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Probiotic Potentials of Lactic Acid Bacteria Isolated From Some Nigerian Indigenous Fermented Foods

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ABSTRACT

Lactic Acid Bacteria (LAB) are responsible for the fermentation of many indigenous foods in Nigeria and may possess probiotic properties which can attribute to the gastrointestinal health of humans and livestock. LAB isolated from various indigenous fermented foods were assayed for antagonistic activities against some clinical enteric bacteria. The survivability of the isolates in the gastrointestinal tracts was determined by testing their tolerance in acid, bile, phenol, simulated gastric and intestinal juice. Isolate's hydrophobicity, aggregative abilities and haemolytic activities were also assayed. In vivo antimicrobial activity was also determined using broiler chicks. Ten of the LAB isolates had antibacterial activities with highest zone of inhibition (28mm) produced by *Lactobacillus fermentum* against *Salmonella enteritidis*. Also, *L. fermentum* along with four others, tolerated pH 1.5 and 0.5% bile salt, simulated gastric and intestinal conditions, 0.4 % phenol, and had percentage hydrophobicity, auto- and co-aggregation that ranged between 38.76 % - 58.06 %, 34.38 % - 54.05 % and 47.21 % - 74.64 % respectively. The LAB were non-haemolytic and demonstrated in-vivo antimicrobial activity against *S. enteritidis* in broiler chicks. *L. fermentum* and four other LAB isolated in this study, possess probiotic properties and can be used as prophylaxis against gastrointestinal diseases.

Keywords: Probiotics, lactic acid bacteria, Fermentation, Nigerian indigenous foods, gastrointestinal diseases

1.0 Introduction:

Traditional fermentation of cereals, tubers, legumes dairy and non-dairy milk has been used to produce nutritionally rich foods with high sensory qualities in Nigeria. Cereal grains including maize (*Zea maiz*), sorghum (*Sorghum bicolor*) and millet (*Pennisetum typhoideum*) are used for products such as 'ogi', a pap meal that serves as breakfast for many and more importantly, as weaning foods for infants; and 'kunnun zaki', a refreshing and nutritious food drink. Cassava tubers (*Manihot esculenta*) are used to produce 'gari', 'lafun' and 'fufu' which are staple foods. Locust beans (*Parkia biglobosa*), melon seeds (*Colocynthis citrillus* L.) and seeds of African oil bean tree (*Pentaclethra macrophylla)* are used for food condiments known as 'iru', 'ogiri' and 'ugba' respectively. Nunu and maishanu are fermented products of dairy milk.

Lactic acid bacteria (LAB) play major roles in the fermentation processes that lead to many of the food products. LAB break down sugar in foods to produce lactic acid wholly as obtained in homolactic fermentation, or together with other products such as ethanol, carbon dioxide, acetic acid etc., by heterofermenters. Several researchers have reported the isolation of a variety of LAB as major fermenting organisms in many of the food products. *Lactobacillus plantarum, L. pentosus, L. cellobiosus, Pediococcus pentosaceus* and *Leuconostoc mesenteroides* were reportedly involved in the fermentation of cereals for ogi and kunnu zaki production (Nwachukwu *et al.*, 2010). Likewise, Banwo *et al.* (2012) isolated *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus pentosus, Pediococcus pentosaceus, Pediococcus acidilactici, Enterococcus faecium*, from ogi, wara, gari and nunu. Oyedeji et al. (2013) also reported that *Lactobacillus plantarum, L. lactis, L. coprophillus, L. acidophilus, L. brevis*. and *Leuconostoc mesenteroides* were among the organisms responsible for the fermentation of cassava pulp in *fufu* production while *L. cellobiosus, L. plantarum, Lc. Lactis, L. acidophilus* and *Lc. paramesenteroides* were found in *ogi*.

LAB create acids in foods which not only give it the characteristic pleasant flavour and aroma, but also preserve the foods by eliminating or inhibiting organisms that could cause spoilage or constitute safety concern (Hitendra *et al.*, 2016). Lactic acid fermentation improves the availability of micronutrients in cereals by decreasing the antinutrients, such as phytate and tannins (Hotz and Gibson, 2007). Exopolysaccharides forming LAB such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* used as starter in the production of yoghurt, improve texture, avoid syneresis and increase the viscosity of foods (Ruas-Madiedo *et al*., 2002)

LAB also produce ethanol and bacteriocins, each of which contribute to the improved shelf life and safety of fermented foods. Elshaghabee *et al.* (2016) reported the production of high amount of ethanol from *Lactobacillus fermentum* and *Weisella confusa*. Bacteriocins are classified as ribosome-synthesized biologically active proteins or protein complexes with antimicrobial activity against closely related species.

The ability to control intestinal pathogens through the production of antibacterial substances such as lactic acid, ethanol, hydrogen peroxide and bacteriocins together with other properties including competition with pathogens for nutrients and adhesion sites, inactivation of pathogen's toxins and metabolites, and immunomodulation through the stimulation of nonspecific immunity (Kore *et al.*, 2012), qualify many LAB with the ability to adhere to the intestinal mucosa of the host, as probiotic organisms. In addition, such LAB may also have anti-carcinogenic activity and may help to improve lactose tolerance. LAB with probiotic activities have been isolated from indigenous fermented foods e.g., *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Weisella confusa* from ogi (Adesulu-Dahunsi *et al*., 2018).

Apart from being consumed in fermented milks and other fermented foods (Mduduzi *et al*., 2016), probiotics are packaged in form of drugs and retailed in pharmaceutical outlets as food supplements. They however come with high prices and unaffordable for low income earners. This study aims to identify probiotic lactic acid bacteria in Nigerian indigenous foods with a view

to recommend such for regular consumption and for possible packaging as food supplements in order to harness the health benefits available.

2.0 Methodology:

2.1 Isolation and biochemical characterization of organisms

Organisms were isolated from Fura (millet gruel), Nunu (fermented (yoghurt-like) milk), Maishanu (buttermilk byproduct of nunu production), Kunu- Zaki (fermented millet beverage), Ogi (maize gruel) and Cassava pulp on De Mann Rogosa and Sharpe (MRS) Agar; using the pour plate techniques. Plates were prepared in duplicate for aerobic and anaerobic incubation at 37±2 ℃ for 48 - 72 hours. Organisms were characterized by their morphology and physiology (Fawole and Oso, 2004).

2.2 Antimicrobial Activity of Lactic Acid Bacteria Isolates

Cell-free supernatant of MRS broth cultures of the lactic acid bacteria isolates were used to challenge some test organisms using the agar well diffusion and broth dilution methods (Balouiri, *et al*., 2016).

2.3 Antibiotic Susceptibility Test

The antibiotic susceptibility was determined for both the test organisms and the LAB isolates using the disk diffusion method described by Charteris *et al*. (1998).

