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PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA (*LAB*) ISOLATED FROM WHOLEGRAIN MILLET SOURDOUGHS

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Abstract
This research was carried out to characterise lactic acid bacteria (LAB) isolated from wholegrain millet sourdoughs and to provide scientific data on the health functionality of the microorganisms responsible for sourdoughs fermentation. A total of 127 LAB isolates were observed on de Man Rogosa Sharpe (MRS) agar, while sixteen of the LAB isolates were characterized with respect to dominance in the fermentation process with percentage occurrence of 63% for Lactobacillus plantarum, Lactobacillus pentosus (12%), Pediococcus pentosaceous (19%) and Lactobacillus brevis (6%) using API 50 CHL. Lactic acid bacteria were assessed for probiotic attributes with respect to their in vitro health promoting effects, in terms of ability to tolerate low acidic pH, different bile salt concentrations, antimicrobial and antibiotic activities under conditions simulating the human gastrointestinal tract (GIT). The isolates displayed a good tolerance to acidic environment of Hydrochloric acid (pH. 3.0) with survival rate between 2.0 - 227.0 and tolerance to bile salt at 0.3% concentration. Furthermore, they exhibited clear zones of inhibition (2.0 mm– 14.0 mm) against all the tested pathogens and revealed strong resistance to four of the antibiotics; gentamycin, ampicillin, rifampicin and norfloxacin.

Keywords: sourdough, gastrointestinal tract, lactic acid bacteria, probiotics.

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1. Introduction

Millets have received very little research attention and relative importance both in the developing and developed World. Hence, they are classed among crops regarded as neglected and under–utilized species (NUS). They serve as source of energy and protein for about 130 million people in sub – Saharan Africa. (Obilana, 2002). Nutritionally, they can be compared with most cereals, data shows that they are superior or equivalent to most cereals especially rice and wheat, high in fibre and gluten free (Van Wyk and Gericks, 2000; Obilana, 2002). Sourdough microflora is composed of a stable association of lactic acid bacteria and yeast which have been known to improve baking properties of flour especially non-wheat flours (Coda *et al.*, 2010). Spontaneous sourdough is rich in fermentable carbohydrate and possesses an initial pH of 5.0 to 6.2, which is rather low. It therefore, allows a spontaneous development of characteristic LAB derived from the cereals or flours, which depend on the flour, preparation and sourdough production technology applied.

During spontaneous fermentation, the LAB quickly dominates the gram negative enterobacteria without a significant difference between wheat and rye (Stolz, 2003). Among LABs isolated from millet sourdoughs are *Pediococcus pentosaceous*, *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Pediococcus stilesli* 161 (Coda, 2010).

Lactic acid bacteria are Gram positive, acid tolerant, non- spore forming, catalase negative, rod or cocci shaped bacteria. They produce lactic acid as a major or sole product of fermentative metabolism (Holzapfel *et al*., 2001). They are of importance in food technology and have been reported to be isolated from cereal based foods (Lei and Jakobsen, 2004; Edema and Sanni, 2008; Kalui *et al*., 2009). They also produce a variety of antimicrobial compounds which include ethanol, formic acid, acetone, hydrogen peroxide, diacetyl and bacteriocin which play a crucial role in the preservation and microbial safety of fermented foods, thus, promoting the stability of the final products of fermentation (Oliveira *et al*., 2008). The lactic acid fermentations provide foods with a variety of flavours, aromas, and textures, safe and with a

long shelf life.
Associated with fermentation are probiotics which have been shown to have health benefits in the human body (Lankaputhra and Shah, 1995). Probiotics are live microorganisms in food stuff comprising lactic acid bacteria and yeast such that when ingested are able to reach the GIT in sufficient numbers and confer health benefits to the host (Osuntoki *et al*., 2007). Several health benefits have been confirmed to be associated with consumption of probiotic organisms which are; promotion of intestinal lactose digestion, decreased duration of diarrhoea, increased nutritional value through better digestibility and increased absorption of vitamins and minerals, prevention of colon cancer, reduction of certain allergies and serum cholesterol concentrations (Orrhage and Nord, 2000). This has mediated the consistent research in the area of probiotics. Recent research prospects however, tends to harness local substrate for beneficial probiotic attributes. One of the most important criteria for the selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive the bile concentrations encountered in the intestine (Desai, 2008). The aim of this study was to isolate lactic acid bacteria from finger millet, pearl millet, black and white fonio sourdoughs. Also to evaluate *in vitro,* their growth pattern in acidic environment, tolerance to bile salts, antimicrobial and antibiotic resistance ability.

