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Antimicrobial properties, safety, and probiotic attributes of lactic acid bacteria isolated from Sauerkraut

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Abstract:

Background: According to the World Health Organization, probiotics have been defined as live microorganisms that when administered in the right amount provide health benefits to the host. This study aims to isolate lactic acid bacteria (LAB) from Sauerkraut and determine their anti-pathogenic potential and suitability as probiotics.
Methodology: Lactic acid bacteria (LAB) were isolated from fermented cabbage obtained from an open market in Akure, Ondo State, Nigeria by inoculating the prepared cabbage suspension on triplet plates of Rogosa agar (*Lactobacillus* selecting agar) and incubating in an anaerobic jar with gaspaks at 37°C for 72 hours. The LAB isolates were presumptively identified phenotypically by colony morphology, Gram stain reaction, and catalase test. The antibacterial activity of the LAB isolates was then carried out using agar overlay and agar-well diffusion methods. The most efficient LAB isolate was selected based on its strong antibacterial activity, confirmed by 16S rRNA sequencing and further evaluated for probiotic activities including bile salt resistance, survival in low pH, hydrophobicity of the cell surface, auto-aggregation, and co-aggregation. The putative probiotic LAB isolate was also evaluated for its safety using *in vitro* tests (antibiotic susceptibility testing, haemolysis, and DNase tests) and *in vivo* assays (sub-acute oral toxicity tests in male albino rats).
Results: A total of 5 LAB isolates were preliminarily identified from the cabbage. The LAB isolate that showed strong antibacterial activity was confirmed as *Levilactobacillus brevis.* The isolate showed 83.3% survival rate in low pH of 3 and 85.9% in 0.3% bile concentration indicating potential tolerance to gastrointestinal conditions. The cell surface hydrophobicity assay showed 51% and auto-aggregation of 60% which represents the adhesion properties of the isolate. The sub-acute oral toxicity evaluation of the putative probiotic strain in Wister albino rats showed no altered health condition.
Conclusion: The findings in this study suggest that *Levilactobacillus brevis* isolated from Sauerkraut is suitable as probiotics and could be applied in the pharmaceutical and food industry.

Keywords: probiotics; safety assessment; *Levilactobacillus brevis*; sauerkraut; cabbage

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Propriétés antimicrobiennes, innocuité et attributs probiotiques des bactéries lactiques isolées de la choucroute

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Résumé:

Contexte: Selon l'Organisation mondiale de la santé, les probiotiques ont été définis comme des micro-organismes vivants qui, lorsqu'ils sont administrés en quantité appropriée, procurent des avantages pour la santé de l'hôte. Cette étude vise à isoler les bactéries lactiques (LAB) de la choucroute et à déterminer leur potentiel anti-pathogène et leur aptitude en tant que probiotiques.
Méthodologie: Des bactéries lactiques (LAB) ont été isolées à partir de chou fermenté obtenu sur un marché libre à Akure, dans l'État d'Ondo, au Nigeria, en inoculant la suspension de chou préparée sur des plaques triplet de gélose Rogosa (*Lactobacillus* sélectionnant de la gélose) et en incubant dans un bocal anaérobie avec gaspaks à 37°C pendant 72 heures. Les isolats de LAB ont été présumément identifiés phénotypiquement par la

morphologie des colonies, la réaction de coloration de Gram et le test de catalase. L'activité antibactérienne des isolats de LAB a ensuite été réalisée en utilisant des méthodes de superposition d'agar et de diffusion en puits d'agar. L'isolat de LAB le plus efficace a été sélectionné en fonction de sa forte activité antibactérienne, confirmée par le séquençage de l'ARNr 16S et évaluée plus avant pour les activités probiotiques, notamment la résistance aux sels biliaires, la survie à un pH bas, l'hydrophobicité de la surface cellulaire, l'auto-agrégation et la co-agrégation. L'isolat putatif de LAB probiotique a également été évalué pour sa sécurité à l'aide de tests in vitro (tests de sensibilité aux antibiotiques, hémolyse et tests de DNase) et in vivo (tests de toxicité orale subaiguë chez des rats albinos mâles).
Résultats: Un total de 5 isolats de LAB ont été préalablement identifiés à partir du chou. L'isolat LAB qui a montré une forte activité antibactérienne a été confirmé comme étant *Levilactobacillus brevis*. L'isolat a montré un taux de survie de 83,3% à un pH faible de 3 et de 85,9% à une concentration de bile de 0,3% indiquant une tolérance potentielle aux conditions gastro-intestinales. Le test d'hydrophobicité de la surface cellulaire a montré 51% et une auto-agrégation de 60%, ce qui représente les propriétés d'adhérence de l'isolat. L'évaluation de la toxicité orale subaiguë de la souche probiotique putative chez les rats albinos Wister n'a montré aucun état de santé altéré.
Conclusion: Les résultats de cette étude suggèrent que le *Levilactobacillus brevis* isolé de la choucroute convient comme probiotique et pourrait être appliqué dans l'industrie pharmaceutique et alimentaire.