2.4 Determination of pH and Titratable Acidity of Bacterial Isolates

The pH of the bacterial isolates in broth culture was determined using a standardized pH meter. Titratable acidity was determined by titrating the broth culture against 0.1N NaOH solution using phenolphthalein indicator. Uninoculated MRS broth served as the control. The percent titratable acidity was calculated using the following formular (GEA, 2006);

$$\%\ Titratable\ Acidity\ (TA) = \frac{mL_{NaOH}\ X\ N_{NaOH}\ X\ 90}{V\ X1000}\ X\ 100$$

Where:

$mL_{(NaOH)}$: Titre value

$N_{(NaOH)}$: Normality of titrant

V: volume of bacteria suspension

Acid and Bile Salt Tolerance Test

Overnight broth cultures of the LAB isolates were centrifuged at 4000 x g for 15mins for the collection of bacterial cells. The bacterial cells were washed twice with sterile Phosphate Buffer Saline and adjusted to 10^8 CFU/ml. Acid tolerance was determined by adding 100 µl of the cell suspension into a test tube containing 10 ml of fresh MRS broth which has been adjusted to pH 1.5 and 3.5 (using 1M HCl). For bile salt tolerance, test tubes containing 10ml fresh MRS broth that had been adjusted with 0.3% and 0.5% (w/v) bile salt were inoculated with 100 µl of cell suspension. The tubes were incubated at 37 ˚C for 6 hours and the viability of the cells was determined after 3- and 6-hours incubation by culturing on MRS agar (Jose *et al*., 2015).

2.6 Simulated Gastric and Intestinal Juice Survivability Test

The resistance of LAB isolates to simulated gastric and intestinal conditions was determined as described by Wang *et al*. (2020). Gastric juice was simulated by dissolving 0.35 g of pepsin in 100 ml of 2% saline and adjusted to pH 2.5 with 1M HCl. For artificial intestinal juice 0.1 g trypsin, 1.8 g bile salts, 1.1 g NaHCO3 and 0.2 g NaCl were dissolved in 100 ml sterile distilled water and adjusted to pH 8.0 with 1M NaOH. The simulated gastric and intestinal juices were sterilized using 0.22 µm membrane filter. After sterilization, 100 µl of overnight broth cultures of LAB isolates were inoculated into 10 ml of each of the simulated gastrointestinal juices and incubated at 37 ˚C for 6 hours. Viability of the cells was determined after 0-, 3- and 6-hours incubation by culturing on MRS agar.

2.7 Phenol Tolerance

Phenol tolerance ability of LAB isolates was determined by inoculating overnight broth cultures into MRS broth containing gradient concentration (0.1–0.4%) of phenol at 1% (v/v) in test tubes, and incubating at 37 ˚C for 24 hours. Strain viability was assessed by measuring the absorbance using spectrophotometer at 620 nm after incubation (Hoque *et al*., 2010).

2.8 Hydrophobicity Assay

The cell surface hydrophobicity of LAB isolates was evaluated according to the method of Abbasiliasi *et al*. (2017). The hydrocarbons used were dichloromethane and xylene. Day old broth cultures of LAB isolates were centrifuged at 4000 x g for 15 minutes, the pellets were washed twice

with sterile Phosphate Buffer Saline (PBS) (pH 7.4), optical densities of the bacteria were measured at 620 nm and adjusted to an optical density of 0.6 (A_0). LAB cell suspension, was mixed separately with the hydrocarbons at 3:1 (v/v) and vortexed for 1 min. The mixture was subsequently allowed to separate into 2 phases by standing for 30 min. The OD of the aqueous phase was measured at 620 nm using a spectrophotometer (A_1). Bacterial affinity to the solvent (hydrophobicity) was expressed in percentage using the formula:

$$\% \text{ hydrophobicity} = 1 - \frac{A_1}{A_0}\ x\ 100$$

Where:
A_0 – Optical density of bacteria prior to treatment with hydrocarbons
A_1 – Optical density of bacteria after treatment with hydrocarbons

2.9 Aggregative Abilities of LAB Strains

Auto-aggregative and co-aggregative abilities of the LAB isolates were determined using the methods of Polak-Berecka *et al*. (2014). For auto-aggregation, LAB strains were pelleted, washed and resuspended in PBS (pH 7.4) as described in 2.8 above; 5 mL was vortexed for 10 seconds, and absorbance measured at 620 nm (A_0). Absorbance was measured again after 2 hours of incubation at 37°C (A_{2h}). The auto-aggregation percentage was calculated using the formula:

$$\% \text{ autoaggregation} = 1 - \frac{A_{2h}}{A_0}\ x\ 100$$

Where:
A_0 – Optical density of bacteria before incubation
A_{2h} – Optical density of bacteria after incubation
The coaggregation ability of the LAB isolates with some pathogens was determine by mixing the LAB suspension and the pathogen cell suspension v/v, vortexed, and incubated for 2 h at 37°C. The absorbance of the mixture was measured at 620 nm before (A_{mix0}) and after incubation (A_{mix2h}). Percentage co-aggregation was calculated using the formula:

$$\% \text{ autoaggregation} = 1 - \frac{A_{mix2h}}{A_{mix0}}\ x\ 100$$

Where:
A_{mix0} – Optical density of bacteria before incubation
$Amix_{2h}$ – Optical density of bacteria after incubation

2.10 Haemolytic Activities of LAB

The haemolytic activity was determined by streaking fresh cultures of LAB isolates on blood agar plates and incubating at 37°C for 24 hours. *Staphylococcus aureus* was used as the control. Haemolysis was indicated by clear zones (blood lyses) around the colonies (β-hemolysis), or green-hued zones around the colonies (α-hemolysis), or bacterial growth with no effect on the blood agar (γ-hemolysis) (Wang *et al*., 2016).

2.11 Assessment of the *in vivo* Antimicrobial Potential of LAB Isolates using Broiler Chicks

This was performed as described by Chen *et al*. (2012). Day old chicks were randomly divided into groups of ten each and were gavage fed as follow; group A which was the blank control, received sterile Phosphate Buffer Saline (PBS) (pH 7.4) at 0.2 ml/chick once daily; group B, the normal control, were given sterile PBS (pH 7.4) at 0.2 ml/chick during day1- day3 and 0.2 ml of 10^8 CFU *Salmonella enteritidis*/chick on day4 only. Group C, the treatment groups, separately received 0.2 ml/chick of 10^9 CFU LAB isolates with high antibacterial activities, resistance to gastric conditions, hydrophobicity, auto-aggregation, co-aggregation and with α or γ haemolytic potential, once daily for 3 days, and 0.2 ml of 10^8 CFU *Salmonella enteritidis*/chick on day4. Three chicks/group and for each of the LAB treatments were randomly withdrawn for sampling at 1-, 4- and 7-days post *Salmonella enteritidis* challenge, and were euthanized via cervical dislocation. Samples were taken from the crop, gizzard, cecum contents, spleen and liver. These samples were serially diluted with PBS and plated on *Salmonella-Shigella* Agar. The number of *Salmonella enteritidis* were counted after 48h incubation at 37 °C and expressed as CFU. The CFU recovered from the samples of LAB treated chicks were compared with those of the blank and the normal controls.