2. Materials and methods

Sample collection and processing

Four species of millet (finger, pearl, black and white fonio) were all bought from a local market in Zaria, Kaduna state, North Central Nigeria. The grains were pulverized using an attrition mill (ATLAS, YL 90L – 4) the samples packaged in air tight containers and kept in the refrigerator at 4 ^{o}C for further analyses. The millet sourdoughs were prepared by mixing individual flour with water in the ratio 1:1 (w/v) in a glass bowl and stirred manually using a glass stirring rod and allowed to stand at a temperature of 28 ± 2 ^{o}C for 48 h for fermentation to occur.

pH determination

This was determined using a pH meter (PEC MEDICAL). The pH electrode was rinsed with distilled water, dipped directly into the sourdoughs and the pH was read.

Determination of titratable acidity

This was done according to the method described by Edema and Sanni (2008). Ten gram of each sourdough was weighed into a 250 ml beaker. Ninety millilitre of distilled water was added and homogenized. Then, 10 ml of the resultant mixture was titrated against 0.1M NaOH solution to a faint pink colour using 1% phenolphthalein as indicator. The acid equivalent was calculated as the amount of NaOH consumed per gram in millilitres.

Determination of diacetyl production

The method used to evaluate the diacetyl production were those described by Edema and Sanni (2010). Ten grams of prepared sourdough was weighed, mixed with 90 ml tap water and homogenized. Twenty millilitres each of the homogenized mixture was measured and 7.5 ml of 1 M hydroxylamine solution was added in two flasks (one flask was for residual titration). Both flasks were titrated against 0.1 N HCl solution to a greenish yellow end point using bromophenol blue as indicator. The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated as follows;

$$DP = \frac{(R - S)\,(100E)}{W}$$

Where;

DP = percentage of diacetyl (mg)

R = ml of 0.I N HCl consumed in residual titration

S = ml of 0.I N HCl consumed in normal titration of sample

E = equivalence factor of HCl to diacetyl

W = volume of sample

Determination of hydrogen peroxide production

Hydrogen peroxide production was determined using the method described by AOAC (2005). Ten grams of individual sourdough was weighed and mixed with 90 ml tap water and

homogenized. Twenty five millilitres of homogenized mixture was mixed with 25 ml of dilute 10% H_2SO_4. The resulting mixture was titrated with 0.1 N potassium permanganate solution ($KMNO_4$) until a pale pink colour which persisted for 15 sec before de-colorization was observed. Each milliliter of 0.1 N $KMNO_4$ is equivalent to 1.701 mg of H_2O_2. The volume of hydrogen peroxide produced was calculated as follows:

$$H_2O_2 = \frac{KMnO_4 \text{ (ml)} \times KMnO4 \text{ (N)} \times M.E \times 100}{H_2SO_4 \text{ (ml)} \times \text{volume of sample}}$$

Enumeration of lactic acid bacteria in the sourdoughs

Enumeration of LABs were determined as described by Pederson *et al.* (2004) and Asmahan and Muna, (2009). Twenty five grams of each sourdough was mixed with 225 ml sterile peptone water and serially diluted in distilled water. One millilitre aliquots of each dilution was pour plated with de Man Rogosa Sharpe (MRS) agar (Oxoid Hampshire, England) and incubated anaerobically at 37 ^oC for 48 h. A Gas Pak system (AN0025A, Basingstoke, and Hants, UK) was used throughout the experiment to obtain an anaerobic environment. All colonies were counted and recorded as colony forming units (CFU) per gram. After incubation, colonies were streaked in three successions on sterile MRS agar plate under aseptic condition to obtain discrete colonies. The pure isolates were kept on agar slants and stored at 4 ^oC in a refrigerator. All experiments were done in duplicate. The isolates were gram stained and subjected to various biochemical tests of catalase production, oxidase, endospore and sugar fermentation tests. Final phenotypic characterization was done using API 50 CHL system (Bio merieux SA Marcy1'Etoile, France) according to manufacturer's instruction.

Determination of LAB survival under acidic conditions

The method used to evaluate the survival of probiotic cultures under acidic conditions was that described by Desai (2008). Young cultures of 18-24 h were grown in MRS broth medium at 37 ^oC for 20 h, with the pH adjusted to 3.0, 2.5, 2.0 and 1.5 using 5 N HCl. It was then incubated at 37 ^oC for 3 h. Samples were then taken every hour for 3 h and the viable numbers of the probiotic cultures were enumerated by pour plate counts using 10-fold serial dilution prepared in 0.1% peptone water.

Determination of LAB tolerance to bile salts

The isolates were examined for their ability to grow in the presence of bile salt according to the method described by Pederson *et al.* (2004) with slight modification. One millilitre of overnight MRS broth culture was added to 9 ml of freshly prepared MRS broth medium. These were then supplemented with 0.1, 0.3, 0.5, 1.0, 2.0 and 5.0% (w/v) of Oxoid bile. The isolates were incubated at 37 ^oC for 24 h. A control was set up by inoculating cells in MRS broth without bile salt. Absorbance readings at 600 nm were recorded and the growth was then characterized as strong growth, weak growth or no growth (Strong growth representing on optical density (OD) comparable to that of non-supplemented culture, weak growth representing at least a doubling of the O.D).