Mots-clés: probiotiques; évaluation de la sécurité; *Levilactobacillus brevis*; choucroute; chou

Introduction:

Microorganisms have historically been an integral part of the human diet, absorbed through naturally fermented foods such as fruits and vegetables and their juices. Lactic acid bacteria (LAB) are common microorganisms that may be found in any environment that is high in carbohydrates, such as plants and other fermented foods (1). The use of preserved and fermented vegetables in human nourishment has been around since ancient times, with sauerkraut being one the most widely consumed vegetable fermented products (2). Sauerkraut, which is a fermented cabbage food with origin in Central Europe, has traditionally been seen as an underutilized natural source of probiotic bacteria that might be used as a starting culture in other fermentation procedures (3).

The health promoting qualities of Sauerkraut have been attributed to its high contents of bioactive components produced as a result of indigenous microbial metabolic activities (4). *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Pediococcus,* and *Enterococcus* are among the known beneficial bacteria that contribute to sauerkraut formation. Most of the beneficial bacteria with a proven history of safe use are probiotic bacteria.

The human gastrointestinal tract (GIT) and fermented dairy products are the two main sources of probiotic bacteria. The strains derived from other sources, such as plant-based meals, however, provide significant options since they might be more helpful and viable for use in comparable non-dairy-based probiotic products (5). Genetically, plant-derived LAB strains are comparable to milk-derived bacteria and have been shown to exhibit extra advantageous traits such as greater tolerance to stress conditions and the ability to ferment a wider range of carbohydrate sources (6,7).

Probiotics are gaining popularity due to their proven safety and positive benefits on human health (8). The majority of probiotics are members of the LAB group, which includes many species such as *Lactobacillus*, *Leuconostoc, Streptococcus*, and *Enterococcus*. Recent growth in the worldwide market for probiotics has been fuelled by increased consumer demand for healthy diets and well-being, prompting food researchers and manufacturers to produce novel probiotic-containing products. However, there has been growing concern regarding the safety of probiotic bacteria which include the potential to cause diseases such as bacteraemia or endocarditis, production or release of toxic metabolites on the gastrointestinal tract, and the presence of transferable antibiotic resistance genes (9).

Conversely, pieces of evidence that probiotic bacteria are causal agents in cases of the diseases highlighted are limited and unclear, and infection caused by probiotics has never been observed in healthy persons (10). The ability to adhere to human mucosal or epithelial cells and cell lines, exert an antimicrobial effect against pathogenic bacteria or fungi, reduce pathogen adhesion to surfaces, stimulate bile salt hydrolase activity, and increase probiotic viability are among the most crucial characteristics of probiotics (11). This study aimed to isolate possibly probiotic LAB from fermented cabbage (Sauerkraut) and assess the possibility of safe use.

Materials and method:

Preparation of Sauerkraut:

The cabbage used in this study was purchased from Shasha market, Akure, Ondo State, Nigeria. The cabbage was washed in sterile distilled water, and shredded into bits before being placed into a clean glass jar, and 2% salt was added to make 200 ml volume. The shredded cabbage was comple-

tely submerged in distilled water and sealed to ensure anaerobic conditions. The setup was kept at room temperature (ambient temperature of 18-22^{o}C) to ferment for 7 days.

Isolation of and characterization of LAB:

The isolation of probiotic strains from fermented cabbage was carried out according to the method previously described by Jagannath et al., (12). The fermented cabbage was crushed into a slurry in a warring blender. One millimeter was aseptically collected and diluted serially in saline solution. Thereafter, 100µL of each of the diluted suspension (10^{-1} to 10^{-6}) was separately inoculated on triplet plates of Rogosa agar media (De Man Rogosa and Sharpe *Lactobacillus* Selecting Agar, pH 5.4 ± 0.2, Oxoid, CM0627) and spread using a sterile spreader. The inoculated plates were incubated under anaerobic conditions using an anaerobic gas jar containing gaspaks (AnaeroGen™ 3.5L, ThermoScientific, AM0035, UK) at 37^{o}C for 72 hours. The LAB isolates were presumptively identified phenotypically by colony morphology, Gram staining, and catalase test.

Antibacterial & antagonistic activities of LAB

Agar overlay assay:

The indicator bacteria (*Salmonella* Typhi, *Escherichia coli* and *Staphylococcus aureus*) were grown on nutrient agar at 37°C for 24-hour. The inhibitory activity of the selected LAB isolates against the indicator organisms was done using the agar overlay technique described by Hockett and Baltrus (13). The LAB isolates were spot-plated on MRS agar and incubated for 24 hat 37˚C under anaerobic conditions using a gas generating system (AnaeroGen™ 3.5L, Oxoid, AM0035, UK). Cultures of the pathogens were grown on NA agar for 18 hours, and about 3-5 colonies were picked and suspended in sterile saline solution and the turbidity was adjusted to 0.5 McFarland standard equivalent of $1x10^{8}$ CFU/ml. One hundred microliters of the adjusted suspension of indicator strains were seeded in semi-solid Mueller Hinton agar. The MRS agar containing pre-spotted LAB isolate was overlaid with 10 ml soft MH agar containing 100µL seeded indicator organism and incubated aerobically at 37˚C for 24 hours. Clear zones of inhibition of more than 1 mm extending laterally from the border of the LAB colonies after 24 hours incubation was considered positive antagonistic (antibacterial) activity.

