins and suspended in phosphate buffered saline (PBS) to 0.5 optical density (O.D.) units at 600 nm. Percent autoaggregation ability was calculated as:

1- (O.D. upper suspension/O.D. total bacterial suspension) × 100.

2.3.8 Determination of upper gastric transit tolerance

Methods of Charteris *et al*. [21] and Sourabh *et al*. [19] were adopted. To 0.2ml of washed cells suspension in a 2.0ml capacity microfuge tubes were admixed 1.0ml of stimulated gastric juice and 0.3ml NaCl (0.5w/v). The content was vortexed using mrc Vortex mixer at speed 5 for 10 seconds and incubated at 37^{o}C for 3 hours. During the assay 0.1ml of the aliquot was removed at 1 minute, 90 minutes and 180 minutes and plated on MRS agar, incubated at 37^{o}C for 24- 48hours to determine the viable count of the organisms.

2.3.9 Growth at different temperature

The LAB were grown in 10 ml of MRS broth and tubes incubated at 30^{o}C and 37^{o}C for 48 hours. The test was performed in triplicate for each selected strain. 0.1 ml from each serially diluted tube were cultured on MRS agar plates, incubated at 37ºC micro aerobically for 48 h and followed by determination of viable count.

2.4 Safety Assessment of Selected LAB Strains

2.4.1 DNAase test

The selected LAB isolates was streaked on DNAase agar medium (Oxoid) and incubated at 30 $\pm$ 2^{o}C for 48 hours to check for the production of DNAase enzyme. After incubation, a clear pinkish zone around the colonies was considered positive for DNAase production [22].

2.4.2 Gelatinase Activity

Gelatinase activity was investigated as described by De La Cruz and Torres [23]. A 24 hour old culture was spot-inoculated into nutrient gelatine agar (Oxoid, England). The plates were incubated anaerobically

for 48 hour at 37°C after which they were flooded with ammonium sulphate solution and observed for clear zones surrounding colonies (positive reaction for gelatine hydrolysis).

2.4.3 Exopolysaccharide Production Assay (EPS)

Pure cultures of the LAB isolates were point inoculated on MRS agar supplemented with 0.2 g/l of sodium azide, 0.12 g/l of bromocresol purple and 2% (w/v) sucrose. The plates were incubated at 37^{o}C for 24 to 48 hours, plates with yellow colouration were positive for EPS production [24].

2.5 Characterization and Identification of Isolated Bacteria.

Characterization of LAB isolates with probiotic potentials was carried out using morphological, physiological, biochemical tests (API 50CH kits) and identification using PIB Win software.

3.0 RESULTS

All Gram positive, catalase negative rods and cocci (preliminary screening of LAB isolates from malted and spontaneously fermented acha) yielded a total of 40 LAB isolates were subjected to screening for their probiotic potential.

The result of the antimicrobial activity of the LAB isolates against selected pathogens is as shown in table 1. A high number (90%) of the LAB isolates produced metabolites that inhibited the growth of *Pseudomonas aeruginosa*, the highest zone of inhibition was observed in isolate L14 (19.5mm) and the least was in L26 (2.0mm). *Staphylococcus aureus* was susceptible to metabolites produced by all the LAB isolates, the zone of inhibition ranged from 1.0mm to 17.0mm in L28 and L118 respectively. *Salmonella* sp. was susceptible to metabolites produced by 87.5% of LAB isolates with zones of inhibition ranging from 0.5mm (L218) and 11mm (L211). *Escherichia coli* had 75% susceptibility to metabolites secreted and 25% of produced metabolite was not active against *E.coli,* the zones of inhibition ranged from 2.0mm (L25) to 14.5mm (L116). *Bacillus* sp. had 80% susceptibility and the diameter of the zone of inhibition ranged from 3.0mm (L25) to 11.5mm (L19).

The antibiotic susceptibility pattern of the LABs to different antibiotics is shown in table 2. Isolates L117, L215 and L218 showed resistance to all the antibiotics, while the other LAB's were either highly susceptible or partially susceptible to the antibiotics.

The LAB isolates with high antimicrobial activity against all the test pathogens and with good antibiotics susceptibility pattern were further selected for screening of their probiotic potential. Of these 14 LAB isolates were selected; L12, L13, L15, L17, L19, L113, L115, L116 L117, L118, L22, L211, L213 and L214.

The antibiotic susceptibility pattern of the LABs to different antibiotics is shown in table 2. Isolates L117, L215 and L218 showed resistance to all the antibiotics, while the other LAB's were either highly susceptible or partially susceptible to the antibiotics.The LAB isolates with high antimicrobial activity against all the test pathogens and with good antibiotics susceptibility pattern were further selected for screening of their probiotic potential. Of these 14 LAB isolates were selected; L12, L13, L15, L17, L19, L113, L115, L116 L117, L118, L22, L211, L213 and L214.