Determination of ability of LAB to resist pathogens

Antimicrobial activity of the isolates was done according to the methods described by Soleimani *et al*. (2010) using the agar-well diffusion method. Bacteria cultures of *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium* were obtained from Wesley Guild Hospital an arm of Obafemi Awolowo University Teaching Hospital, Ilesha, Osun State, Nigeria. The bacteria cultures were grown in nutrient broth at 37 ^{o} C for 24 h. Also LAB isolates were grown overnight in MRS broth, centrifuged at 10,000 revolutions per min for 10 min and washed with sterile distilled water. Muellar-Hinton agar plates were prepared and the bacteria cultures were spread on the prepared agar plates with sterile

effusion. The wells were made and filled with 100 µl of LAB isolate supernatant. The inoculated plates were incubated at 37 ^{o}C for 24 h and the diameter of the zone of inhibition was measured in millimetre.

Determination of Antibiotic resistance of LAB

Antibiotic resistance of isolates was determined using the disc diffusion method. Ten microlitres of the LAB isolate was spread on the surface of Muellar - Hinton agar plates with sterile cotton swab. The antibiotic discs (Ampiclox (20 µg), erythromycin (15 µg), ciprofloxacin (10 µg), gentamycin (10 µg), amoxicillin (20 µg), streptomycin (30 µg), levofloxacin (20 µg), chloramphenicol (30 µg), norfloxacin (10 µg) and rifampicin (20 µg) were placed on the surface of the agar and the plates were incubated at 37 ^{o}C for 24 h. Resistance against antibiotics was assessed by measuring the diameter of the zone of inhibition in millimetre (mm). A clear zone around the discs showed resistance while lack of clear zone shows susceptibility of the isolates to the antibiotics tested (Tambekar and Bhutada, 2010).

Statistical Analysis

All the experiments were carried out in triplicate and data obtained were analyzed using analysis of variance (ANOVA) and Duncan's new multiple range test using a 5% significance level (SPSS version 19 computer software).

3. Results and Discussion

Physicochemical Properties

The pH and TTA of the sourdoughs at the end of 48 h spontaneous fermentation under ambient temperature of 28 ± 2^{o} C ranged from 3.70 -3.92 and 1.00 ml- 2.13 ml respectively as shown in Table 1. This low pH and high TTA may be due to the fermentative transformation of carbohydrate to lactic and acetic acid by the microorganisms present.

The values obtained for diacetyl (234.4 mg-437.6 mg) and hydrogen peroxide (0.50 mM-1.62 Mm) were also high and these properties can be said to give fermented product a longer shelf stable quality, unique aroma and flavour. The low acidic environment obtained created a suitable environment for LAB because they can survive under high acidic condition and thus have the ability to produce high level of lactic acid (Adebayo–Tayom and Onilude, 2008) Also, yeast must have fed on the carbohydrate, provided vitamins and other growth factors for LAB a relationship between LAB and yeast which is common in fermented foods (Corsetti *et al*., 2001; Edema and Sanni, 2008). Diacetyl, hydrogen peroxide, organic acids and bacteriocin are some metabolites that are responsible for the unique flavour, aroma, antimicrobial activity and long shelf stable quality of fermented products, which may also account for the strong aroma of the sourdough breads during baking.

Microbial composition of sourdoughs

The microflora of the millet sourdoughs were found to be majorly lactic acid bacteria and yeast species which was probably due to the low pH and high diacetyl property obtained in the sourdoughs as a result of fermentation which had some inhibitory activity against other organisms. A total of 127 LAB isolates were identified on the MRS agar plates and 74 yeast isolates on the MEA agar respectively. The isolates were observed microscopically to be well defined gram – positive, non-motile, cocci and rod shaped which were distributed either in chains or individually. Biochemical tests also revealed catalase negative, oxidase negative, non-spore forming isolates which are typical characteristics of LAB. On the basis of fermentation pattern of API 50 CHL, 16 isolates were characterized as *Lactobacillus plantarum* (10), *Pediococcus pentosaceous* (3), *Lactobacillus pentosus* (2) and *Lactobacillus brevis* (1) as shown in Table 2.

Acid tolerance ability of LAB isolates

At pH 3.0 which is the set standard for acid tolerance of probiotic cultures all the isolates were able to survive. This may be explained that the pH was not too harsh to cause destruction of the LAB isolates *L. plantarum* having highest survival at the 3rd hour (Table 3). It was observed that the longer the staying

period of the isolates, the less their tolerance to the acidic environment of HCl.