Agar-well diffusion test:

Agar–well diffusion assay was carried out to determine the zone of inhibition of the antibacterial agent in the cell-free culture supernatant (CFCS) of LAB isolates against the indicator organisms. Lactobacilli that showed a zone of inhibition in the agar overlay assay were selected and cultured overnight in MRS broth at 37˚C for 18 hr. After incubation, the culture was centrifuged at 10,000 rpm for 10 min at 4˚C and further filtered through a 0.22 µm sterile syringe filter (PES, AXIVA) to obtain a CFSC. Overnight cultures of the indicator bacteria were inoculated in BHI broth and incubated for 3 h at 37˚C. Thereafter, 0.5 McFarland standard suspensions of the culture were prepared.

One hundred microliters of the prepared suspension were mixed with 10ml of melted and cooled semi-solid BHI agar and poured into the respective Petri dishes. The semi-solid BHI agar was allowed to set and wells of 6mm diameter were aseptically made using a sterile cork-borer. Bases of the wells were sealed with 30µl melted semi-solid BHI agar. Wells were filled with 100µl of CFCS and then kept in the refrigerator for 3-4 hours to ensure the diffusion of antimicrobial compounds within the media. The plates were hereafter incubated for 18 hours at 37˚C. The diameters of the zone of inhibition that extends radially from the edge of the wells were taken and recorded. Unspent MRS broth was used as a negative control.

Molecular identification of the efficient LAB

Based on its potential probiotic attributes, the efficient LAB isolate was identified by 16S rRNA sequencing. Extraction (isolation) of DNA and PCR (polymerase chain reaction) amplification was carried out using the universal primers 27F-5'AGA GTT TGA TCC TGG CTC AG-3' and 1492R-5'GGT TAC CTT GTT ACG ACT TT-3'. The reaction was carried out in 25 µL of the reaction mixture, which contained forward and reverse primers (10 pmol), 0.5 µL of DNA template (50–100 ng), dNTP (0.2mM), and polymerase (0.5U). Optimum conditions for the PCR involved an initial denaturation step for 5 min at 95°C followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, extension for 5 min at 72°C, and final extension for 7 mins at 72°C. The PCR products were confirmed on 1% agarose gel electrophoresis. The PCR products were further sequenced and the obtained sequences were compared using BLAST (basic local alignment search tool).

Evaluation of probiotic attributes of LAB

Cell surface hydrophobicity:

The *in vitro* bacterial cell surface hydrophobicity of LAB isolate-3 (cbak3) was evaluated by measuring the microbial cell adhesion to hydrocarbons (xylene) according to the method described by Rokana et al., (14).

The young cultures in MRS broth were centrifuged at 6,000 rpm for 10 min, washed twice with PBS, and re-suspended in PBS buffer. A cell suspension of about 3ml was blended with 2 ml of xylene and incubated at 37 °C for 3 h without shaking the separation of the aqueous and organic phases. One millimeter of the aqueous phase was removed carefully and the absorbance (A_1) was measured at 600 nm. The cell hydrophobicity assay was measured (in percentage) using the formula; cell surface hydrophobicity % = $[1-A_t/A_0] \times 100$, where A_0 and A_t are the absorbance before and after extraction with organic solvents, respectively.

Acid and bile tolerance assay:

The tolerance of the LAB isolates to both acidic pH and bile salts was carried out using the methodology described previously by Fadare et al., (5) with little modification (pH adjusted to 3.0). Young cultures of the strains were inoculated in 50 ml of MRS broth with an adjusted pH value of 3.0 using 1 M HCl, and 50 ml MRS with 0.3% (w/v) bile salt (Himedia, India), respectively. The inoculum size was adjusted by comparing the turbidity with 0.5 McFarland standards equivalent to ~1.5 x 10^8 CFU/ml. The samples were incubated at 37°C. At time intervals of 0, 1, 2, and 3 h, a 100 µL aliquot was taken to determine the total viable cells by the standard plate count method. The survival rate was determined using the formula; survival rate (%) = Biomass at time (B_t)/Biomass at initial time (B_o) × 100.

Auto-aggregation assay:

The ability of the LAB isolate to auto-aggregate was tested using the method previously described by Zommiti et al., (16). The overnight culture was harvested by centrifugation (at 6000 rpm for 10 min) and washed with PBS twice and re-suspended in PBS buffer. The sample was allowed to stand while incubating anaerobically at 37°C. Afterward, the upper suspension was checked for absorbance at 600 nm at time intervals of 0, 1h, 2h, and 3h. The auto aggregation was measured in percentage using the formula; auto aggregation % =$[1-A_t/A_0] \times 100$, where where A_t represents the absorbance at time t = 3 h and A_0 the absorbance at t = 0.

