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Evaluation of probiotic and antimicrobial properties of *Lactobacillus* species isolated from decaying *Musa acuminata*

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Abstract

The increasing interest in probiotics has heightened the need to explore natural sources of beneficial microorganisms. While dairy products have long been a staple for probiotic isolation, plant sources offer a diverse array of novel strains of probiotics with antimicrobial properties. This study aimed to evaluate the probiotic potentials and antimicrobial activity of *Lactobacillus* species isolated from decaying *Musa acuminate* (banana). Ten different decaying banana samples were obtained from different banana vendors. *Lactobacillus* was isolated in deMan Rogosa Sharpe (MRS) agar containing 0.5% $CaCO_3$ and was identified based on cultural, biochemical and molecular characteristics. The isolated *Lactobacillus* species were screened for their probiotic properties using different parameters. The antibacterial activity of the effective *Lactobacillus* species was evaluated using agar well diffusion. The analyses of their probiotic properties showed that the isolated *Lactobacillus* species exhibited good tolerance to acidic pH, high tolerance to sodium chloride and 0.3 bile salt concentration and were sensitive to proteolytic enzymes. The result of antibacterial activity showed that the isolates exhibited a wide spectrum of antimicrobial activity against gram-positive and negative bacteria in the agar well diffusion test. The gene sequence analysis identified the isolates as *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus fermentum* and *Lactobacillus rhamnosus*. This study has shown that the banana is a potential source of probiotic *Lactobacillus* species with broad-spectrum antimicrobial properties.

Keywords: Probiotics; *Lactobacillus; Musa acuminate*; Decay Banana; Antimicrobial Resistance

1. Introduction

Probiotics which can be defined as live microorganisms that provide a health benefit on the host when administered in adequate amounts [1] have garnered significant attention in the field of functional food and human health. These natural products have steadily become more popular in recent years, and this is because microorganisms have become resistant to antibiotics, and only by returning to the more natural products, we can build immunity to them. In recent years, probiotics have been used to suppress harmful pathogens and extend the shelf life of foods. Probiotics have been documented to show various health benefits such as maintaining of a healthy balance of gut microbiota [2] wound healing effect and immunomodulatory effect [3].

Among the wide variety of probiotic microorganisms are *Lactobacillus* species. *Lactobacillus* is a group of beneficial bacteria that naturally resides in the digestive, urinary, and genital tracts without causing harm. They are among the groups of lactic acid bacteria that have been extensively studied due to their significant health benefits, including the promotion of gut health, enhancement of the immune system, food fermentation and preservation and antimicrobial activity against pathogenic organisms [4]. *Lactobacillus* strains have been isolated from dairy products, fermented foods

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and the gastro intestinal tract. However, there is an increasing need to explore alternative natural sources to diversify the range of probiotic strains available and to meet the growing demand for non-dairy probiotics. The industrial importance of *Lactobacillus* species is generally recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces [5].

The process of isolation and identification of bacteria from natural sources have revealed a diverse range of *Lactobacillus* strains with antimicrobial properties. However, while considerable attention has been directed towards *Lactobacillus* isolated from dairy products, fermented foods, and in the gastrointestinal tracts, less emphasis has been placed on decaying plant matter as a reservoir of novel strains. Decaying fruits, in particular, provide a distinctive microenvironment that fosters microbial competition, potentially leading to the emergence of strains with enhanced antimicrobial potentials [6].

Musa acuminate (Banana) which is one of the most widely consumed fruits globally, undergo rapid microbial colonization during decay, creating a rich substrate for the isolation of diverse microbial species. Decaying bananas provide a unique, cost-effective source for isolating *Lactobacillus* species. Bananas are rich in fermentable sugars like glucose, fructose, and sucrose, decaying bananas create an ideal environment for the proliferation of these bacteria. These sugars act as substrates for fermentation and production of organic acid which aids in growth of *Lactobacillus* species [7].

Compared to other sources such as dairy or fermented foods, decaying bananas offer several advantages for *Lactobacillus* isolation. They are widely available, sustainable, and reduce food waste, particularly in tropical regions where bananas are abundant. The presence of prebiotics in bananas, such as fructo-oligosaccharides (FOS), promote the growth of probiotics bacteria. In the present study, decaying bananas present an underexplored source for isolating *Lactobacillus* species with probiotics and antimicrobial properties. This study aims to isolate, characterize and assess the probiotic and antimicrobial activity of *Lactobacillus* species obtained from decaying bananas. Knowledge of these properties could contribute to the development of plant-based probiotics with antimicrobial activities.