3.0 Results and Discussion

3.1 Isolated bacteria

Fourteen bacteria that were gram positive, non-stringy in KOH, catalase negative, non-motile and non-spore forming were selected out of the twenty-eight morphologically different isolates from the samples, as potential LAB (Table 1). The bacteria were identified based on their biochemical characteristics (Table 2). *Lactobacillus fermentum* was the most dominant LAB isolated from the various food sources. Others were *Enterococcus feacalis, Pediococcus pentosaceus, Lactococcus lactis, L. plantarum, L. brevis, L. salivarius* and *L. paracasei*. This is an indication that the food samples used have lactic acid bacteria as part of the fermenting organisms and may serve as sources of the bacteria for use as starter in food fermentation. Similar studies have reported isolation of *Lactobacillus fermentum* from ogi, wara, fufu, gari, millet dough and nono (Ogunshe *et al*., 2007; Owusu-Kwarteng *et al*., 2015; Ikwuemesi, 2021). Other lactic acid bacteria including *Lactococcus lactis, L. plantarum* isolated in this study, were also found in many cereals and cassava based fermented foods (Oyewole and Odunfa, 1990; Amoa-Awua *et al*., 1996; Kostinek *et al*., 2005; Nwachukwu *et al*., 2010)

3.2 Antagonistic activity of LAB isolates on test organisms

Among the fourteen LAB isolates, only ten showed antimicrobial activity against the test organisms (Table 3). Six of the test organisms used were inhibited by *Pediococcus pentosaceus*, five each by *E. faecalis* and *L. brevis*, and four by *L. plantarum*. The highest antimicrobial activity in terms of inhibition zone (28 mm) and broth culture dilution (82%) was however demonstrated by *L. fermentum* against *Salmonella enteritidis*. The other lactic acid bacteria also exhibited different levels of activity against the test organisms. Some LAB isolates from the Nigerian indigenous fermented foods samples have antimicrobial activities which may qualify them as probiotics. In consistence with findings in this study, *L. fermentum L. brevis, L. plantarum* and *P. pentosaceus* were among the strains of LAB that demonstrated high antimicrobial activities against *Escherichia coli* O157: H7, *Enterococcus faecalis, Salmonella typhimurium, Salmonella enteritidis, Listeria monocytogene, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus* spp. reported previously (Reuben *et al*., 2020; Popoola *et al*., 2021). In addition, the antibacterial activities of the LAB isolates compared favorably with that of some commercial antibiotics including Ampiclox, Zinnacef, Amoxacillin, Erythromycin and Augmentin as shown in the sensitivity test (Table 4), and may serve as better drugs to treat gastrointestinal diseases caused by the organisms.

3.3 pH and Titratable Acidity of the culture filtrates of the LAB isolates

As presented in Table 5, the pH values and Titratable Acidity of the culture filtrates of the LAB isolates that showed antagonistic activity against the test organisms indicated high acidity. And this may be partly responsible for the antagonistic activities observed. Enan *et al*. (2018) also attributed the antimicrobial activities of LAB to primary metabolites including lactic, acetic, formic and benzoic acids.

Table 1: Morphological characteristics of isolated bacteria

	Colonial Morphology										Cellular Morphology				
Isolates	**Shape**	**Size**	**Pigmentation**	**Margin**	**Elevation**	**Colony Surf.**	**Optical Cha.**	**Consistency**	**Appearance**	**Gram Rxn.**	**Cell shape**	**Arrangement**	**Cell Size**	**Spore stain**	**Motility**
L5	I	M	W	E	F	Ro	O	B	D	+	Rd	Sg	M	-	-
L6	C	L	Cr	E	R	Sm	O	B	G	+	Cc	Cl	S	-	-
L7	C	S	Cr	Lo	F	Sm	O	Mo	D	+	Cc	Ch	S	-	-
L9	C	L	W	E	F	Sm	O	Mo	G	+	Cc	Ch	S	-	-
L10	C	M	Cr	E	F	Sm	O	V	D	+	Rd	Sg	M	-	-
L12	C	L	W	E	R	Ro	O	B	D	+	Cc	Sg	M	-	-
L16	C	M	W	U	R	Ro	O	V	D	+	Rd	Cl	S	-	-
L17	I	M	W	E	F	Ro	O	B	D	+	Rd	Sg	M	-	-
L18	I	S	Cr	Lo	F	Sm	O	B	D	+	Rd	Cl	S	-	-
L20	C	M	Cr	E	Co	Ro	O	V	D	+	Rd	Ch	S	-	-
L22	C	S	Cr	E	F	Sm	O	V	D	+	Cc	Cl	S	-	-
L23	C	S	W	E	R	Sm	O	Mo	G	+	Rd	Sg	S	-	-
L25	C	L	Cr	E	F	Ro	O	Mo	D	+	Rd	Sg	M	-	-
L27	C	M	Cr	E	F	Sm	T	Mo	G	+	Rd	Sg	S	-	-

C: Circular, I: Irregular, S: Small, M: Medium, L: Large, W: White, Cr: Cream, Entire, L: Lobate, E: Entire; U: Umbonate, R: Raised, F: Flat, Co: Convex, Sm: Smooth, Ro: Rough, Tp: Transparent, T: Transluscent, O: Opaque, B: Butyrous, Mo, Moist, V: Viscid, +: G: Glistening, D: Dull, +: Positive, -: Negative, Rd: Rod, Cc: Cocci, Ch: Chains, Cl: Clusters, P: Pairs, Sg: Single.