Co-aggregation assay:

The ability of the LAB isolates and pathogenic indicator organisms (*Escherichia coli* and *Staphylococcus aureus*) to co-aggregate was determined using the method described by del Re et al., (17). The overnight culture was harvested by centrifugation at 6000 rpm for 10 min and washed with PBS twice and re-suspended in PBS buffer. An equal volume of the LAB isolates and pathogenic indicator suspension was prepared with the turbidity equivalent to 0.5 MacFarland standard and allowed to stand for a while. The upper suspension was checked for absorbance at 600 nm at time intervals of 0, 1, 2, and 3 h. Percent co-aggregation was calculated using the equation of Nagaoka et al., (18); co-aggregation % = $[1 - A_t/A_0] \times 100$, where A_t represents the absorbance at time t = 1, 2 or 3 h, and A_0 is the absorbance at t = 0.

Evaluation of the safety of the LAB isolate:

Antimicrobial susceptibility test of LAB:

The AST of the suspected *Lactobacillus* spp. was performed using the Kirby-Bauer disc diffusion technique. The procedure for the experiment was carried out as specified in the Clinical and Laboratory Standard Institute (19) guideline. Mueller–Hinton agar and Brain heart infusion agar (i. e. MHA and BHI in 1:1) were used as previously described (20). The following antibiotic discs were tested; ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), gentamicin (10µg), cefuroxime (30µg), erythromycin (15µg), ciprofloxacin (5µg) and vancomycin (30µg).

Suspensions of overnight cultures of the test organisms were prepared with turbidity equivalent to 0.5 McFarland standards in sterile normal saline. The bacterial suspensions were aseptically inoculated on the prepared medium using a sterile swab stick, incubated anaerobically at 37°C for 24 h and then 72 h. The diameter of the zone of inhibition was measured and recorded to the nearest millimeter using a ruler. The susceptibility status was determined using the zone size interpretative chart as provided by CLSI (19).

Haemolysis

The method described by Yadav et al., (21) was used to assess the LAB isolates' haemolytic activity. The isolates tested were streaked onto blood agar plates containing 5% (w/v) human blood and incubated at 37°C for 24 h. The plates were examined for α-, β-, and non-haemolytic activity after incubation.

DNase activity:

The DNase activity of the LAB isolates was determined using the method as previously described by Shuhadha et al., (22). Briefly, the LAB isolates were streaked onto DNase agar medium (Himedia, India) to test for the production of the DNase enzyme. The plates were then incubated at 37°C for 24 h and observed for clear zones showing DNase activity.

***In vivo* sub-acute oral toxicity assay in laboratory animals**

The procedures involving animals in this study were carried out following the guidelines of the Ondo State health research ethics committee. Eight male Wistar rats of the subspecies *Rattus norvegicus* aged 6-7 weeks old, and weighing 68-76 g were obtained from the animal house of Elizade University. The rats were randomly grouped into two (A & B), comprising four rats per group, and allowed to acclimatize for 14 days before beginning the toxicological assay.

The sub-acute oral toxicity assay was a 28-day repeated-dose oral toxicity assay carried out according to the OECD guidelines. The *L. brevis* was revived and prepared for administration using the method previously described by Pradhan et al., (10) with few modifications. Briefly, the bacterial isolates were sub-cultured and finally harvested by centrifugation at 4500 rpm for 15 min after 16 h of incubation at 37^{o}C. The centrifuged culture was thereafter washed thrice in sterile phosphate-buffered saline (PBS). The pellet obtained was then re-suspended in sterile saline and the turbidity was adjusted to 0.5 McFarland standard equivalent to ~1.5 x 10^{8} CFU/ml). Rats in group A were administered oro-gastrically with 500 µL of the bacterial suspension daily for 28 days, while the control group (B) was fed with sterile saline. During the treatment period, daily observations for any changes in the animal's activity were made, and body weight increase was assessed weekly. All rats were sacrificed on the 29th day.

Following the period of toxicity test, blood samples were collected on the 29th day into appropriate vials for haematological analysis. The haematology tests, which include packed cell volume (PCV), haemoglobin concentration (HB), red blood cell count (RBC), erythrocyte sedimentation rate (ESR), and white blood cell (WBC) counts were carried out according to the standard procedure. After the physical evaluation, the rats were sacrificed, the abdominal cavities were opened, the intestines removed and preserved in 10% formalin for histopathological analysis. The tissue sections were prepared on a slide and stained with haematoxylin and eosin. The integrity of the sectioned intestines was examined using a microscope. The intestines of the control group were compared with the treated group.

Statistical analysis

All tests were conducted in triplicates and data were presented as mean and standard deviation, and analysed using the Statistical Package for the Social Sciences (SPSS) version 21.0. To determine significant differences between means, a one-way analysis of variance (ANOVA) was used, with a significant difference of $p < 0.05$.