Table 1: Physicochemical property of wholegrain millet sourdoughs

Sourdough Samples	pH	TTA (ml)	Diacetyl (mg)	H_2O_2 (mM)
Pearl millet	3.92^{a}±0.10	2.13^{c}±0.28	234.4^{a} ± 0.28	1.25^{b} ± .21
Finger Millet	3.87^{a}±0.10	2.01^{c}±0.13	437.6^{b} ± 0.42	1.58^{b} ± 0.22
Brown fonio	3.70^{a}±0.02	1.45^{b}±0.13	260.7^{a} ± 0.42	1.62^{c} ± 0.02
White Fonio	3.72^{a}±0.27	1.00^{a}±0.14	280.9^{a} ± 0.40	0.50^{a} ± 0.02

Mean value with different superscript on the same column are significantly different ($p<0.05$)
Values are means ± SD of triplicate measurement.
TTA = Titratable acidity. Please confirm if the unit for TTA is ml

Table 2: Characterization of LAB using API 50 CH System

Isolates	O	GLY	ERY	DARA	LARA	RIB	DXY	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG
Lp2P	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	v	-	v
Lb3P	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	v	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	v	-	v
Lp4P	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	-		-	-	v	-	v
Lp5P	-	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-		-	-	v	-	v
Ls2F	-	v	-	-	+	+	+	-	v	-	+	+	+	+	+	+	+	+	v	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	v	v	+	v	-	-		v	v	v	-	v
Ls3F	-	v	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-	+	v	v	v	-	v
Pd4F	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	v	+	+	+	-	-	+	-	-	-	-	+	-	-	+		-	-	v	-	-
Lp5F	-	-	-	-	+	+	+	-	-	-	-	+	v	+	+	+	+	+	+	+	+	+	v	+	+	+	-	-	+	-	-	-	+	-	-	-	+	-	-	+	-	-		-	-	v	-	v
Lp2W	-	-	-	-	+	+	+	-	+	+	+	+	+	+	-	v	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	v	v
Lp3W	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	v		-	v
Pd4W	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	v	-	-	-	+	+	-	+	+	+	+	+	+	v	+	+	+	-	+	-	-	-	+	-	-	+	-	-	-	-	-	v	-	-
Lp5W	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-	-	-	-	-	v	-	v
Lp2B	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+		+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	v	-	v
Lp3B	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	v	-	v
Pd4B	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	v	+	+	+	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	+	v	-	-	v
Lp5B	-	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	v	-	v

Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B= Strains of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= Strains of *Pediococcus pentosaceous*; Ls2F, Ls3F= Strains of *Lactobacillus pentosus*; Lb3P= Strain of *Lactobacillus brevis*, + = Positive, - = negative, v = variable

O= Temoin; GLY= Glycerol; ERY= Erythritol; DARA = D-arabinose; LARA= L-arabinose; RIB = D-ribose; DXLY= D-xylose; LXYL= L-xylose; ADO= D-adonitol; MDX= Methyl-Bd-xylopyranoside; GAL= D-galactose; GLU= D-glucose; FRU= D-fructose; MNE= D-mannose; SBE= L-sorbose; RHA= L-rhamnose; DUL= dulcitol; INO= inositol; MAN= D-mannitol; SOR= D-sorbitol; MDM= Methyl-αD-Mannopyranoside; MDG=Methyl-αD-Glucopyranoside; NAG= N-acetylglusamine; AMY= Amygdaine; ARB= Arbutine; ESC= Esculine citrate de fer; SAL= Salicine; CEL= D-cellobiose; MAL= D-maltose; LAC= D-lacose (origine bovine); MEL= D-melibiose; SAC= D- saccharose; TRE= D-Trehalose; INU= Inuline; MLZ= D- melezitose; RAF= D-raffinose; AMD= Amidon; GLYG= Glycogene; XLT= Xylitol; GEN= Gentibiose; TUR= D-Turanose; LYX= D-Lyxose; TAG= D-Tagatose; DFUG= D-fucose; LFUC= L-fucose; DARL= D-arabitol; LARL= L-arabitol; *GNT= potassium 2-cetogluconate; 2KG=potassium2-CetoGluconate;5KG=potassium5-CetoGluconate

Probiotics should therefore, be able to withstand at least pH 3.0 and colonize the GIT, thereby help to change the composition of the normal intestinal microflora from a potentially harmful composition to a microflora that would be beneficial to the host in order to be good source of probiotics (Ouwehand *et al.*, 2002; Huang and Adams 2004).

At pH 2.5, result shown on Table 4 revealed that the survival rate was similar to the trend observed at pH 3.0. However, only two of the isolates *L. brevis* and *L. pentosus* were able to tolerate the 3 h incubation time at this pH.

It was observed that at pH 2.0 some of the *L. plantarum* survived at 2 h while none of the isolates was able to survive 3 h incubation period (Table 5). At pH 1.5 after 0 h of incubation, the decline in viability was very sharp. *Lactobacillus plantarum* and *L. brevis* (Lp2P and Lb3P) isolates survived for 1 h, while *L. pentosus* (Ls3F) only was able to survive 1-2 h incubation time, and none of the isolate was alive after 3 h.

Generally, from this study, there was a regular trend in the responses of all the isolates at the 3 h exposure time to HCl which is similar to what obtains in the human stomach.