TABLE 1: ANTIMICROBIAL ACTIVITY OF LAB ISOLATES AGAINST SELECTED PATHOGENS

	DIAMETER OF ZONE OF INHIBITION (mm)				
Isolate Code	*Pseudemonas aeruginosa*	*Staph. aureus*	*Salmonella*	*E.coli*	*Bacillus* sp.
L11	11.5^{h}	9.5^{h}	2.5^{n}	7.5^{f}	9.0^{d}
L12	9.5^{j}	1.5^{v}	9.5^{c}	8.0^{e}	8.0^{f}
L13	11.5^{h}	7.5^{k}	7.5^{f}	4.5^{k}	6.5^{i}
L14	19.5^{a}	5.0^{p}	5.5^{j}	10.5^{b}	3.0^{o}
L15	9.5^{j}	7.5^{k}	6.5^{h}	4.0^{l}	3.0^{o}
L16	8.5^{l}	7.5^{k}	7.0^{g}	3.5^{m}	5.0^{k}
L17	14.5^{c}	10.0^{g}	8.5^{e}	7.5^{f}	10.0^{c}
L18	8.0^{m}	8.0^{j}	9.0^{d}	2.5^{o}	—
L19	9.5^{j}	11.5^{f}	3.0^{m}	5.0^{j}	11.5^{a}
L110	12.5^{f}	12.0^{e}	1.5^{p}	9.5^{d}	5.0^{k}
L111	3.5^{t}	2.0^{u}	—	—	—
L112	—	2.5^{t}	—	—	—
L113	15.0^{b}	13.0^{c}	7.0^{g}	9.5^{d}	10.5^{b}
L114	9.5^{j}	6.5^{m}	2.0^{o}	3.0^{n}	3.5^{n}
L115	7.5^{n}	7.0^{l}	3.5^{l}	7.5^{f}	7.0^{h}
L116	13.0^{e}	13.5^{b}	6.0^{i}	14.5^{a}	10.5^{b}
L117	12.5^{f}	12.5^{d}	3.0^{m}	10.0^{c}	7.5^{g}
L118	13.5^{d}	17.0^{a}	6.5^{h}	9.5^{d}	10.5^{b}
L21	8.5^{l}	6.5^{m}	3.0^{m}	5.0^{j}	7.5^{g}
L22	12.0^{g}	3.5^{s}	6.5^{h}	7.0^{g}	6.5^{i}
L23	5.5^{r}	5.0^{p}	3.0^{m}	5.5^{i}	5.0^{k}
L24	10.0^{i}	3.5^{s}	2.0^{o}	6.5^{h}	5.0^{k}
L25	4.5^{s}	6.0^{n}	—	2.0^{p}	3.0^{o}
L26	2.0^{v}	8.0^{j}	0.5^{q}	3.0^{n}	4.0^{m}
L27	7.5^{n}	5.0^{p}	—	5.5^{i}	8.5^{e}
L28	3.0^{u}	1.0^{w}	—	—	—
L29	—	1.0^{w}	—	4.0^{l}	—
L210	9.5^{j}	7.0^{l}	3.5^{l}	——	—
L211	5.5r	4.0^{r}	11.0^{a}	4.5^{k}	4.5^{l}
L212	6.0^{q}	5.0^{p}	9.0^{d}	5.5^{i}	8.5^{e}
L213	9.5^{j}	5.5^{o}	10.5^{b}	5.5^{i}	6.0^{j}
L214	9.0^{k}	7.0^{l}	9.0^{d}	3.0^{n}	3.5^{n}
L215	4.5^{s}	4.5^{q}	2.0^{o}	5.0^{j}	7.0^{h}
L216	7.0^{o}	3.5^{s}	4.5^{k}	—	8.0^{f}
L217	—	2.5^{t}	3.0^{m}	1.0^{q}	—
L218	—	2.0^{u}	0.5^{q}	—	—
L219	7.5^{n}	5.5^{o}	8.5^{e}	—	3.5^{n}
L220	6.5^{p}	8.5^{i}	7.0^{g}	—	3.5^{n}
L221	7.0^{o}	5.0^{p}	6.5^{h}	—	3.5^{n}
L222	7.0^{o}	4.5^{q}	5.5^{j}	—	4.5^{l}

Mean are based on duplicate reading. Mean within the same column of selected pathogen with different superscripts are significantly different using the Duncan multiple range test at $p \leq 0.05$.