Table 2: Biochemical Characteristics of Isolated Bacteria

Isolate	Catalase	Citrate	Urease	Indole	Methyl Red	Voges	Arginine	Nitrate	Starch	Growth at	Growth at	H_2S	Gas	Reaction	Glucose	Lactose	Maltose	Sucrose	Mannitol	Xylose	D-Arabinose	L-Arabinose	Ribose	Mannose	Probable Organisms
L5	-	-	-	-	-	+	+	-	-	+	-	-	+	AK	AG	+	+	+	-	-	+	-	+	+	*Lactobacillus fermentum* LF1
L6	-	+	-	+	-	+	+	-	-	+	+	+	+	KK	A	+	+	+	+	-	-	-	+	+	*Enterococcus faecalis* EC1
L7	-	-	-	-	-	-	-	-	-	+	+	-	-	KK	A	+	+	+	-	-	+	+	+	+	*Pediococcus pentosaceus*
L9	-	+	-	-	-	-	+	-	-	-	-	-	+	KA	AG	+	+	+	+	-	-	-	+	+	*Lactococcus lactis*
L10	-	+	-	-	+	+	+	-	-	+	+	+	+	AK	A	+	+	+	+	-	-	-	+	+	*Enterococcus faecalis* EC2
L12	-	-	-	-	-	-	+	+	-	-	+	-	-	KA	A	+	+	+	+	-	-	-	+	+	*Lactobacillus plantarum* LP1
L16	-	-	-	-	-	+	+	-	-	+	-	-	+	AK	AG	+	+	+	-	-	-	+	+	+	*Lactobacillus fermentum* LF2
L17	-	-	-	-	-	-	+	-	-	-	+	-	+	AK	AG	+	+	+	-	-	-	-	+	-	*Lactobacillus brevis* LB1
L18	-	-	-	-	-	+	+	-	-	+	-	-	+	AA	AG	+	+	+	-	+	+	-	+	+	*Lactobacillus fermentum* LF3
L20	-	-	-	-	+	-	+	-	-	-	+	-	+	KA	AG	+	+	+	-	-	-	-	+	-	*Lactobacillus brevis* LB2
L22	-	-	-	+	-	+	-	-	-	+	-	+	-	AK	A	+	+	+	+	-	-	-	-	+	*Lactobacillus salivarius*
L23	-	-	-	-	-	-	+	+	-	-	-	-	-	AK	A	+	+	+	+	-	-	+	+	+	*Lactobacillus plantarum* LP2
L25	-	-	-	-	-	+	-	-	-	-	-	-	-	AK	A	+	+	+	+	-	-	-	+	+	*Lactobacillus paracasei*
L27	-	-	-	-	-	-	+	-	-	+	-	-	+	AK	AG	+	+	+	-	-	-	-	+	+	*Lactobacillus fermentum* LF4

+: Positive, -: Negative, AK: Acid/Alkaline, KK: Alkaline/Alkaline, KA: Alkaline/Acid, AA: Acid/Acid, A: Acid, AG: Acid and gas

Table 3: Antagonistic activity of the LAB isolates on test organisms

Isolates	**Zone of inhibition on agar (mm) and percentage dilution of broth culture (in parenthesis)**								
	Y.E	S.L	E.C	B.S	S.E	S.A	K.O	C.F	C.A
L. fermentum (LF1)	-	-	-	-	++++ (57)	-	++++ (53)	-	-
E. faecalis (EC1)	++++ (46)	-	-	-	-	-	-	++++ (6)	-
P. pentosaceus	++++ (47)	-	-	++ (27)	+++ (23)	++ (19)	-	++++ (42)	+++ (33)
L. lactis	++ (29)	-	-	-	+ (16)	+++ (36)	-	-	-
L. plantarum **(LP1)**	-	+++ (18)	-	-	++++ (34)	+++ (22)	-	++++ (42)	-
L. plantarum **(LP2)**	-	-	-	-	-	-	-	-	-
E. faecalis (EC2)	-	++++ (57)	+++ (45)	++ (31)	-	-	-	++ (32)	++ (23)
L. fermentum (LF2)	-	+++ (18)	-	-	++ (24)	-	-	-	-
L. brevis (LB1)	-	-	-	-	-	-	-	-	-
L. fermentum (LF3)	-	-	-	-	-	-	-	-	-
L. brevis (LB2)	+++ (44)	-	+++ (31)	+++ (24)	+++ (30)	-	-	+++ (18)	-
L. salivarius	-	-	-	-	-	-	-	-	-
L. paracasei	-	-	-	-	+++ (23)	-	+++ (31)	-	-
L. fermentum **(LF4)**	-	-	++ (25)	-	+++ (33)	++ (22)	-	-	-

Values are presented as + and – signs for agar diffusion test as follow: "-" denotes 0 (no inhibition), "+" denotes ≤ 5; "++" denotes > 5 to ≤ 10; "+++" denotes > 10 to ≤ 20; "++++" denotes > 20; while values in parenthesis are percentage clearance in the broth dilution method. **Test organisms:** Y.E; *Yersinia enterocolitica*, S.L; *Serratia liquifaciens*, E.C; *Eshcherichia coli*, B.S; *Bacillus subtilis*, S.E; *Salmonella enteritidis*, S.A; *Staphylococcus aureus*, *K.O; Klebsiella oxytoca, C.F; Citrobacter freundii*, C.A; *Candida albicans*

Table 4: Sensitivity of Test Organisms to Standard Antibiotics

Test organisms	**Zone of inhibition (mm)**													
	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E	OFX	CH	SP	AU
Y.E	++++	-	-	-	-	-	++++	-	-	-	+++	-	++++	-
S.L	-	-	-	-		-	++++	-	-	-	-	+++	++++	-
E.C	-	-	-	-	-	-	-	-	-	-	-	+++	-	-
B.S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.E	++++	-	-	-	-	-	-	-	-	-	-	++++	-	-
S.A	++++	++++	-	-	-	-	-	-	++++	-	-	-	-	-
K.O	-	-	-	-	-	-	-	++++	-	-	-	-	-	-
E.CC	++++	-	-	-	-	-	++++	-	+++	-	-	+++	++++	-
C.F	-	-	-	-	-	-	+++	-	-	-	-	-	-	-

Values are presented as + and – signs for agar diffusion test as follow: "-" denotes 0 (no inhibition), "+" denotes ≤ 5; "++" denotes > 5 to ≤ 10; "+++" denotes > 10 to ≤ 20; "++++" denotes > 20; **Test organisms:** S.A; *Staphylococcus aureus*, B.S; *Bacillus subtilis*, E.C; *Escherichia coli*, S.L; *Serratia liquifacien*, K.O; *Klebsiella oxytoca*, E.CC; *Enterococcus cloacae*, C.F; *Citrobacter freundii*, Y.E; *Yersinia enterocolitica*, S.E; *Salmonella enteridis*. **Antibiotics:** PEF; Pefloxacin, CN; Gentamycin, APX; Ampiclox, Z; Zinnacef, AM; Amoxacillin, R; Rocephin, CPX; Ciprofloxacin, S; Streptomycin, SXT; Septrin, E; Erythromycin, OFX; Ofloxacin, CH; Chloranphenicol, SP; Sparfloxacin and AU; Augmentin

Table 5: pH and titratable acidity of culture filtrates the LAB isolates

Isolates	**pH**	**Titratable acidity (%)**
Lactobacillus fermentum (LF1)	4.16	0.896
Enterococcus faecalis (EF1)	6.10	0.421
Pediococcus pentosaceus -	4.38	0.893
Lactococcus lactis	5.48	0.775
Lactobacillus plantarum (LP1)	4.77	0.836
Lactobacillus fermentum (LF2)	5.04	0.764
Lactobacillus brevis (LB2)	4.89	0.802
Lactobacillus plantarum (LP2)	4.24	0.886
Lactobacllus paracasei	4.19	0.895
Lactobacillus fermentum (LF3)	4.55	0.881