Table 3: Survival of LAB isolates in HCl solution at pH 3.0

pH 3.0	Total plate counts (log_{10})			
Isolates	**0 h**	**1 h**	**2 h**	**3 h**
Lp2P	58.0^{c}	49.3^{c}	30.0^{cd}	-
Lb3P	74.3^{bc}	61.0^{bc}	69.0^{abcd}	-
Lp4P	68.03^{bc}	59.0^{bc}	28.0^{cd}	-
Lp5P	91.0^{abc}	68.0^{bc}	38.0^{bcd}	7.0^{b}
Ls2F	135.0^{abc}	56.0^{bc}	139.0^{a}	-
Ls3F	144.0^{abc}	85.0^{abc}	57.0^{bcd}	1.00^{b}
Pd4F	167.0^{abc}	56.0^{bc}	10.0^{d}	-
Lp5F	64.0^{c}	44.0^{c}	38.0^{bcd}	6.00^{b}
Lp2W	126.3^{abc}	134.0^{abc}	104.0^{abc}	21.0^{a}
Lp3W	153.0^{abc}	158.0^{ab}	112.0^{ab}	2.00^{b}
Pd4W	196.0^{ab}	178.0^{a}	66.0^{abcd}	-
Lp5W	164.0^{abc}	108^{abc}	102.0^{abc}	-
Lp2B	209.0^{a}	102.0^{abc}	44.0^{bcd}	4.00^{b}
Lp3B	166.0^{abcd}	92.0^{abc}	39.0^{bcd}	2.00^{b}
Pd4B	188.0^{abc}	96.0^{abc}	40.0^{bcd}	-
Lp5B	107.0^{abc}	86.0^{abc}	30.0^{cd}	-

Means with different superscripts on the same column are significantly different ($p<0.05$). Values are means ± SD of triplicate measurement.
Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B= Isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= isolates of *Pediococcus pentosaceus*; Ls2F, Ls3F= isolates of *Lactobacillus pentosus*; Lb3P= isolate of *Lactobacillus brevis*.

Table 4: Survival of LAB isolates in HCl solution at pH 2.5

pH 2.5	Total plate counts (log_{10})			
Isolates	**0 h**	**1 h**	**2 h**	**3 h**
Lp2P	55.0^{b}	85.0^{ab}	40.0^{cd}	-
Lb3P	86.0^{b}	22.0^{c}	25.0^{de}	2.00^{a}
Lp4P	75.0^{b}	43.0^{bc}	17.0^{de}	-
Lp5P	140.0^{b}	118.0^{a}	14.0^{de}	-
Ls2F	219.0^{a}	102.3^{a}	73.0^{b}	-
Ls3F	248.0^{a}	112.0^{a}	100.0^{a}	2.00^{a}
Pd4F	92.0^{b}	64.0^{abc}	16.0^{de}	-
Lp5F	95.0^{b}	68.0^{abc}	38.0^{cd}	-
Lp2W	53.0^{b}	40.0^{bc}	10.0^{e}	-
Lp3W	86.0^{b}	70.0^{abc}	25.0^{de}	-
Pd4W	109.0^{b}	91.0^{abc}	63.0^{bc}	-
Lp5W	106.0^{a}	90.0^{ab}	79.0^{ab}	-
Lp2B	75.0^{b}	17.0^{c}	-	-
Lp3B	94.0^{b}	19.0^{c}	1.00^{e}	-
Pd4B	104.0^{b}	88.0^{ab}	1.00^{e}	-
Lp5B	98.0^{b}	68.0^{abc}	8.00^{e}	-

Means with different superscripts on the same column are significantly different ($p<0.05$).
Values are means ± SD of triplicate measurement.
Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B= isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= isolates of *Pediococcus pentosaceus*; Ls2F, Ls3F= isolates of *Lactobacillus pentosus*; Lb3P= isolate of *Lactobacillus brevis*.

Table 5: Survival of LAB isolates in HCl solution at pH 2.0

pH 2.0	Total plate counts (log_{10})			
Isolates	**0 h**	**1 h**	**2 h**	**3 h**
Lp2P	86.0^{ab}	23.0^{ab}	-	-
Lb3P	86.0^{ab}	5.0^{b}	-	-
Lp4P	104.0^{a}	21.0^{ab}	-	-
Lp5P	94.0^{ab}	22.0^{ab}	1.00^{b}	-
Ls2F	59.0^{de}	3.0^{a}	-	-
Ls3F	45.0^{efg}	80.0^{ab}	-	-
Pd4F	47.0^{efg}	13.0^{ab}	-	-
Lp5F	40.0^{fg}	21.0^{ab}	-	-
Lp2W	32.0^{g}	1.00^{b}	-	-
Lp3W	71.0^{bd}	11.0^{ab}	6.00^{a}	-
Pd4W	88.0^{abc}	13.0^{ab}	-	-
Lp5W	81.0^{ab}	30.0^{a}	-	-
Lp2B	48.0^{efg}	6.0^{b}	1.00^{b}	-
Lp3B	52.0^{ef}	5.0^{b}	1.00^{b}	
Pd4B	41.0^{fg}	-	-	-
Lp5B	61.0^{de}	21.0^{ab}	1.00^{b}	-

Means with different superscripts on the same column are significantly different ($p<0.05$).
Values are means ± SD of triplicate measurement.
Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B= isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= isolates of *Pediococcus pentosaceus*; Ls2F, Ls3F= isolates of *Lactobacillus pentosus*; Lb3P= isolate of *Lactobacillus brevis*.