KEY: —: No Zone of Inhibition

TABLE 2: ANTIBIOTICS SUSCEPTIBILITY OF LAB ISOLATES FROM SPONTANEOUSLY FERMENTED ACHA

Isolate Code	Diameter of Zone of Inhibition (mm)							
	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG
L11	—	—	—	17.0	24.0	—	—	25.0
L12	—	2.2	3.5	16.5	23.5	—	—	21.0
L13	—	—	—	20.0	25.5	—	26.0	33.0
L14	5.5	23.0	4.5	18.0	23.5	7.0	—	21.5
L15	—	—	—	17.5	29.0	—	30.5	36.0
L16	6.0	26.5	7.5	24.5	25.0	4.0	—	24.5
L17	—	—	—	15.5	24.5	—	11.0	28.0
L18	35.0	41.0	7.0	21.0	24.5	6.0	—	42.5
L19	6.0	23.5	7.0	20.5	19.0	7.5	13.0	23.0
L110	—	—	—	12.5	28.5	—	12.0	26.5
L111	7.5	23.0	9.5	20.5	24.0	6.0	—	19.0
L112	7.0	21.0	8.5	17.5	22.0	5.5	—	21.5
L113	5.5	20.0	3.5	14.5	25.5	12.0	9.0	21.5
L114	5.0	21.0	5.5	18.5	28.0	4.5	6.0	20.5
L115	41.0	30.0	28.0	41.0	44.0	38.0	30.5	39.5
L116	5.5	23.0	6.5	17.0	23.5	—	—	21.5
L117	—	—	—	—	—	—	—	—
L118	7.5	21.5	2.5	12.0	24.5	5.0	5.0	22.5
L21	38.0	—	12.0	32.0	37.5	29	38	38.0
L22	15.0	19.0	8.0	15.0	18.0	10	11	22.5
L23	33.0	10.0	13.5	29.5	32.0	—	30.0	39.5
L24	8.5	19.5	8.5	18.0	17.5	9.0	10.5	20.0
L25	10.0	20.5	7.0	18.0	18.0	9.5	10.5	18.5
L26	18.5	24.5	8.5	20.0	19.5	10.0	12.5	18.0
L27	12.5	20.0	8.5	19.5	18.0	9.5	11.5	22.5
L28	18.0	25.5	11.0	19.0	20.5	13.0	14.5	26.0
L29	6.0	22.5	10.0	20.0	20.5	5.5	—	21.5
L210	5.5	24.0	6.5	19.0	23.5	5.0	—	20.5
L211	6.5	21.5	8.0	18.5	22.5	—	—	18.0
L212	33.0	10.5	12.0	16.5	26.5	—	30.0	38.0
L213	35.0	30.0	26.5	36.5	38.5	31.5	29.0	39.0
L214	45.0	44.0	14.5	38.0	41.0	37.0	40.0	49.0
L215	—	—	—	—	—	—	—	—
L216	21.0	26.0	19.0	25.0	22.5	10.0	16.0	27.0
L217	31.0	13.5	13.5	33.0	36.5	29.0	26.0	37.0
L218	—	—	—	—	—	—	—	—
L219	36.0	13.5	12.0	33.5	37.0	29.5	31.5	41.0
L220	32.0	25.5	14.0	21.5	35.5	20.5	17.0	28.0
L221	35.0	15.0	12.5	34.0	37.5	33.0	34.0	42.0
L222	37.0	29.5	27.5	38.5	41.0	36.0	33.5	42.0

KEY: Ceftazidime (CAZ); Cefuroxime (CRX); Gentamycin (GEN); Ceftriaxone (CTR); Erythromycin (ERY); Cloxicillin (CXC); Ofloxacin(OFL); Amoxycillin/ Clavulinate (AUG)

The ability of the selected LAB to tolerance sodium chloride at 2% to 10 % (w/v) concentrations is shown in table 3. Tolerance and growth in sodium chloride was high between 2% and 6%, for all the LAB isolates. At 8% concentration, isolates L12, L13, and L22 showed no growth, slightly turbid growth was observed in isolates L15, L17, L19, L116 and L214, while at 10% concentration, tolerance to NaCl was observed among isolates L115, L211 and L213.

Figure 1 shows the growth response of LAB isolates at different pH range of 2 to 6. As the acidity decreases or pH increases, the growth of the LAB isolates increased. All the LAB isolates grew well at pH 6 except for isolate L13. At pH 4, there was high growth in all the LAB isolates except in L13 which showed slight growth. At pH 5 and 6, the tolerance to pH was high except for isolate L13 which had a very low growth.