Results:

In this study, five LAB isolates were recovered from fermented cabbage (Sauerkraut). The pH of the fermented cabbage after 7 days of fermentation was observed to drop from the initial mean value of 6.8±0.23 to a mean value of 3.9±0.15, at temperature of 25^{o}C.

Antibacterial activity of LAB isolates

The five isolates showed antimicrobial activity against the test pathogens in an agar-overlay method, but only the isolates designated as cbak2 and cbak3 showed a zone of inhibition when the cell-free culture supernatant was tested in an agar diffusion method (Table 1). On the other hand, the pH-neutralized cell-free culture supernatants of the two probiotic LAB showed no inhibitory activity against the test pathogens.

16s rRNA sequencing identification

The sequence analysis of cbak3 isolate using the Basic local alignment search tool (BLAST) program detected 97.64% sequence identity with known species (*Levilactobacillus brevis*). The strain has been deposited into the National Center for Biotechnology Information (NCBI) database with accession number OM262042.

Table 1: Antibacterial activity of cell-free supernatants of the 5 LAB isolates against pathogens

Indicator organisms	**Mean zone diameter of inhibition (mm)**				
	Cbak1	**Cbak2**	**Cbak3**	**Cbak4**	**Cbak5**
Salmonella Typhi	0 ± 0.00	1.2 ± 0.21	14 ± 0.02	0.00	0.00
Staphylococcus aureus	0 ± 0.00	3.1 ± 0.04	16 ± 0.32	0.00	0.00
Escherichia coli	0 ± 0.00	0.8 ± 0.32	14 ± 0.05	0.00	0.00

Values are the mean (± SD) of triplicate samples (n=3); cbak= cabbage sample

Table 2: Mean viable count of the lactic acid bacterial isolates

Strain	**Bile (0.3%) tolerance**			**Acid tolerance (pH=3)**		
	0 hour	**3-hour**	**Survival rate (%)**	**0 hour**	**3-hour**	**Survival rate (%)**
L. brevis	8.28 ± 0.04	7.11 ± 0.16	85.9	8.54 ± 0.02	7.11 ± 0.21	83.3

Values are the mean (± SD) of triplicate samples (n=3)

Table 3: Cell surface hydrophobicity, auto-aggregation and co-aggregation of LAB isolate

Bacteria	**CSH (%)**	**Auto-aggregation (%)**	**Co-aggregagtion (%)**		
			***S.* Typhi**	***S. aureus***	***E. coli***
L. brevis	53.3 ±1.73	60.0 ± 2.99	48.3 ±1.73	65.3 ± 2.85	51.7 ±1.73

Values are the mean (± SD) of triplicate assay; CSH = cell surface hydrophobicity

Probiotic attributes of LAB isolate

The LAB cbak3 isolate that showed strong antibacterial activity was further evaluated for its probiotic and safety status. The *L. brevis* isolate showed appreciable levels of tolerance to 0.3% bile salt and acidic pH 2.0 after 3 exposures to both conditions (Table 2). *L. brevis* showed 85.9% and 83.3% survival rates in 0.3% bile salt and low pH of 2.0 respectively.

The *L. brevis* had a median value (33% < 66%) for both the cell surface hydrophobicity and auto-aggregation assays (Table 3). As indicated, *L. brevis* showed a higher co-aggregation rate with *S. aureus* (65.3±2.85%) compared to the percentage values obtained for *E. coli* (51.7±1.73%) and *Salmonella* Typhi (48.3±1.73%).

Safety evaluation results

As shown in Table 4, *L. brevis* showed resistance to ciprofloxacin, cefuroxime, and vancomycin, but sensitive to erythromycin, gentamicin, ampicillin, tetracycline, and chloramphenicol.

Levilactobacillus brevis showed no haemolytic and DNase activities. With respect to the *in vivo* toxicity test, no death in the rats (both the experimental and control group) was reported. No sign of ill- ness was observed as the experimental rats remained active and healthy throughout the experiment. Compared to the control, a significant increase in the average weight (82.2g to 101.96g) of the rats administered with *L. brevis* was observed, showing a weight gain of 19.3% (Table 5).

The values obtained for the WBC and RBC in group A were significantly different compared to the control group. However, the values for each parameter assayed did not exceed the normal range (Table 6). As shown in Table 7, the white blood cell differential counts for the experimental rats were significantly lower ($p < 0.05$) for monocytes and higher for lymphocytes compared to that of the control group. However, the values did not exceed the normal range.