There was a substantial decrease in the count of viable survivors of all isolates during the 3rd h incubation period at all the pH conditions, with most isolates having no survivor. This is similar to the findings of Desai, (2008) and Sahadeva *et al*. (2011) that worked on probiotics. In addition, it can be compared to Pederson *et al*. (2004) which showed that none of the three isolates of *Lb. amylolyticus* examined could survive synthetic stomach juice of pH 2.5 for 30 min, whereas, *Lb. panis* and *Lb. pontis* from the same study were able to survive this harsh condition.

At pH 2.0 and 1.5, the low pH environment might have inhibited the growth and metabolic activity of the cells thus rendering the cells vulnerable and reducing their viability. Mandal *et al*. (2006) also confirmed that the viability count of bacteria declined when exposed to simulated gastric juice of pH 1.5 after an incubation period of 3 h. The loss of viability at pH ≤ 2.0 after the first hour suggested that most cells were probably killed by severe pH of HCl. Zavaglin *et al*. (2002) reported that an acid such as HCl found in the human stomach is a

strong oxidizer, thus it can oxidize many biomolecules of cells such as fatty acids, proteins, cholesterol, DNA and destroy them. Low pH environment can inhibit the metabolism, reduce the growth and viability of LAB. Thus the isolates that were able to survive this low pH can be considered for probiotics.

Table 6: Survival of LAB isolates in HCl solution at pH 1.5

pH 1.5	Total plate counts (log_{10})			
Isolates	0 h	1 h	2 h	3 h
Lp2P	14.0[a]	3.0[a]	-	-
Lb3P	11.0[ab]	1.0[a]	-	-
Lp4P	11.0[ab]	-	-	-
Lp5P	4.0[ab]	-	-	-
Ls2F	3.00[ab]	-	-	-
Ls3F	10.0[ab]	3.0[a]	2.0[a]	-
Pd4F	6.0[ab]	-	-	-
Lp5F	-	-	-	-
Lp2W	3.0[ab]	-	-	-
Lp3W	-	-	-	-
Pd4W	-	-	-	-
Lp5W	3.00[ab]	-	-	-
Lp2B	2.0[b]	-	-	-
Lp3B	5.0[ab]	-	-	-
Pd4B	7.0[ab]	-	-	-
Lp5B	1.0[b]	-	-	-

Means with different superscripts across the column are significantly different ($p<0.05$).
Values are means ± SD of triplicate measurement.
Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B= isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= isolates of *Pediococcus pentosaceous*; Ls2F, Ls3F= isolates of *Lactobacillus pentosus*; Lb3P= isolate of *Lactobacillus brevis*.

Tolerance of LAB isolates to bile salt

All the isolates demonstrated strong growth tolerance against bile salt except *Lactobacillus plantarum* (Lp3B) which demonstrated weak growth to all the bile concentrations. At 0.5% concentration, ten of the isolates showed strong growth while at 2% only three isolates survived. The result also showed that as bile concentration increased, resistance decreased. Considering survival in 0.3% bile salt concentration which is set as standard by other researchers, all but one of the isolates showed high survival rate and all the isolates which survived 3 h at pH 3 in HCl, also exhibited strong tolerance to 0.3% bile concentration except Lp3B, which showed weak resistance to all the bile concentrations. The variation observed in the tolerance of the isolates to the control (0% bile) could be because resistance to bile salt varies among lactic acid bacteria species and even among strains (Kacem and Kaid- Harche, 2008). Sahadeva *et al*. (2011) also supported variations to bile salt among LAB. The survival of probiotic in bile salt environment in the small intestine is one of the main criteria for *in vitro* selection of potential probiotic bacteria and critical points for microbes (Pederson *et al*., 2004; Hawaz, 2014). These results have also been reported by Kacem and Kaid (2008) and Abriouel *et al.* (2012). Exposures to bile acid, brings about cellular homeostasis disruption causing the dissociation of lipid bilayer and integral protein of the cell membranes, thereby resulting in leakage of bacterial content and untimely death. This could be responsible for the weak growth observed at 1.0% and 2.0% bile salt concentrations.