TABLE 3: GROWTH OF LAB ISOLATES AT DIFFERENT CONCENTRATION OF SODIUM CHLORIDE (NaCl)

	NaCl Concentration (%)				
ISOLATE CODE	2	4	6	8	10
L12	++	++	++	—	—
L13	++	++	+	—	—
L15	++	++	++	++	—
L17	++	+	++	+	—
L19	++	++	++	+	—
L113	++	++	++	++	—
L115	++	++	++	++	+
L116	++	++	++	+	—
L117	++	++	++	++	—
L118	++	+	++	++	—
L22	++	++	++	—	—
L211	++	++	++	++	+
L213	++	+	++	++	+
L214	++	++	++	+	—

KEY: ++ (Very turbid) + (slightly turbid) — (no growth)

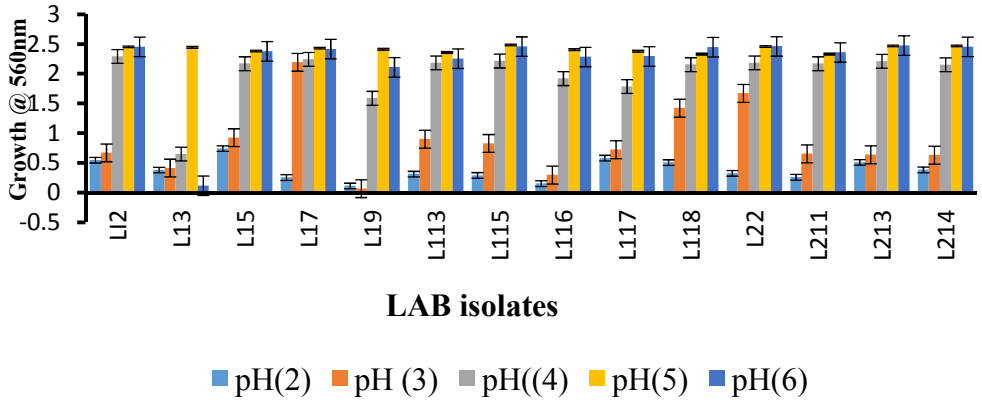

FIG 1: Effect of different Hydrogen ion Concentration (pH) on growth of LAB isolates

Tolerance of the LAB isolates to bile salt of different concentrations (0.05%, 0.1%, 0.3%, 0.6% and 1.0%) after 4 hours holding time is shown in table 4. A decrease in colony count as the bile salt concentration increased and tolerance to bile salt was observed up to 1% concentration except for isolate L115 which had no growth after 0.3% bile concentration. Isolate L118 tolerated all the bile salt concentrations with little or no growth as the bile salt concentration increases.

The gastric tolerance assay for the LAB isolates at 1, 90 and 180 minutes holding time before plating showed that the LAB isolates tolerated gastric juice with a sharp decrease by 90minutes incubation time(Table 5). This was followed by an increase in the colony count after 180minutes of incubation except for isolates L12 and L17 which decreased throughout the 180minutes assay time and isolate L118 which increased in count throughout the assay time.

Generally, high hydrophobicity values were not observed in the LAB isolates, except for isolate L117**.** Table 6 shows the result of auto aggregation and hydrophobicity assays determined spectrophotometrically and expressed as a percentage. The auto aggregation varied from 0.88% to 93.71%, with the highest for the LAB isolate obtained in L19 (93.71%) and the least in isolate L118 (0.88%). The microbial adherence to hydrocarbon (MATH) or hydrophobicity assay of the LAB isolate ranged from 2.08% to 43.39% with the highest obtained in isolate L117 (43.39%) and the least obtained in isolate L17 (2.08%).

The safety assessment of the isolates is shown in Table 7. All the LAB isolates were negative for the production of the enzyme DNAase and gelatinase, and were all positive for exopolysaccharide production except isolates L13 and L19 which were not exopolysaccharide producers.

TABLE 4: TOTAL VIABLE COUNTS OF LAB ISOLATES AFTER FOUR HOURS INCUBATION IN BILE SALT.

		Viable colony count (cfu/ml)				
		Bile salt concentration (%)				
Isolate code		0.05	0.10	0.30	0.60	1.00
LAB	L12	TNTC	TNTC	1.2×10^{3}	3.9×10^{2}	3.8×10^{2}
	L13	TNTC	TNTC	1.1×10^{3}	1.0×10^{3}	8.8×10^{2}
	L15	4.6×10^{4}	1.2×10^{4}	2.4×10^{4}	NG	NG
	L17	TNTC	TNTC	1.4×10^{4}	1.4×10^{4}	1.0×10^{4}
	L19	TNTC	TNTC	TNTC	TNTC	TNTC
	L113	TNTC	TNTC	TNTC	TNTC	TNTC
	L115	TNTC	TNTC	TNTC	2.1×10^{4}	2.0×10^{4}
	L116	TNTC	TNTC	TNTC	TNTC	TNTC
	L117	TNTC	TNTC	TNTC	TNTC	TNTC
	L118	1.9×10^{4}	1.9×10^{4}	1.6×10^{4}	1.5×10^{4}	1.1×10^{4}
	L22	TNTC	1.1×10^{4}	8.9×10^{3}	6.0×10^{3}	4.6×10^{3}
	L211	TNTC	TNTC	TNTC	TNTC	TNTC
	L213	TNTC	TNTC	TNTC	TNTC	TNTC
	L214	TNTC	TNTC	TNTC	TNTC	TNTC