2. Materials and Methods

2.1. Sample Collection

Decaying bananas were selected as the source for isolating *Lactobacillus* strains due to their high sugar content and the microbial activity that occurs during the decay process. Ten Banana samples in the advanced stages of decay, showing visible signs of spoilage such as browning and soft texture were purchased from fruit sellers in Eke Awka market. The samples were then transported to the microbiology laboratory and processed within 24 hours to ensure viability of the microorganism.

2.2. Isolation of *Lactobacillus* Species

The isolation of *Lactobacillus* from decaying banana samples was carried out as previously described by [8]. Approximately 10 grams of each decaying portion was cut out from different areas of the banana showing visible signs of decay and were homogenized in 90 Ml of distilled water using a stomacher to ensure a uniform suspension of microorganisms. Ten-fold serial dilutions were prepared by transferring 1 Ml of the homogenized banana samples into 9 Ml of distilled water. 0.1mL from 10^{-5} dilution were inoculated onto MRS agar using the pour plate method and incubated at 37 °C for 72 hours under microaerophilic conditions. Colonies were sub-cultured onto nutrient agar for further purification.

2.3. Morphological and Biochemical characterization

After incubation, colonies with characteristic *Lactobacillus* morphology, such as; small, circular, oval, creamy, white colonies on MRS agar were selected for further analysis. The isolates were subjected to Gram staining to confirm the presence of Gram-positive rods, a key characteristic of *Lactobacillus* species. Biochemical tests, such as the catalase, citrate, oxidase, motility and sugar fermentation test were carried out as described in Bergy's Manual of Determinative Bacteriology [9]. The confirmed *Lactobacillus* isolates were further preserved in 15% glycerol stock and stored at -20^0C which was finally subjected to molecular characterization using 16s rDNA analyses.

2.3.1. Molecular identification of Lactobacillus sp. by 16s rDNA

16s rDNA sequencing was used as a tool to identify and confirmed the bacterial strain. PCR was used to amplify the 16s ribosomal DNA and was determined by direct sequencing. The confirmed strains were then screened for probiotic properties.

2.4. Screening for Probiotic Properties of Isolated *Lactobacillus.*

2.4.1. Sodium Chloride (NaCl) Tolerance Test

Sodium chloride tolerance was carried out as described by [10]. *Lactobacillus* species were inoculated into 10 mL sterile MRS broths in test tubes containing three different concentrations of NaCl (2% to 10% (w/v)) and incubated at 37 ^{o}C for 48 h. Growth was monitored by visual observation of the test tubes. The influence of NaCl concentrations on the degree of inhibition of bacterial growth was recorded. Positive control experiments were made of tubes containing lactic acid bacterial cultures without NaCl, while negative control experiments were made of tubes with added NaCl but without lactic acid bacterial cultures.

2.4.2. Bile salt tolerance Test

The bile tolerance test was conducted following the protocol of [11]. One hundred microliters of overnight grown culture of each *Lactobacillus* isolates were inoculated in freshly prepared MRS broth containing 0.3% bile salts (Oxoid). Test isolates (*Lactobacillus* sp.) were also inoculated in MRS broth without bile salt, which acts as a control. Both the test tubes (with and without bile) containing test isolates were incubated at 37 ^{o}C for 4 h, and their growth at a different time interval was noted by measuring the absorbance of MRS broth at 600 nm.

2.4.3. Tolerance to Acidic pH

Tolerance of the *Lactobacillus* species to acidic pH was performed as described by [12]. 1 mL of each *Lactobacillus* sp. at 1×10^{5} cfu/ml was inoculated into sterile 10Ml de Man Rogosa and Sharpe broth tubes. The tubes were adjusted to pH values of 2.0 to 9.0 using hydrochloric acid and sodium hydroxide. 1 mL inoculum from each tube was inoculated into de Man Rogosa and Sharpe agar medium using the pour plate technique and incubated anaerobically at 37 ^{o}C for 48 hours. The Presence or absence of growth of lactic acid bacteria on MRS broth was used to designate the isolate as pH tolerant. Un-inoculated sterile MRS broth served as the control.