Tolerance of the isolates to bile salt at different concentrations

Table 7: Bile salt tolerances of LAB isolates

Isolates	Different concentrations of bile salt (%)					
	Control	0.1	0.3	0.5	1.0	2.0
Lp2P	++	++	++	+	+	++
Lb3P	++	++	++	++	+	+
Lp4P	++	++	++	++	++	+
Lp5P	++	++	++	++	+	+
Ls2F	++	++	++	++	++	+
Ls3F	++	++	++	+	++	+
Pd4F	++	++	++	++	+	+
Lp5F	++	++	++	++	+	+
Lp2W	++	++	++	++	+	+
Lp3W	++	++	++	++	+	+
Pd4W	++	++	++	+	+	+
Lp5W	++	++	++	+	+	+
Lp2B	++	++	++	+	+	+
Lp3B	++	+	+	+	+	+
Pd4B	++	++	++	++	++	++
Lp5B	++	++	++	++	+	++

++ = strong growth; + weak growth.
Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W, Lp5F, Lp2B, Lp3B, Lp5B, = isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B = isolates of *Pediococcus pentosaceous*; Ls2F, Ls3F = isolates of *Lactobacillus pentosus*; Lb3P = isolates of *Lactobacillus brevis*.

Antimicrobial activity of LAB against selected pathogens

Results showed that the LAB isolates were able to inhibit the growth of the tested pathogens and this was probably achieved by their ability to produce organic acids, high amount of hydrogen peroxide and diacetyl which helped to increase the acidity of the medium and thereby inhibit the growth of pathogenic bacteria. It has also been reported that LAB produce bacteriocins which have a bacteriostatic effect against pathogenic organism. The high inhibition zones obtained were similar to the findings of Soleimani *et al*. 2010 which reported 13 mm inhibition zones for *L. plantarum* against *S. aureus.* Other studies have also shown inhibition of similar pathogens by LAB isolated during spontaneous fermentations (Olasupo, 1997; Sanni *et al.*, 1999; Edema and Sanni, 2008). Kaboosi (2011) however, reported that probiotic organisms had no activity against *E.coli* and *Pseudomonas aeruginosa* but showed bacteriostatic and bactericidal effect on other pathogens.

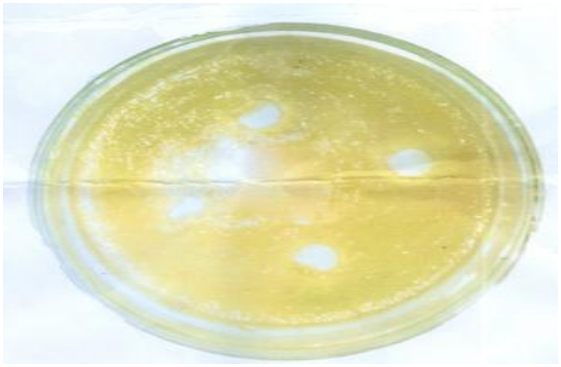

Figure 1: Agar well diffusion assay showing antimicrobial resistance of isolates

Sensitivity pattern of LAB isolates to antibiotics

The 16 identified isolates of LAB were tested for resistance to ten commonly used antibiotics and the isolates were found to be resistant to gentamycin, ampicillin, rifampicin and norfloxacin. Resistance was determined using the standard disc diffusion method and the zone of inhibition measured in millimetre using a vernier calliper. *Lactobacillus pentosus* was resistant to all the tested antibiotics.