KEY: TNTC (Too Numerous to Count) NG (No growth)

TABLE 5: GASTRIC TRANSIT TOLERANCE ASSAY

	Assay Time (minutes)/ Total viable count (× 10^3 cfu/ml)			
Isolate code	Before assay	1	90	180
L12	2.7	2.4	1.2	1.1
L13	TNTC	3.4	1.5	2.1
L15	TNTC	3.0	0.7	1.9
L17	TNTC	TNTC	3.9	TNTC
L19	TNTC	0.6	2.5	2.1
L113	TNTC	1.6	0.6	2.4
L115	TNTC	1.4	0.7	0.9
L116	TNTC	4.5	1.3	2.5
L117	TNTC	2.2	1.3	0.8
L118	1.3	1.2	2.2	4.3
L22	TNTC	1.7	1.2	5.1
L211	TNTC	1.9	0.6	2.0
L213	TNTC	0.7	0.3	2.4
L214	TNTC	2.1	0.6	0.8

KEY: TNTC (Too Numerous To Count)

TABLE 6: AUTOAGGREGATION AND HYDROPHOBICITY ASSAY OF THE LAB ISOLATES.

	Isolate code	Autoaggregation %	Hydrophobicity %
LAB	L12	89.85	9.92
	L13	83.38	3.76
	L15	88.13	2.23
	L17	70.08	2.08
	L19	93.71	6.61
	L113	44.55	8.06
	L115	25.88	6.87
	L116	56.08	16.70
	L117	33.07	43.39
	L118	0.88	4.89
	L22	47.55	4.21
	L211	45.40	7.32
	L213	51.22	6.01
	L214	26.24	7.93

TABLE 7: SAFETY ASSESSMENT OF LAB AND YEAST ISOLATES

	Isolate code	Gelatinase production	DNAase test	Exopolysaccharide production
LAB	L12	—	—	+
	L13	—	—	—
	L15	—	—	+
	L17	—	—	+
	L19	—	—	—
	L22	—	—	+
	L113	—	—	+
	L115	—	—	+
	L116	—	—	+
	L117	—	—	+
	L118	—	—	+
	L211	—	—	+
	L213	—	—	+
	L214	—	—	+

KEY: + (positive) — (negative)

IDENTIFICATION OF LAB ISOLATES

The result of the *in vitro* probiotic screening indicated that the 14 LAB isolates had good probiotic potential based on their antimicrobial activity against selected pathogens and tolerance for high concentration of sodium chloride, bile salt, low pH, survival in the gastrointestinal tract (GIT), the adherence to hydrocarbon, autoaggregation assay they were subjected to. They were also regarded as safe for been gelatinase and DNAase negative. Their ability to produce exopolysaccharide (EPS) was an advantage for their selection.

Based on the morphological, biochemical and physiological characteristics, the 14 LAB isolates were identified as *Lactobacillus plantarum* (L15, L17, L117, L214), *Lactobacillus casei* (L113, L116, L213), *Lactobacillus sp*. (L13, L19, L22, L211), *Enterococcus sp*. (L12, L115) and *Pediococcus* sp. (L118) as shown in Table 8.

TABLE 8: IDENTIFICATION OF SELECTED POTENTIAL PROBIOTIC LAB

Isolate Code	Probable Identity	No of Occurrence	% Occurrence
L15,L17, L117,L214	*Lactobacillus plantarum*	4	28.57
L113, L116, L213	*Lactobacillus casei*	3	21.43
L13, L19,L22,L211	*Lactobacillus sp.*	4	28.57
L12, L115	*Enterococcus* sp	2	14.29
L18	*Pediococcus* sp.	1	7.14

4.0 DISCUSSION

Antimicrobial activity is an important property for probiotic selection. Mahnaz *et al*., [13], Oluwajoba *et al.,* [16] and Hawaz [25] reported the antimicrobial properties of *Lactobacillus* spp. which is due to different factors including the production of organic acids and bacteriocins. Oluwajoba *et al*., [16] further reported that LABs produce peptides having inhibitory properties against strains of closely related species. The cell free culture supernatant of *Lactobacillu*s spp. showed great deal of antimicrobial activity against the selected food pathogens; *Salmonella* sp. *Escherichia coli, Bacillus sp., Pseudomonas aeruginosa* and *Staphylococcus aureus*. *S.aureus* was completely susceptible to the metabolite, so were other pathogens, this result is comparable to that obtained by Oluwajoba *et al.,* [16], who reported that *Staphylococcus aureus* in particular was susceptible to metabolites produced by all the LAB isolated. The capacity to produce different antimicrobial compounds may be one of the critical properties for effective competitive exclusion of pathogen survival and an expression of a probiotic effect for the host [25, 26].