3.4 Probiotic activities of the LAB isolates

3.4.1 Acid and Bile Tolerance

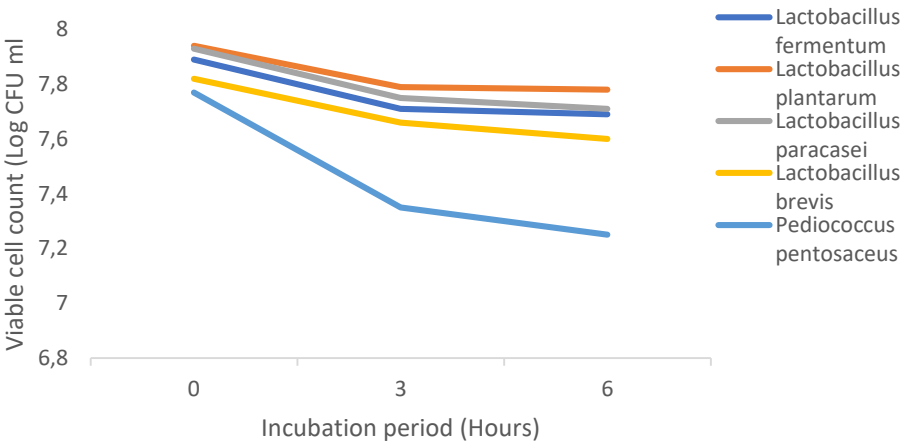
Among the LAB isolates screened, *Lactobacillus fementum* (LF1), *L. brevis* (LB2), *L. plantarum* (LP2), *L. paracasei* and *Pediococcus pentosaceus* were able to tolerate low pH of 1.5 and 3.5, and bile concentration of 0.3 and 0.5% with a decrease in viable colony count not more than 0.6 log CFU/mL and 0.3 log CFU/mL respectively (Figures 1 and 2). Acid and Bile Tolerance are among the properties that could qualify the isolates as probiotics. Similar results were reported by Ayodeji *et al*. (2017), Olatunde *et al.* (2018) and Popoola *et al*. (2021).

3.4.2 Survivability of the LAB isolates in Simulated Gastric and Intestinal Juice

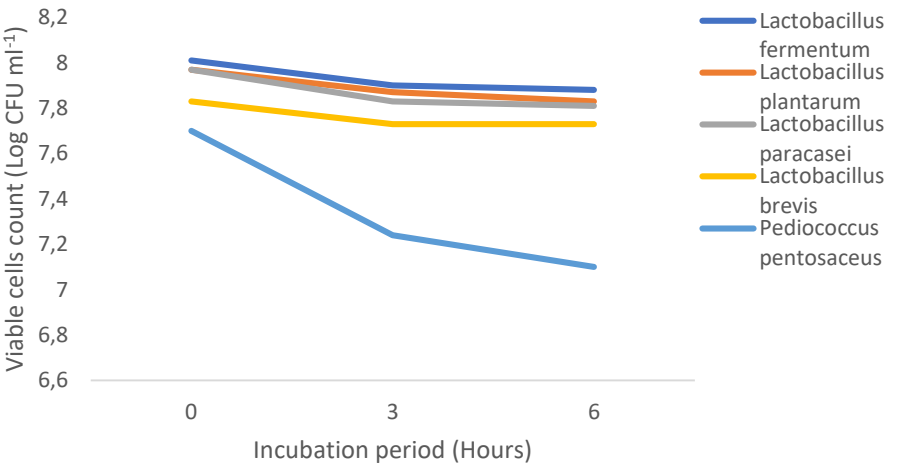
The ability of the LAB strains to survive simulated gastric and intestinal juice is presented in Figures 3. Except for *Pediococcus pentosaceus*, all the LAB strains demonstrated high survivability with a decrease not exceeding 0.3 log CFU/ml after exposure for 6 hours. Similar results were obtained with LAB strains in previous studies (Reuben *et al*., 2020; Watanabe *et al*., 2020; Wang *et al*., 2020).

3.4.3 Phenol Tolerance of the LAB isolates

Phenol is a toxic metabolite of aromatic amino acids, secreted in the GIT by some bacteria and possess bacteriostatic activities (Suskovic, 1997). Phenol tolerance is therefore important for survivability in the GIT. All the LAB isolates tested showed high tolerance to 0.2% phenol while *L. fermentum* tolerated up to 0.4% (Figure 4) giving it an edge over the others. Similar tolerance levels were reported in previous studies (Shehata *et al*., 2016; Reuben *et al*., 2020).

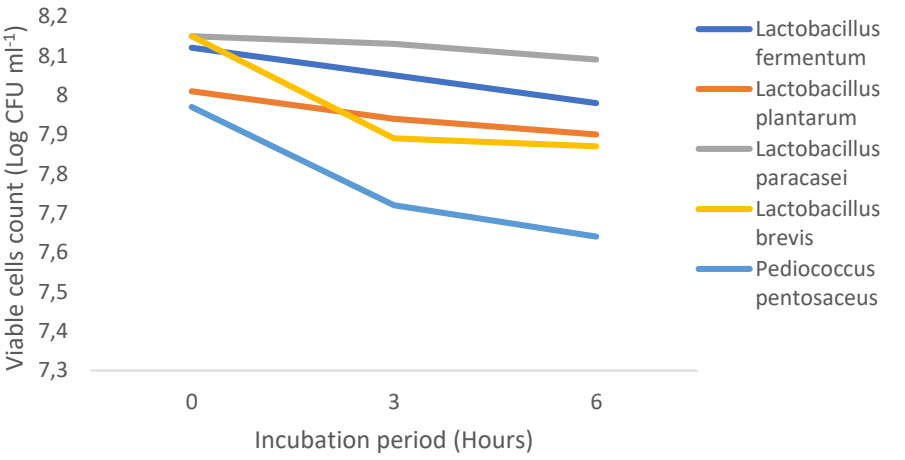


(a)