Table 4: Antibiotic-resistant profile of *Levilactobacillus brevis*

Test Bacteria	**Diameter of zone of inhibition (mm)**							
	CIP (5µg)	**E (15µg)**	**CXM (30µg)**	**GEN (10µg)**	**TET (30µg)**	**AMP (10µg)**	**C (30µg)**	**V (30µg)**
L. brevis	R	S	R	S	S	S	S	R

CIP – Ciprofloxacin, E – Erythromycin, CXM – Cefuroxime, GEN – Gentamicin, TET – Tetracycline, AMP – Ampicillin, C – Chloramphenicol. V – Vancomycin; R – Resistant; S – Susceptible; I - Intermediate

Table 5: Effect of putative probiotic bacteria (*L. brevis*) on the weight of Wister albino rats

Group	**Weight gain/week (g)**					***WG (%)**
	0	**1**	**2**	**3**	**4**	
A (*L. brevis*)	82.20±1.05^{a}	86.11±3.19^{a}	92.25±2.05^{c}	97.04±1.16^{d}	101.96±2.18^{d}	19.3
C (Control)	84.30±1.10^{c}	87.49±3.14^{d}	91.66±2.24^{b}	94.22±1.52^{b}	96.68±2.12^{b}	12.8

Values were expressed as mean ± SD of 4 determinations; Values with different superscripts in a row were significantly different ($p < 0.05$); WG = Percentage weight gain after treatment

Table 6: Effect of *L. brevis* on the haematological parameters of Wister albino rats

Group	**Blood Parameter**				
	WBC ($\times10^9$/mm^3)	**PCV (%)**	**HB (%)**	**RBC (/mm^3)**	**PLT ($\times10^3$/mm^3)**
A (*L. brevis*)	9.53±0.11^{d}	42.67±0.58^{a}	14.20±0.20^{a}	39.97±0.19^{a}	11.50±0.17^{a}
B (Control)	7.73±0.07^{b}	46.00±0.00^{b}	15.17±0.33^{b}	35.03±0.17^{b}	11.73±0.24^{a}
*Normal range	4.4 – 14.8	36 – 54	10.4 – 16.5	38 – 66.8	6.38 – 11.8

Values were expressed as mean ± SD of 4 determinations in each group; Values with different superscripts in a column were significantly different ($p < 0.05$); WBC= White blood cell; HB = Haemoglobin; PCV = Park cell volume; RBC = Red blood cell; *Delwatta et al., (23)

Table 7: Effect of *L. brevis* on WBC differential counts of rats

Group	**Parameter**				
	Lymphocytes (%)	**Neutrophils (%)**	**Monocytes (%)**	**Eosinophils (%)**	**Basophils (%)**
A (*L. brevis*)	75.6 ± 0.58^{c}	21.0 ± 1.00^{a}	2.0 ± 0.00^{a}	1.0 ± 0.00^{a}	0.0 ± 0.00^{a}
B (Control)	73.6 ± 0.58^{b}	20.7 ± 0.58^{a}	4.6 ± 0.58^{c}	1.0 ± 1.00^{a}	0.0 ± 0.00^{a}
*Normal range	65 – 85	13 – 36	0 – 5	0 – 6	0 – 1

Values with different superscripts in a row were significantly different ($p < 0.05$). *Delwatta et al., (23)

The histopathology of the intestine of the experimental rats compared with the control group shows no negative pathology architecture. Mucosal epithelia are intact with adequate thickness. The villi are prominent and the integrity is preserved. There was no necrosis, haemorrhage, or kariolysis of the intestinal villi. The intestinal villi projections are well-formed with adequate arterial and venous supply in both the experimental group (a) and the control (b) with adequate projections (Fig. 1a and b).

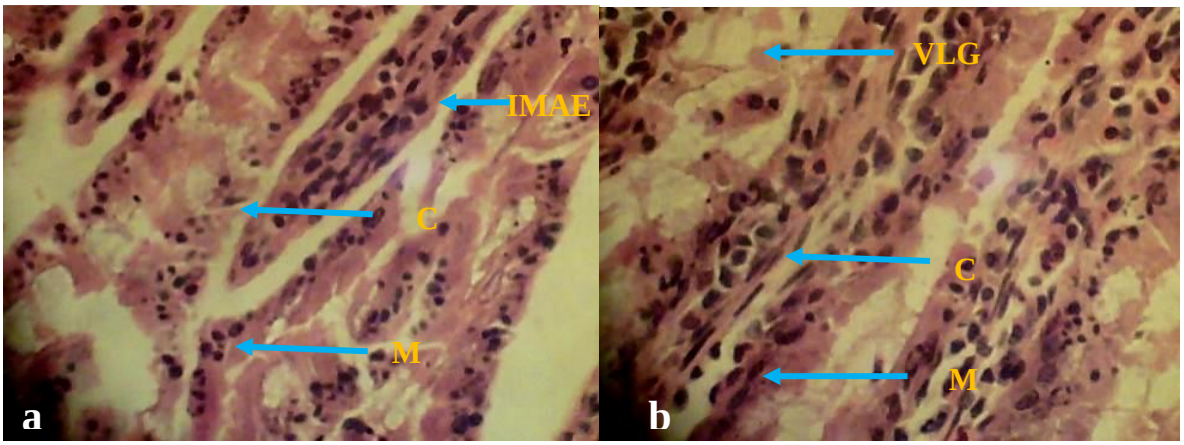

Fig. 1. Photomicrograph of the small intestines of the experimental rats and the control group. **a.** Experimental group administered with 500 µL aliquot of *L. brevis* isolate once daily, **b. C**ontrol group administered with sterile saline solution.
Key: IMAE – Intact mucosal absorptive enterocytes, **VLG** – Villi lining goblets, **C** – Crypt, **M** - Mucosal