The LAB isolates were sensitive to amoxyllin clavulanate and resistant to gentamycin but showed a variation in resistance to ceflazidime, cefurozine, ceftriazone, erythromycin, cloxillin and ofloxicine. This finding compares with that of Pundir *et al*. [14] who stated that resistance to who stated that resistance to wide spectrum of antibiotics implied that if such isolated probiotics are induced in patients treated with antibiotics therapy it may be helpful in faster recovery of patients due to rapid establishment of desirable microbial flora. However, findings in this current study is in contrast with the report of Hoque *et al*. [15] who in their experiment on *Lactobacillus* spp. isolated from yoghurt observed some antibiotics such as Gentamycin and amoxicillin can drastically drop *Lactobacillus* spp. from the intestinal microflora, this was possibly due to wide use of antibiotics in veterinary medicine and Agriculture which could be contributing to the dissemination of resistance.

The use of LAB isolates as probiotic require that they survive acidic conditions in the stomach, resist bile salt, gastric tolerance and possess antimicrobial/antagonistic effect against pathogenic bacteria as reported earlier [13, 16, 19, 25, 27].

NaCl is an inhibitory substance which may inhibit the growth of certain types of bacteria [15],high salt concentration can improve the flavour of fermented food, and also inhibit the growth of pathogenic bacteria [28]. The result of this research showed that *Lactobacillus* spp. isolated from fermented acha were able to tolerate 2-10% of NaCl which is agreement with the report of Elezete and Carlos [29] that *Lactobacillus* spp. isolated from fermented yoghurt were able to tolerate 1-9 % of NaCl and Qing *et al*.,[28] who reported the growth of *Lactobacillus plantarum* in NaCl concentration of 0-10% from fermented soybean paste and Thakkar *et al*.[27] whose LAB isolates tolerated 8- 12% NaCl concentration. At these high concentrations, bacteria cells would experience loss of cell pressure, which will in turn affect the physiology, enzymes and metabolism of the cells. A concentration of 1- 6.5% is recommended for LAB, ability to withstand or tolerate this stress condition of higher concentration of NaCl make the isolates preferred as good probiotic as tolerance to high salt concentrations initiates metabolism which produces acid that further inhibits the growth of undesirable microorganisms[27].

Resistance to low pH is a major selection criteria for probiotic. The pH of the stomach after meal ranges from pH 1 to pH 4. For the probiotic to reach the small intestine, they have to pass through stressful conditions [25]. The results obtained in this study showed that the LAB isolates could tolerate extreme pH of 2- 3 this is in agreement with the report of Hawaz [25] that the pH of the stomach could be as low as pH1, and Thakkar *et al*., [27], who reported that the threshold point to state acid resistance in their research was set at pH 2 and pH 3 for 4 hours incubation, as it simulates bacterial residency in the stomach but for most *in-vitro* assay pH 3.0 is the preferred.

The relevant physiological concentrations of human bile range from 0.1 to 0.3% [30] and 0.5% [31] and staying time is proposed to be 4 hours [32]. Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% (w/v) maximum in healthy men. In this research the LAB isolates were able to grow at the different concentrations of bile used with decrease in viable count of cells. This is in contrast to the findings of Hoque *et al*., [15], Agaliya and Jeevaratnam [17] stated that various *Lactobacilli* could grow in presence of 0.05, 0.1 and 0.3% of bile but no growth in higher percentages of 0.6 and 1%. The result obtained in this study is in agreement with Sourabh *et al.* [19] who reported a variable tolerance to low pH (2.0 - 3.0) and high bile concentrations (0.2 – 2.0%)..

The gastric transit tolerance was monitored from 0 to 90 minutes and then to 180 minutes. The reason for 90 minutes of incubation time in acidic broth is that the time from entrance to release from the stomach is 90 minutes [17, 33]. The result obtained is in agreement with Agaliya and Jeevaratnam [17] who reported that all the isolates were able to survive conditions mimicking the gastrointestinal environment.

On tolerating the upper gastrointestinal transit, the next challenge an effective probiotic should surmount is adherence to the small intestine. Cell surface hydrophobicity and autoaggregation are also considered very important factors in the adhesion and proliferation of microorganism in the intestinal epithelial cells [19, 34]. Several workers have suggested that the ability of beneficial microorganisms to aggregate and adhere aids in colonization of the gut and in the establishment of a barrier which prevents enteric pathogens from establishing an infection [20].