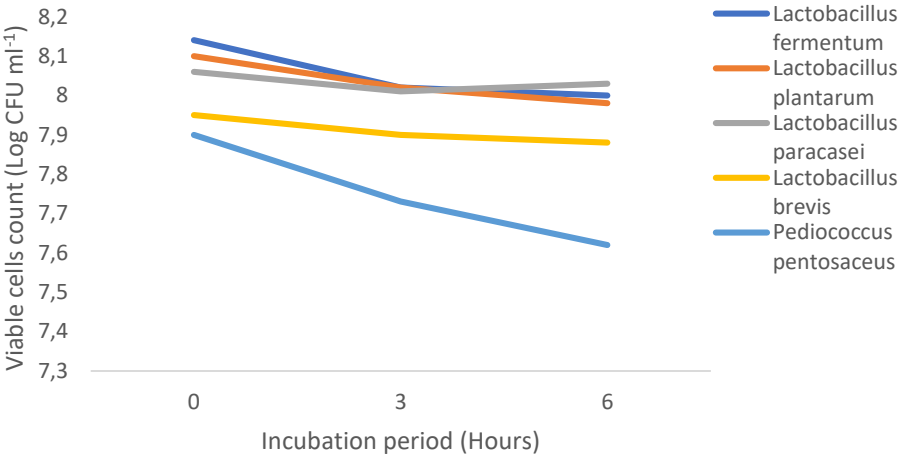


(b)

Fig. 1: Acid tolerance of LAB isolates (a) pH 1.5 (b) pH 3.5. The bacterial isolates were exposed to hydrochloric acid to mimic the acidity of the stomach for a period of 6 hours. Viable cell counts were obtained after culturing on MRS agar

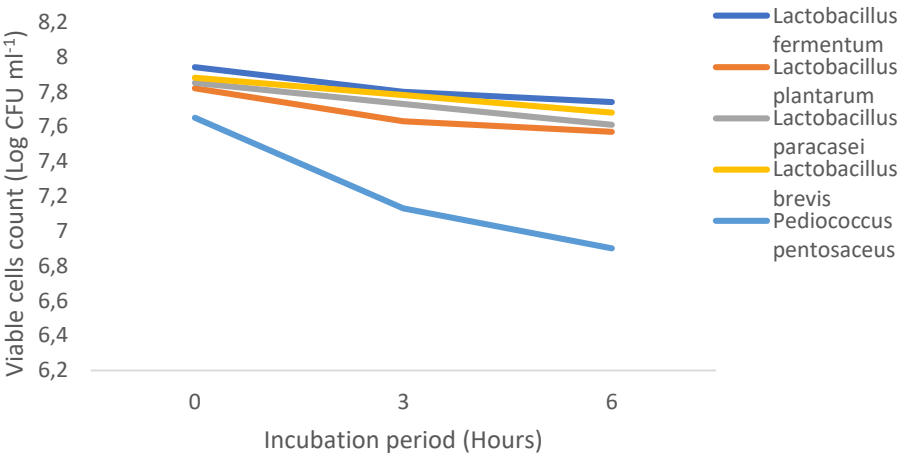


(a)

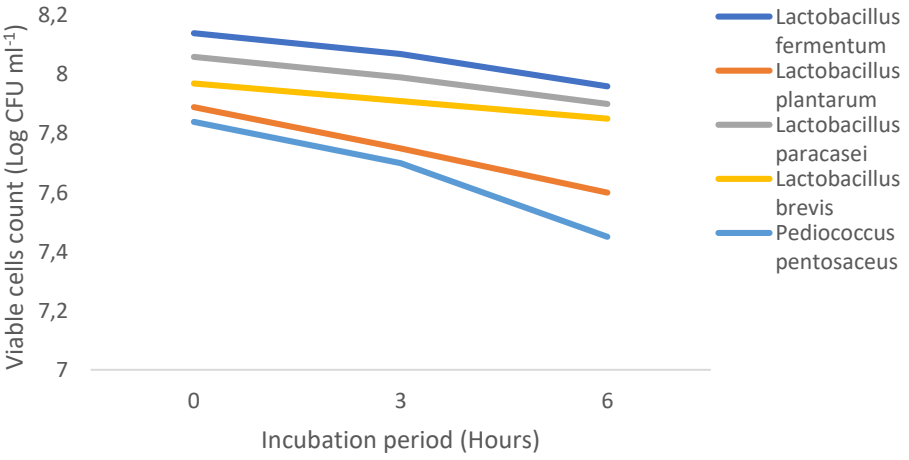


(b)

Fig. 2: Tolerance of LAB isolates to bile salts at (a) 0.3% (b) 0.5%. The bacterial isolates were exposed to bile salts as it occurs in the intestine for a period of 6 hours. Samples were withdrawn periodically and cultured on MRS agar to obtain viable cell counts.



(a)



(b)

Fig. 3: Survivability of LAB isolates in (a) simulated gastric juice and (b) simulated intestinal juice. The general gastrointestinal condition was simulated and survivability determined by viable cell counts on MRS agar.

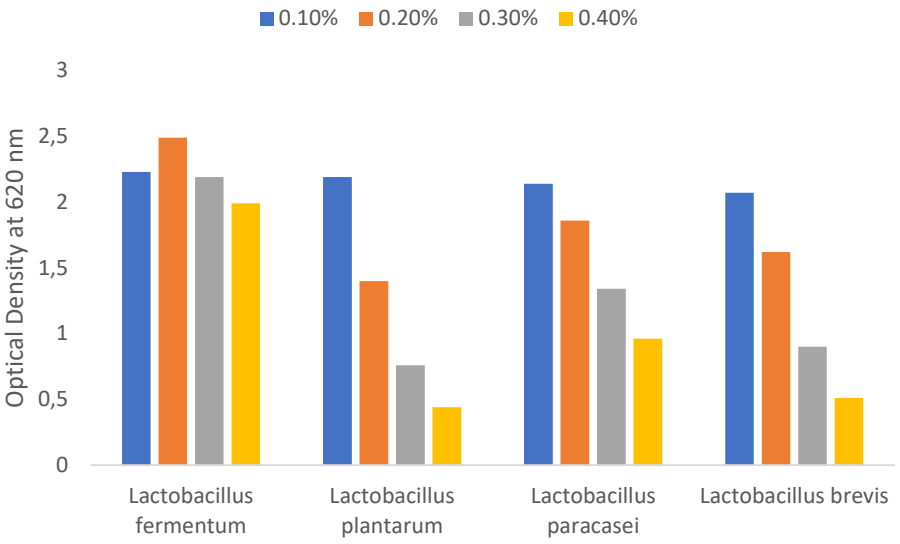


Fig. 4: Fig. 4: Phenol tolerance of LAB isolates. Survivability is also indicated by the ability to tolerate phenol which is a toxic metabolite of the normal gastrointestinal bacterial flora. The optical density after exposure to phenol was used as the measure of survivability.

3.4.4 Hydrophobicity Abilities of the LAB Isolates

Hydrophobicity is one of the important properties that define probiotic organisms. LAB strains that possess high hydrophobicity have good binding properties and are able to form barriers that prevent colonization of pathogens (Wang *et al*., 2020). The percentage hydrophobicity of the LAB isolates in this study ranged from 38.76 ± 0.55% to 58.06 ± 0.35% (Figure 5) with highest obtained for *L. fermentum* in both dichloromethane and xylene. Again, *L. fermentum* stands out as a better probiotic organism with this high hydrophobicity property. Reuben *et al.* (2020) and Popoola *et al*. (2021) also reported similar percentage hydrophobicity for LAB strains in their studies.