2.4.4. Sensitivity to Temperature Test

The isolated *Lactobacillus* species were inoculated into 10 mL sterile de Man Rogosa and Sharpe broth in test tubes and incubated anaerobically at varying temperatures from 15- 45 ^{o}C for 48-72 hours. Thereafter, 1 mL inoculum was cultured on de Man Rogosa and Sharpe agar plates by pour plate method and incubated at 37 ^{o}C for 48 h. The growth of *Lactobacillus* on de Man Rogosa and Sharpe agar plates were used to designate isolates as temperature tolerant [12].

2.5. Assay for Antibacterial Activity of isolated *Lactobacillus* sp.

Antibacterial activity of the *Lactobacillus* isolates was determined by the agar well diffusion assay as described by [13] against test organisms (*Staphylococcus aureus, Salmonella typhi* and *Escherichia coli*). A 0.2 mL of the test organism which were previously incubated in nutrient broth for 24 h, were aseptically introduced into the sterile Petri dishes. The sterilized Mueller-Hinton agar medium at 45 ^{0}C was poured into the Petri dishes. Wells were made on the agar plates using a sterile cork borer of 5 mm in diameter. A 0.1 ml of the *Lactobacillus* sp. were placed into each well. A negative control was 0.1ml of the broth without organisms. Plates were incubated at 37 ^{o}C for 48 hours and diameters of the growth inhibition zones were measured using a ruler calibrated in millimeter. Each experiment was replicated three times and the results were expressed as average values. Isolates showing the widest zone of inhibition above 1 mm against the target microorganism was determined to have antimicrobial activity.

2.6. Enzymes Treatment

Sensitivity to enzymatic proteolytic action was tested by treatment of the isolated *Lactobacillus* sp. with different enzymes (trypsin, pepsin and proteinase K) and incubated at 37^{0}C for 2 hours. Buffer without an enzyme was used as a negative control. The agar well diffusion method was applied to measure the antimicrobial activity as described by [12].

2.7. Data Analysis

All the measurements were performed in triplicate, and the results were expressed as mean standard deviation. Data collected were subjected to two-way ANOVA analysis using sigma plot version 12 statistical software. All data analysis was performed with origin 8.6 and SPSS 19.0.

3. Result

3.1. Morphological and Biochemical Characterization of *Lactobacillus*

The result of morphological observation showed four isolates with distinct cellular (whitish, creamy, circular, flat, raised colonies) on MRS agar plates. The isolates when Gram stained, found Gram-positive rod shaped. The biochemical result shows that the four isolates were negative to catalase test, oxidase test, urease and citrate test. (Table 1). The isolates were able to ferment the given sugars except isolate B_2 and isolate B_3 that were negative to xylose sugar (table 2)

Table 1 Morphological and Biochemical Characteristics of *Lactobacillus* species

Parameters	**Observation**			
Morphological characteristics	**Isolate B1**	**Isolate B2**	**Isolate B3**	**Isolate B4**
Cell morphology on MRS agar	**short rod**	**rod in pairs**	**short Rod**	**rod**
Colour	creamy	White	creamy	creamy
Form	circular	irregular	circular	circular
Pigmentation	-ve	-ve	-ve	-ve
Surface	smooth	Smooth	smooth	smooth
Elevation	flat	Raised	raised	flat
Gram staining	+ve	+ve	+ve	+ve
Biochemical Characteristics				
Catalase test	–ve	-ve	–ve	–ve
Oxidse test	–ve	–ve	–ve	–ve
Citrate test	–ve	–ve	–ve	–ve
Urease test	–ve	–ve	–ve	–ve

Keyword: '+ve' indicates a positive result, '–ve' indicates a negative result, MRS =Man Rogosa Sharpe; Isolate B1 to B4 = isolated Lactobacillus species

Table 2 Sugar Fermentation Test of *Lactobacillus*

Lactobacillus species	**Glucose**	**Lactose**	**Xylose**	**Mannitol**	**Sorbitol**	**Inositol**
Isolate B_1	+	+	+	+/-	+	+
Isolate B_2	+	+	+/-	+	+	+
Isolate B_3	+	+	-	+	+	+/-
Isolate B_4	+	+	-	+	+	+/-

+/- slightly fermented; + strongly fermented (colour changes to yellow); - no fermentation (purple colour)

3.2. Species Identification of the Isolated *Lactobacillus* sp

Ribosomal 16S rDNA sequencing allowed the identification of 4 *Lactobacillus spp.* Similarity searches using sequenced ribosomal DNA fragments retrieved species whose genomic sequences matches *Lactobacillus casei strain LC3* (isolate

B_1), *Lactobacillus plantarum strain MF1298* (isolate B_2), *Lactobacillus fermentum* strain *17-6* (isolate B_3) *and Lactobacillus rhamnosus* strain *WE1-30* (isolate B_4) (Table 3).