The outcome of the adhesion and autoaggregation in LAB isolates was up to 93.71% which is in line with the findings of Syal and Vohra [20] who reported that auto aggregation above 80% is considered to be strong, Isolates possessing high hydrophobicity would exhibit good adhesion property to the intestinal tract cell lines [19]. Although hydrophobicity values for most of the isolates were low, isolates having good autoaggregation ability in conjunction with the good hydrophobicity values can strongly be related to the adhesion ability of these microorganisms. Though these traits are independent of each other; they are still related to adhesion property of a particular microbe [35]. Autoaggregation ability has been more strongly associated to adhesion as compared to hydrophobicity [34] therefore, good amount of autoaggregation ability in spite of low hydrophobicity values may account for adherence property of the LAB isolates.

Safety is one of the most recommended criteria in the FAO/ WHO [36] guidelines on evaluation and selection for probiotics to be used as food or feed supplement. In this study all the isolates were gelatinase and DNAase negative. This agrees with the report of Syal and Vohra [20] that microorganisms should not produce this enzymes so as to be used as probiotic in food and feed supplement. Extracellular DNAse provides growth advantage for pathogens by increasing the pool of nucleotides due to DNA hydrolysis which aids the spread of pathogens by liquefying pus and aiding the evasion of the innate immune response through the degradation of the neutrophil extracellular traps [37].

Exopolysaccharide (EPS) are thought to play a role in the protection against desiccation, toxic compounds, bacteriophages, osmotic stress, and to permit adhesion to solid surfaces and biofilm formation [20]. Findings of this research showed production of Exopolysaccharide by the LAB isolates except for a few LAB species which did not produce EPS. This attributes of the LAB isolates may confer on them immunostimulatory properties and the phosphates group in EPS play an important role in the activation of macrophages and lymphocytes [38].

REFERENCES

1. Chelule PK, Mokoena MP, Gqaleni N. Advantages of Traditional Lactic Acid Bacteria Fermentation of Food in Africa. Curr. Res. Tech. Edu. Topics Appl. Microbiol. Microbial Biotech. A Merdez Vilas (Ed) 2010 p. 1160-1167.
2. Oyewole OA, Isah P. Locally Fermented Foods in Nigeria and their Significance to National Economy: a Review. J. Recent Adv. Agric. 2012; 1(4): 92-102.
3. Sharma N, Handa S, Gupta A. A Comprehensive Study of Different Traditional Fermented Foods/Beverages of Himachal Pradesh to Evaluate Their Nutrition Impact on Health and Rich Biodiversity of Fermenting Microorganisms. Inter. J. Res. Appl. Nat. Soc. Sci. 2013; 1(3): 19-28.
4. Chukwu O, Abdul-Kadir AJ. Proximate Chemical Composition of Acha (*Digitaria exilis* and *Digitaria iburua*) Grains. J. Food Tech.2008; 6 (5): 214-216.
5. William CF, Dennis CW. *Food Microbiology.* Fourth edition. McGraw Hill India; 2011.
6. Ugwuona FU, Awogbenja MD, Ogara JI. Quality Evaluation of Soy-Acha Mixes for infant feeding. Indian J. Sci. Res. 2012; 3(1) 43-50.
7. Motarjemi Y. Impact of small scale fermentation technology on food safety in developing countries. Inter. J. Food Microbiol. 2002; 75: 213-229.
8. FAO/WHO. Guidelines for the Evaluation of Probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. London. Ontario, Canada; 2002
9. Azizpour K, Bahrambeygi S, Mahmoodpour S, Azizpour A. History and Basic of Probiotics. Res. J. Bio. Sci. 2009; 4 (4):409-426.
10. Junior WLGA, Ferrari IS, de Souza JV, Barbosa AY, da Costa MM, Menezes DR, Dias FS. Principal Criteria for Selection of Lactic Acid Bacteria for Potential Use as Probiotics in foods. Afr. J. Microbiol. Res.2015; 9(10): 671-686.