3.4.5 Aggregative Abilities of the LAB Isolates

Auto-aggregative ability was highest with *L. fermentum* (54.05 ± 0.53%) and lowest with *L. brevis* (34.38 ± 0.61%). Except for *L. brevis* the percentage auto-aggregation of the isolates was above the 40% benchmark reported by Wang *et al*., 2020. Also, *L. fermentum* (LF1) had the highest percentage co-aggregation with *Salmonella enteritidis* at 74.31 ± 0.33% while *L. plantarum* (47.74 ± 0.61%) had the lowest. High co-aggregation with *Salmonella enteritidis* was also reported by Reuben *et al.* (2020). Todorov *et al*. (2011) established a relationship between hydrophobicity and aggregation, similar to findings in this study regarding *L. fermentum* which has the highest hydrophobicity and corresponding aggregative abilities.

3.4.6 Antibiotics Susceptibility of the LAB Isolates

The safety of potential probiotic organism is measured by their resistance to a number of commonly used human and veterinary antibiotics due to the likelihood of transfer of antibiotic resistance to pathogens (Szukowska and Gwiazdowska, 2021). The LAB isolates in this study exhibited varying degree of susceptibility to the antibiotics tested (Table 9). This indicate that they may not carry resistance genes and therefore allaying the possibility of transferring such to pathogens. Similar results were reported by Adesina *et al.* (2016); Choi *et al*. (2018) and Wang *et al*. (2020).

3.4.7 Haemolytic Activity of the LAB Isolates

Haemolysis is one of the other indices used to determine the safety of probiotics (Adimpong *et al*., 2012). In consistence with previous findings (Hussain *et al*., 2008), and indicating potentials as probiotics, all the LAB isolates in this study exhibited non-haemolytic effect on blood agar.

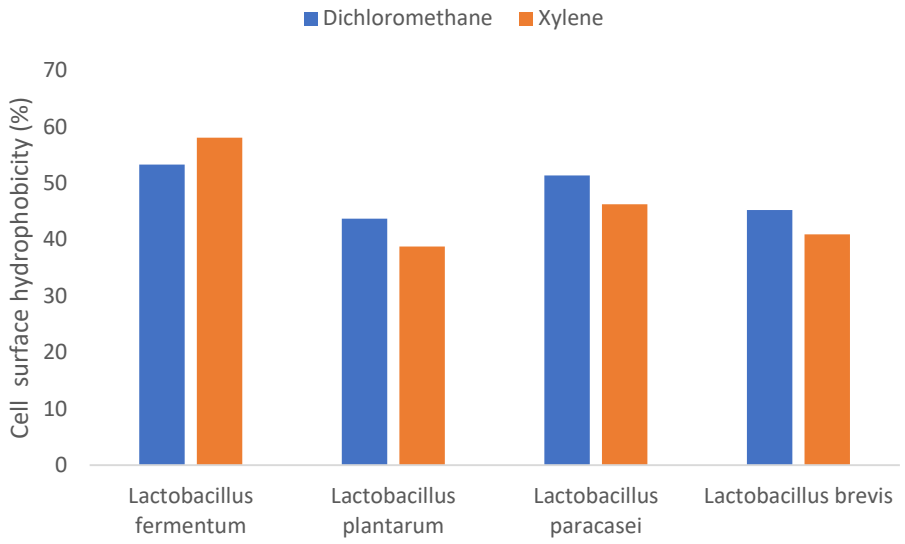


Fig. 5: Cell Surface Hydrophobicity of LAB isolates. Hydrophobicity which is an important property of probiotics, was measured as the affinity of the LAB isolates to the solvent, dichloromethane and xylene. Result showed higher affinity to dichloromethane.

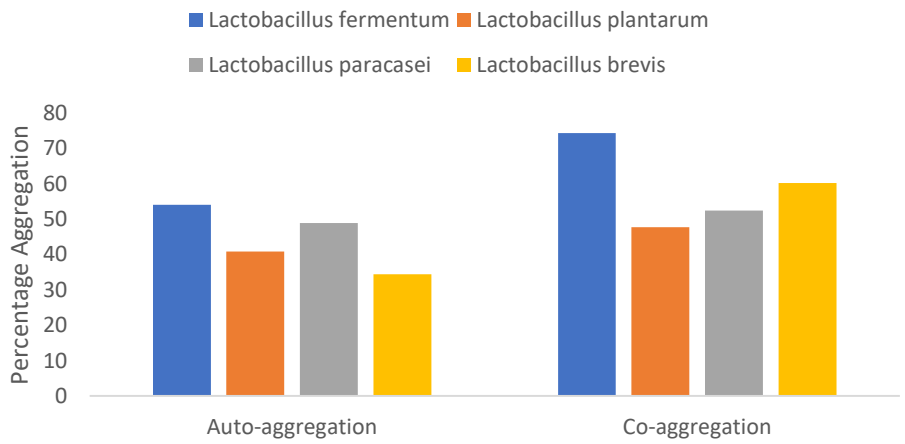


Fig. 6: Auto-aggregative and Co-aggregative ability of LAB isolates. Auto-aggregation was measured to determine the competitive properties of probiotics. Co-aggregation also determine the effectiveness of probiotic LAB against pathogens.

Table 6: Susceptibility of the LAB Isolates to Standard Antibiotics

Antibiotics	Inhibition zone (mm)			
	Lactobacillus fermentum (LF1)	*Lactobacillus brevis* (LB2)	*Lactobacillus plantarum* (LP2)	*Lactobacillus paracasei*
CH (30µg)	8.33 ±1.52	7.33 ± 1.52[b]	27.33 ± 3.78	25.00 ± 3.6
E (10 µg)	17.33 ± 1.52	25.67 ± 3.51	28.00 ± 3.00	7.67 ± 1.52
AU (30µg)	25.67 ± 2.08	18.33 ± 1.15	30.00 ± 1.00	27.33 ± 3.05
CFX (30µg)	25.33 ± 2.08	26.00 ± 0.00	7.00 ± 2.64	18.00 ± 3.00
CTN (25µg)	17.00 ±0.00	17.67 ± 1.52	19.30 ± 2.00	7.33 ±2.51
CN (10µg)	9.00 ± 2.00	6.00 ±1.00	0.00 ± 0.00	6.67 ±2.51
SLF (30µg)	7.00 ± 1.00	21.00 ± 0.00	16.00 ±0.00	25.00 ±1.00
CPX (10 µg)	18.00 ± 2.00	19.00 ± 0.00	25.00 ± 1.00	6.33 ± 1.52
PEF (10µg)	0.00	8.33 ± 0.57	7.00 ± 1.00	8.33 ± 2.51
APX (30µg)	5.00 ± 1.00	28.33 ± 3.51	23.00 ± 1.00	7.33 ± 1.52
AMP (30µg)	18.67 ±2.51	20.00 ± 0.00	0.00	21.33 ± 0.6