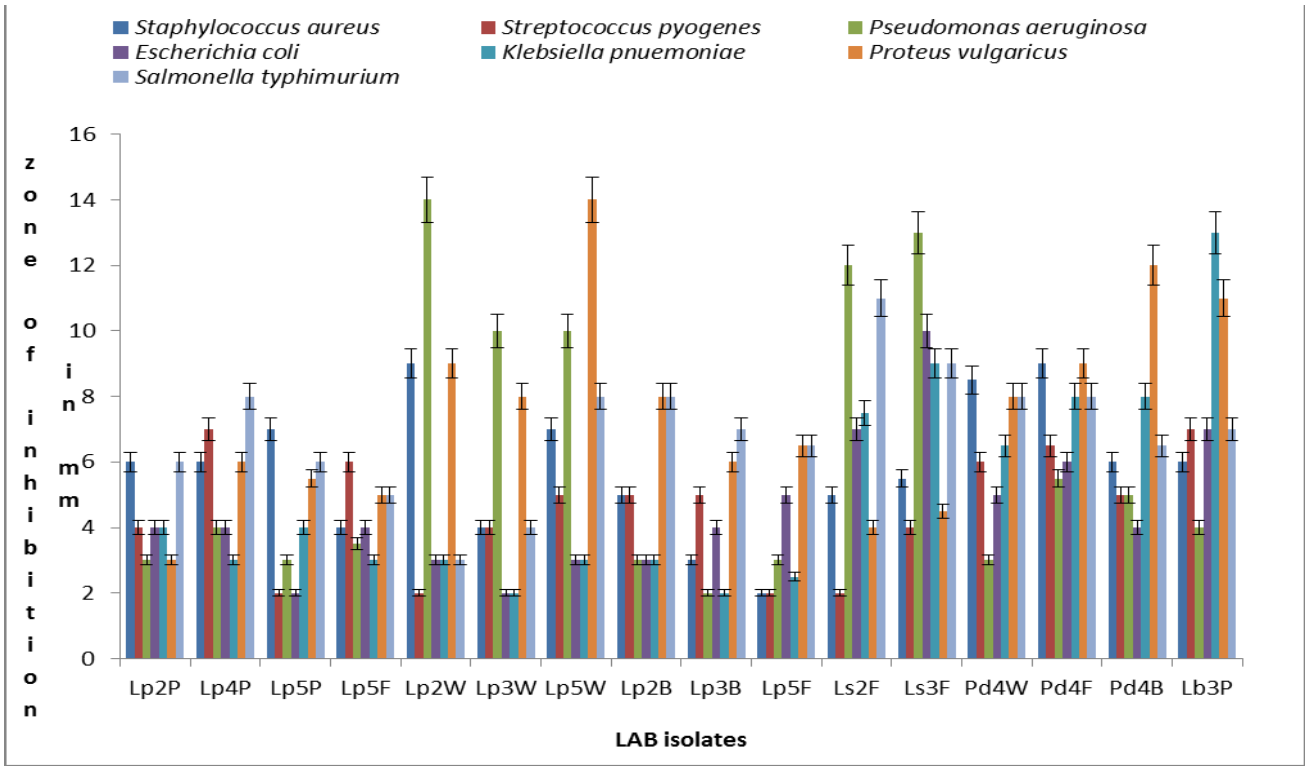

Figure 2: Antimicrobial activity of LAB against pathogenic bacteria

Table 8: Sensitivity pattern of LAB isolates to antibiotic isolates.

	ANTIBIOTICS									
Isolates	CPX	NB	GN	P	ST	RD	E	CH	APX	LEV
Lp2P	S	R	R	S	S	R	S	S	R	R
Lb3P	R	R	R	R	S	R	R	R	R	R
Lp4P	R	R	R	R	S	R	R	R	R	R
Lp5P	R	R	R	R	S	R	S	S	R	S
Ls2F	R	R	R	R	R	R	R	R	R	R
Ls3F	R	R	R	R	R	R	R	R	R	R
Pd4F	R	R	R	R	R	R	R	R	R	R
Lp5F	R	R	R	R	R	R	R	R	R	R
Lp2W	R	R	R	R	R	R	R	R	R	R
Lp3W	R	R	R	R	R	R	R	R	R	R
Pd4W	R	R	R	R	S	R	R	R	R	R
Lp5W	R	R	R	R	S	R	R	R	R	R
Lp2B	R	R	R	R	R	R	R	R	R	R
Lp3B	R	R	R	R	R	R	R	R	R	R
Pd4B	R	R	R	R	S	R	R	R	R	R
Lp5B	R	R	R	R	R	R	R	R	R	R

Lp2P, Lp4P, Lp5P, Lp2W, Lp3W, Lp5W Lp5F, Lp2B, Lp3B, Lp5B = isolates of *Lactobacillus plantarum*; Pd4W, Pd4F, Pd4B= isolates of *Pediococcus pentosaceous*; Ls2F, Ls3F= isolates of *Lactobacillus pentosus*; Lb3P= isolates in of *Lactobacillus brevis*.
CPX= ciprofloxacin, NB=norfloxacin, GN= gentamycin, P= penicillin, ST=streptomycin, RD= rifampicin, E=erythromycin, CH=chloramphenicol, APX= Ampiclox, LEV= levofloxacin
S=Susceptible, R= Resistant.

The 3 identified isolates of *P. pentosaceous* were resistant to all the tested antibiotics except for Streptomycin where Pd4F and Pd4B were found to be susceptible. Among the seven LAB found to be sensitive to streptomycin, four belonged to species of *L. plantarum* and the high sensitivity obtained for streptomycin is similar to the result reported by Gre-goret *et al.* (2013) which reported similar sensitivity to streptomycin, chloramphenicol and erythromycin. De- Angelis *et al*. (2006) also stated sensitivity of *L. plantarum* to 150µg/ml of streptomycin. Generally, studies on antibiotic resistance of lactobacilli indicated that they were usually resistant to major classes of antibiotics such as β-glucans, aminoglycosides, cephalosporin and quinones (Kaur *et al.,* 2012).

4. Conclusion

Lactic acid bacteria that dominated sourdoughs in this study have demonstrated health promoting effects by their ability to withstand *in vitro* the harsh conditions of low pH and bile salt present in the gastrointestinal tract. These isolates also exhibited inhibition zones and resistance against tested pathogenic microorganisms and commonly used antibiotics, which may be an evidence that they are able to colonize and stabilize the intestinal microflora. Thus, these organisms can serve as potential probiotics. The millet sourdoughs also serving as a carrier of these probiotic organisms together with its prebiotic benefit can help promote the GIT stability.

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