Discussion:

Overall, five LAB isolates were successfully recovered from the fermented cabbage, however, only one LAB isolate that showed strong antibacterial activity was selected and identified as *Levilactobacillus brevis*. The putative probiotics in this study were prescreened using antibacterial activity as the primary criterion. As evident in this study, the occurrence of *L. brevis* among other LAB in fermented cabbage (sauerkraut) has been documented (24). *L. brevis* was discovered to have a substantial antagonistic effect on the test foodborne pathogens in this study. In a recent study by Utama et al., (25) *L. brevis* isolated from fermented cabbage was reported to show strong inhibition of *E. coli* and *Salmonella pullorum*. Other studies have also reported strong antimicrobial activity of *L. brevis* isolated from non-vegetable fermented sources against foodborne pathogens (26).

Initially, all five isolates showed high levels of inhibitory activity when tested using the agar-overlay method, but when tested using the agar well diffusion method, *L. brevis* displayed visible and high inhibitory activities against the indicator organisms. This is in tandem with the study conducted by Polak-Berecka et al., (27), where the agar spot test method showed the largest and most distinct inhibitory zones compared to the agar diffusion technique. However, the result obtained in this study using the two methods (agar overlay and agar diffusion) was at variance with the report published by Halder et al., (28), in which the values of results obtained using both methods were in close range. The variation observed in the antibacterial activities between the two methods and as reported by different authors have been attributed to cell density and growth kinetics of the LAB used in the agar overlay methods (29). On the other hand, the volume of cell-free supernatant culture used for the diffusion test has also been considered a potential factor (28). According to Polak-Berecka et al., (27), when live *Lacticaseibacillus rhamnosus* cells were employed instead of cell-free supernatant, the strongest antibacterial activity was seen.

The presence of antimicrobial compounds such as organic acids (lactic, citric, acetic, fumaric, and malic acid), hydrogen peroxide, CO_2, diacetyl, ethanol, reuterin, acetaldehyde, acetoin, ammonia, bacteriocins, bacteriocin-like inhibitory substances (BLIS), and other important metabolites have been attributed to the antibacterial activity of *Lactobacillus* spp (30,31). To guarantee colonization, survival, and metabolic activity within the gut, probiotic isolates must be able to survive bile salts and low pH, otherwise, they will not be able to benefit the host's health and well-being. Tolerance to acidic environments and bile salts have been regarded as two characteristics for a prospective probiotic strain to survive the GIT (32). The pH value of 3.0 and concentration of 0.3% bile salt have been considered standard for such investigation of probiotic strains (33). In the current study, *L. brevis* showed a higher survival rate within the 80% range in both 0.3% bile and acid (pH 3.0) tolerance tests. *Lactobacillus* has been reported to survive and proliferate lavishly in MRS broth enriched with 0.3% bile salt (34), which is similar to our observation. According to Shokryazdan et al., (35), *Lactobacillus* isolates were acid-tolerant for 3 h at pH 3.0. Ehrmann et al., (36) discovered that the acid tolerance level differed across *Lactobacillus* isolates tested under identical conditions, suggesting that tolerance capability may be strain-specific. It is crucial to note, however, that *in vitro* studies involving pH and bile salt tolerance

cannot predict patterns of behavior in the human body. The stomach pH which is usually within the range of 1.2 – 2.0 has been reported to fluctuate depending on the meal consumed. The stomach pH has been shown to rise dramatically close to 5.0 after ingesting yogurt and fermented milk (37).

Cell surface hydrophobicity is another attribute that has been associated with probiotic strain adhesion capacity (5). A correlation between surface hydrophobicity and bacteria capacity to adhere to the intestinal mucosa has been reported (5). In the current study, the hydrophobic character of the surfaces of *L. brevis* was evaluated based on its adhesion to the hydrocarbon phase in a xylene solution. *Lactobacillus brevis* showed a higher hydrophobicity percentage (51%), similar to the result obtained by Kariyawasam et al., (38) who used xylene in their study. Meanwhile, the hydrophobicity of *L. brevis* G1 and *L. brevis* KU15006 in another study ranged from 47% to 48% (39).

It has been demonstrated that pathogens frequently have a high affinity for proteins including collagen, fibrinogen, and mucus because they provide them access to host tissues (40). Auto-aggregation refers to a bacterial strain capacity to interact with itself in a generic manner, which is thought to be a precondition for pathogen colonization and infection of the gastrointestinal tract via adhesive ability. The percentage value of auto-aggregation obtained in the current study was high for *L. brevis*, although the isolate showed values found within the acceptable range (17). Strains of *Lactobacillus* in general have been shown to have a low to moderate auto-aggregation capability (41). As per the co-aggregation test, *L. brevis* showed a higher aggregation percentage with *S. aureus* compared to *E. coli* and *S.* Typhi. A similar pattern of activity has been documented, with the highest levels of co-aggregation reported between the Lactobacillaceae and *E. faecalis* strains and the lowest levels with *S*. Typhimurium and *E. coli* (42). Generally, most LAB which are Gram-positive have been shown to exert low co-aggregating capabilities against Gram-negative bacteria (5,43). It is not clear why this is so, but the structural differences between the cell wall of Gram-negative and Gram-positive bacteria may be a determining factor. Variations by species in the physiology and surface features of cells such as polysaccharide content and adhesin expression have been documented (44). Probiotic bacteria have been shown in several studies to inhibit harmful bacteria from adhering to the intestinal mucosa by creating a physical barrier via auto-aggregation or co-aggregation with the pathogens (45,46).