Values are means ± SD; n = 3. CH; Chloramphenicol, E; Erythromycin, AU; Augmentin, CFX; Cefuroxime, CTN; Ceftriaxone, CN; Gentamycin, SLF; Sulfamethoxazole, CPX; Ciprofloxacin, PEF; Pefloxacin, APX; Ampiclox, and AMP; Ampicillin

3.4.8 *In vivo* Antimicrobial Activities of the LAB Isolates using broiler Chicken

In order to be described as probiotic, an organism should be able to competitively exclude or inhibit pathogens that may cause harm to the host (Reuben *et al*., 2020). *Salmonella enteritidis* is a known pathogen of the GIT of human and a number of farm animals including chickens (Feasey *et al.*, 2016). In this light, *Salmonella enteritidis* was used in the *in vitro* antimicrobial study using broiler chickens. Table 10 shows the number of *Salmonella enteritidis* recovered from the spleen, liver and content of the crop, gizzard and cecum of infected chickens after treatment with LAB isolates. Treatment with culture of LAB isolates especially *L. fermentum* significantly reduced the population of *S. enteritidis* in the various GIT sections, spleen and liver of the infected chickens, and over days of exposure in the case of spleen and liver. This affirms the antagonistic and probiotic activities of the *L. fermentum* isolate in this study. In a similar study, *Lactobacillus plantarum* PZ01, *Lactobacillus salivarius* JM32 and *Pediococcus acidilactici* JH231 were the LAB that reduced the number of *Salmonella* in intestinal content, spleen and liver after a *Salmonella* infection of broiler chicks (Feng *et al*., 2016).

Table 7: Effects of LAB treatment on the count of *Salmonella enteritidis* recovered from some organs of chicks

	Number of *Salmonella enteritidis* cells (log CFU ml^{-1})														
	Crop			**Gizzard**			**Cecum content**			**Spleen**			**Liver**		
	1st day	4th Day	7th Day	1st day	4th day	7th Day	1st day	4th day	7th day	1st day	4th day	7th day	1st day	4th day	7th day
Control	7.55 ± 0.04$^{c;1}$	7.46 ± 0.01$^{d;1}$	7.45 ± 0.02$^{c;1}$	7.75± 0.02$^{c;1}$	7.79± 0.02$^{c;1}$	7.84± 0.01$^{c;1}$	8.16± 0.02$^{d;1}$	8.29± 0.01$^{d;1}$	8.32± 0.01$^{d;1}$	1.98± 0.02$^{d;2}$	2.16± 0.01$^{d;1}$	2.19± 0.01$^{c;1}$	2.11± 0.03$^{d;1}$	2.23± 0.01$^{e;1}$	2.29± 0.01$^{c;1}$
Treatment 1	7.04 ± 0.14$^{a;1}$	6.59 ± 0.26$^{a;2}$	0.00$^{a;3}$	7.22± 0.10$^{a;1}$	6.92± 0.13$^{a;1}$	0.00$^{a;2}$	7.78± 0.02$^{b;1}$	7.39± 0.07$^{a;1}$	7.11± 0.12$^{a;2}$	0.00$^{a;1}$	0.00$^{a;1}$	0.99± 0.09$^{a;1}$	0.00$^{a;1}$	0.00$^{a;1}$	0.00$^{a;1}$
Treatment 2	7.21 ± 0.10$^{a,b;1}$	6.95 ± 0.10$^{a,b;2}$	6.68 ± 0.37$^{b;2}$	7.52± 0.02$^{b;1}$	7.45± 0.05$^{b;1}$	7.09± 0.08$^{b;1}$	7.90± 0.02$^{b;1}$	7.96± 0.02$^{b;1}$	7.74± 0.04$^{c;1}$	1.57± 0.05$^{c;1}$	1.68± 0.02$^{b;1}$	1.07± 0.11$^{a;2}$	0.00$^{a;2}$	1.78± 0.06$^{b;1}$	1.85± 0.02$^{a;1}$
Treatment 3	7.47 ± 0.05$^{b,c;1}$	7.34 ± 0.07$^{c,d;1}$	7.22± 0.16$^{c,d;1}$	7.33± 0.06$^{a;1}$	7.53± 0.05$^{b;1}$	7.66± 0.03$^{c;1}$	8.03± 0.02$^{c;1}$	8.13± 0.01$^{c;1}$	8.20 ±0.01$^{d;1}$	1.90± 0.01$^{d;1}$	1.98± 0.01$^{c;1}$	2.06± 0.01$^{c;1}$	1.98± 0.01$^{c;1}$	2.11± 0.03$^{d;1}$	2.14± 0.02$^{c;1}$
Treatment 4	7.27 ± 0.13$^{a,b;1}$	7.01 ± 0.09$^{b,c;1}$	0.00$^{a;2}$	7.36± 0.02$^{a;1}$	7.62± 0.02$^{b,c;1}$	7.20± 0.05$^{b;1}$	7.79± 0.03$^{a;1}$	7.94± 0.02$^{b;1}$	7.56 ± 0.04$^{b;2}$	1.45± 0.05$^{b;1}$	1.72± 0.03$^{b;1}$	1.49± 0.05$^{b;1}$	1.87± 0.02$^{b;1}$	1.96± 0.01$^{c;1}$	1.95± 0.01$^{b;1}$

Values were compared along the columns between treatment categories and control; and along the rows between days in each of the samples using Duncan multiple intervals test. Values are means ± SD; n = 3. Values with different superscript along the same column^{a-d} and row for each sample$^{1-3}$ are significantly different ($P < 0.05$). Treatment 1: *L. fermentum*, Treatment 2: *L. brevis*, Treatment 3: *L plantarum*, Treatment 4: *L. paracasei*

CONCLUSION AND RECOMMENDATION

Four of the isolated Lactic Acid Bacteria, *Lactobacillus fermentum, L. brevis, L. plantarum* and *L. paracasei* exhibited high antimicrobial activities against the test organisms with wide spectra. The LAB survived the simulated gastrointestinal conditions of low pH, bile salts, phenol and gastrointestinal juices. Their hydrophobicity, auto- and co-aggregative abilities were high. They were found susceptible to commonly used antibiotics and exhibited non-haemolytic effects as a demonstration of their safety. In addition, *L. fermentum* demonstrated high in vivo antimicrobial activity against *S. enteritidis* in chicken. Probiotic activities have therefore been demonstrated by the four LAB isolates. The isolates especially *L. fermentum* can be included in foods, feeds and pharmaceuticals after a more comprehensive *in vivo* and toxicological studies.

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