11. Parvez S, Malik KA, Ah Kang S, Kim HY. 2006. Probiotics and their fermented food products are beneficial for health. J. Appl. Microbiol. 2006; 100:1171-1185.
12. Morelli L. *In vitro* Assessment of Probiotic Bacteria: From Survival to Functionality. Inter. Dairy J. 2007; 17(11): 1278-1283.
13. Mahnaz KI, Che WJ, Khyrunnisa B, Iman Y. Screening of Antibacterial Activity of Lactic Acid Bacteria Isolated from Fermented Vegetables against Food Pathogens. Archives Des Sci. 2012; 65 (6)
14. Pundir RK, Rana S, Kashyap N, Kaur A. Probiotic Potential of Lactic Acid Bacteria Isolated from Food Samples: an *In Vitro* study. J. Appl. Pharm. Sci. 2013; 3(3): 85-93.
15. Hoque MZ, Akter F, Hossain KM, Rahman MS, Billah MM, Islam KMD. Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus* spp. from selective Regional Yoghurts. World J. Dairy Food. Sci. 2010; 5(1): 39-46.
16. Oluwajoba SO, Akinyosoye FA, Oyetayo OV. 2013. *In vitro* Screening and Selection of Probiotic Lactic Acid Bacteria Isolated from Spontaneously Fermenting Kunu-Zaki. Adv. Microbiol. 2013; 3: 309.
17. Agaliya PJ, Jeevaratnam K. Screening of *Lactobacillus plantarum* isolated from fermented idli batter for probiotic properties. Afr. J. Biotech. 2012; 11(65): 12856-12864.
18. Rosenberg M, Gutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbon: a simple method for measuring cell- surface hydrophobicity. FEMS Microbiol.Lett.1980; 9: 29- 33.
19. Sourabh A, Kanwar SS, Sharma OP. Screening of indigenous yeast isolates obtained from traditional fermented foods of Western Himalayas for probiotic attributes. J. Yeast Fungal Res.2011; 2 (8):117- 126.
20. Syal P, Vohra A. Probiotic Attributes of a yeast –like fungus, *Geotrichum klebahnii.* Afr. J. Microbiol. Res. 2014; 8(20): 2037- 2043.
21. Charteris WP, Kelly PM, Morelli L, Collins JK. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotics *Lactobacilli* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 1998; 84(5): 759-768.
22. Gupta H, Malik RK. Incidence of Virulence in Bacteriocin- Producing Enterococcal Isolates. Lait 2007; 87:587-601.
23. De la Cruz TE, Torres JM. Gelatine hydrolysis Test Protocol. American Society for Microbiology. Microbe Library. 2013; 1-9.
24. Sobrun Y, Bhaw-Luximon A, Jhurry D, Puchooa D. Isolation of lactic acid bacteria from sugar cane juice and production of lactic acid from selected improved strains. Adv. Biosci. Biotech. 2012; 3: 398-407.
25. Hawaz E. Isolation and Identification of Probiotic Lactic Acid Bacteria from Curd and *In Vitro* Evaluation of its Growth Inhibition against Pathogenic Bacteria. Afr. J. Microbiol. Res. 2014; 8(13): 1419- 1425.

26. Salminen S, vonWrigh A, Morelli L, Marteau P, Brassart D, deVos VM, Fonden R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T. Demonstration of safety of Probiotics - a Review. Inter. J. Food Microbiol.1998; 44 (1-2): 93-106
27. Thakkar P, Modi HA, Prajapati JB. Isolation, characterization and safety assessment of lactic acid bacterial isolates from fermented food products. Inter. J. Curr. Microbiol. Appl. Sci. 2015; 4(4): 713-725.
28. Qing L, Xiaoli L, Mingsheng D, Jiannzhong Z, Ying W. Adhesion and Probiotic Properties of *Lactobacillus plantarum* Isolated from Chinese Traditional Fermented Soybean paste. Global Adv. Res. J. Food Sci. Tech.2015; 4(1): 001-009.
29. Elezete DFRP, Carlos RS. Biochemical characterization and identification of probiotic *Lactobacillus* for swine. B.CEPPA, Curitiba, 2005; 23:299-310.
30. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan G, Shanahan F, Collins JK. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am. J. Clinical Nutr. 2001; 73:386s-392s.
31. Mathara JM, Schillinger U, Guigas C, Franz C, Kutima PM, Mbugua SK, Shin HK, Holzapfel WH. Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. Inter. J. Food Microbiol.2008; 126:57-64.
32. Mishra V, Prasad MN. Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. Inter. J. Food Microbiol.2005; 103:109-115
33. Chou L, Weimer B. 1999. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci.1999; 82: 23-31
34. Del Re B, Sgorbati B, Miglioli M, Palenzona D. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. 2000; 31: 438-442.
35. Rahman MM, Kim WS, Kumura H, Shimazaki K. Autoaggregation and surface hydrophobicity. World J. Microbiol. Biotech. 2008; 24: 1593-1598.
36. FAO/WHO. Guidelines for the Evaluation of Probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. London. Ontario, Canada. 2002; p.1-11
37. Hasegawa T, Minami M, Okamoto A, Tatsuno I, Isaka M, Ohta M. 2010. Characterization of a Virulence-associated and Cell Wall Located DNase of *Streptococcus pyogenes.* Microbiol. 2010; 186: 184- 190.
38. Uemura JN, Kitazawa H, Kawai Y, Itoh D, Oda M, Saito T. Functional Alteration of Murine Macrophages Stimulated with extracellular Polysaccharides from *Lactobacillus delbrueckii* subsp *bulgaricus* OLL 1073R-1. Food Microbiol. 2003; 20: 267-273.