Table 3 List of *Lactobacillus* Isolates obtained from decaying banana and related species identified by 16s rDNA sequencing

Strain 1D	Bacterial Species	% identity with Reference species in BLAST database	Sources of Isolation	Accession Number
LC3	*Lactobacillus casei*	99	decaying banana	CP049600.1
MF1298	*Lactobacillus plantarum*	99	decaying banana	CP007795.1
17-6	*Lactobacillus fermentum*	99	decaying banana	1KY435814.1
WE1-30	*Lactobacillus rhamnosus*	100	decaying banana	KY041760.1

3.3. Determination of Probiotic Properties of the Isolated *Lactobacillus* species.

3.3.1. Sodium Chloride (NaCl) Tolerance Test

The four isolated *Lactobacillus* from decaying banana where able to tolerate 1- 9% w/v concentration of NaCl in the MRS broth. Figure 1 shows that *L. plantarum* and *L. casei* maintained good growth up to 9% concentration of NaCl and growth declined sharply with increase of NaCl concentration in MRS broth. On the other hand, *L. fermentum* and *L. rhamnosus* maintained good growth up to 8% NaCl concentration. However, upon increasing the NaCl concentration to 10%, growth of *L. fermentum and L. rhamnosus* decreases. None of the isolated *Lactobacillus sp.* showed any growth at 10% concentration of NaCl and above

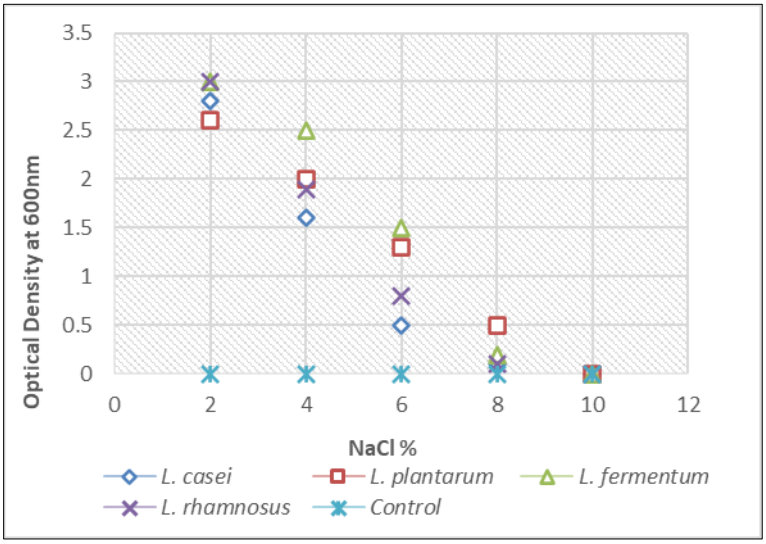

Figure 1 Sodium Chloride (NaCl) Tolerance of the Isolated *Lactobacillus* sp

Growth of *L. casei, L. plantarum, L. fermentum* and *L. rhamnosus* in the presence of 1-10% NaCl Concentration. Values are mean ± standard deviation of three replicates.

3.3.2. Evaluation of Bile Salt resistance

The four *Lactobacillus* strains showed maximum growth when the bile concentration was 0.1%. They both maintained good growth up to 0.3% w/v bile salt concentration in MRS broth. Maximum percentage of growth was observed in *Lactobacillus plantarum and L. casei*. The result displayed in Figure 2 showed that there was a decrease in growth rate of all the isolated *Lactobacillus* strains at increase in bile salt concentration as compared to the control.