The lack of acquired antibiotic resistance genes in bacterial strains authorized for use in human and animal feed is a requirement of the European Food Safety Authority (47). The fundamental worry with antibiotic resistance features is their tendency to spread to other potentially harmful bacteria, possibly causing difficulties and reducing the efficacy of antibiotic therapy. In the current study, *L. brevis* was sensitive to clinically relevant antibiotics including ampicillin, tetracycline, chloramphenicol, gentamicin, and erythromycin which are following the EFSA guidelines (47). Our isolate in line with previous studies showed resistance to ciprofloxacin and vancomycin (48,49). Vancomycin resistance in *L. brevis* isolates has been demonstrated to be typically high, and the resistance is thought to be an inherent trait of the strains (48,50). Additionally, the fact that our strains are of plant origin suggests that they have not come into contact with antibiotics, which might account for the resistance as an inherent trait, in contrast to isolates from humans and animals.

Our study showed that *L. brevis* has non-hemolytic and non-DNAse activities, which comply with the suggested safety features in probiotic selection (51). The pathogenic nature of some groups of bacteria has been attributed to DNase and haemolytic activities (52), and many studies have demonstrated the non-haemolytic and non-DNAse activities of *Lactobacillus* spp. isolated from different sources including humans, animals, and plants (53,54). With regards to the *in vivo* toxicity test, the outcomes of repeated oral administration of *L. brevis* to a group of four albino rats further support the non-pathogenic attribute of the test isolate. To ascertain the safety of a bacterial strain, oral toxicity studies have been considered a standard (9,55). Based on the results of a repeated-dose sub-acute oral toxicity study by Shokryazdan et al., (35), none of the tested *Lactobacillus* strains caused any sign of toxicity when fed to rats at a concentration as high as 1×10^{10} CFU/kg BW/day. The significant weight gain of the albino rat test group observed in this study may be attributable to increase nutrient absorption which may have been aided by the enzyme activity of *L. brevis*. This finding appears to be consistent with a recent work by Oladejo et al., (56), in which hydrolyzed African yam beans with partially purified protease produced by *L. brevis* increased vitamin bioavailability, which accounted for the albino rats' bodyweight gain. However, weight loss caused by some strains of *L. brevis* and other species of *Lacto bacillus* has been reported (57,58), which suggest species and strain specificity (59).

Haematological analysis is vital for assessing toxicological consequences such as inflammation and infection caused by induced treatments (60). In this study, the levels of haemoglobin, packed cell volume, platelet and red blood cells were within the normal range, which could suggest a reduced risk of anaemia. Moreover, WBC count, an indicator for blood infection, was equally within the normal range despite being higher than in the control group. In the treated group, no such abnormality in the differential counts of lymphocytes, neutrophils, monocytes, eosinophils, and basophils was observed. There is a sparse report in the literature showing any abnormality in the blood profiles of healthy albino rats administered with *Lactobacillus* species.

The integrity of the gut was further verified by histology of the intestine. The microscopic observations of the stained tissue of the intestine did not reveal any signs of inflammation, degeneration, or necrosis of the intestinal mucosa, and there was no remarkable difference in the arrangement of epithelial cells between the test and control groups. Although, the histology of the intestine showed that the formation of intestinal finger-like villi differs in maturity, no structural changes in the crypt depth or distortion in the villus structure were observed. Probiotics have been intensively studied for their involvement in maintaining the intestinal barrier, which is thought to be the first line of defense against pathogens entering the digestive system (61,62). Reinforcement of gut barrier function can lead to decreased intestinal inflammation, and several investigations have shown that probiotic strains can restrict inflammatory responses by strengthening gut barrier permeability (63,64).

Conclusion:

Based on the findings of this study, it can be stated that the *L. brevis* isolated from Sauerkraut is potentially safe and useful due to its appealing probiotic features and promising antibacterial activity. This lends credence to the idea that as a probiotic strain, it might be useful in a variety of applications, particularly in the food industry. However, whole strain genomic profiling as well as clinical trials are further required to affirm the suitability of the putative probiotic isolate before it can be applied in human or animal nutrition.

Contribution of authors:

OSF, CHA, AOM and TKB conceptualized and designed the study. Material preparation, data collection, and analysis were carried out by OSF, CHA and AOM. The first and second draft of the manuscript were written by CHA and OSF respectively. All authors discussed the results and contributed to the final manuscript draft for submission.

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