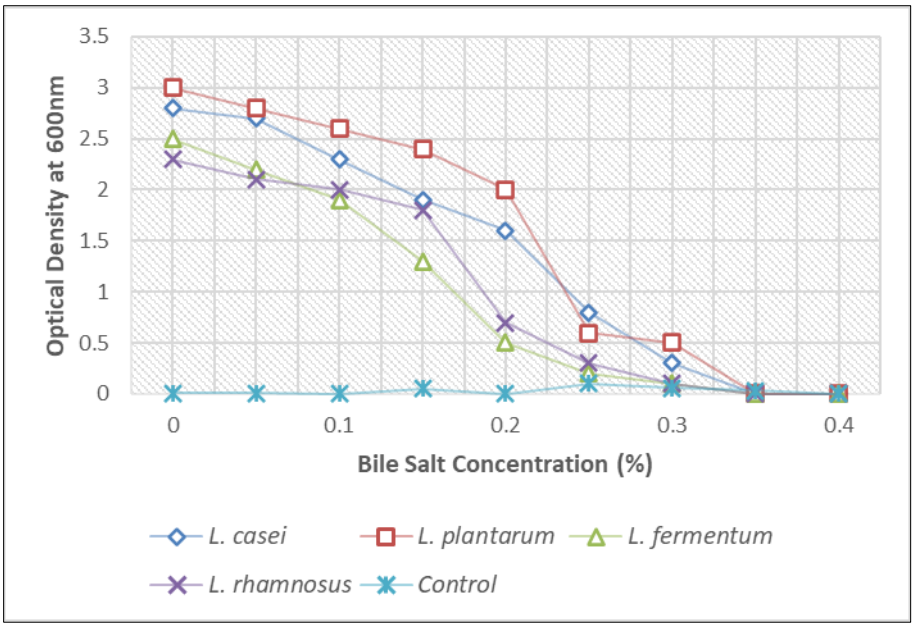

Figure 2 Bile Salt Tolerance of the Isolated *Lactobacillus sp*

Growth of isolated *Lactobacillus species* in the presence of 0.3% bile and their growth comparison with control set (without bile). Values are mean ± standard deviation of three replicates

3.3.3. Tolerance to Acidic pH

Good Probiotic microorganisms must survive in the acidic pH environment in the gastrointestinal tract and arrive at the site of action in a viable physiological state. Different concentrations of growth condition were used to culture the four isolated *Lactobacillus* species*. Lactobacillus fermentum* showed no tolerance at pH 2 and pH 10. *Lactobacillus plantarum and Lactobacillus rhamnosus* showed tolerance at pH 2-9 after 2 hours of incubation at 37°C. Maximum growth rate was observed at pH 5.0 and 6.0 for all the strains. Among the four *Lactobacillus* strains, *L. plantarum* exhibited maximum tolerance. At pH 9, all isolates showed reduced growth, and at pH10 no growth was observed from all the *Lactobacillus* strains. The tolerance to acidic pH of the *Lactobacillus* isolates is shown on (Figure 3).

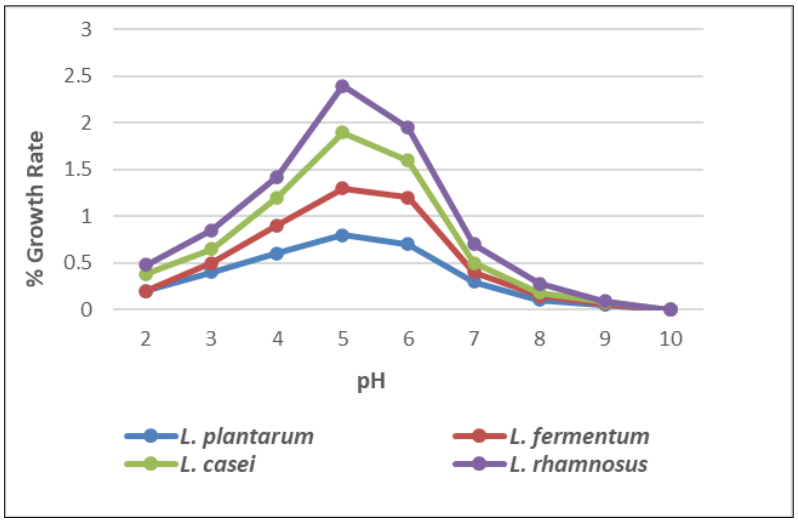

Figure 3 Acid Tolerance by selected *Lactobacillus* strains

Tolerance of the isolated *Lactobacillus sp.* to Acidic pH. Values are mean ± standard deviation of three replicates.

3.4. Antibacterial activity of *Lactobacillus* Strains

The results of antimicrobial activity in figure 4 showed that the four *Lactobacillus* species isolated from decaying banana showed inhibitory effect in the agar well diffusion test against the three food pathogenic organisms chosen as an indicator strains (*S. aureus, S. typhi and E. coli*). *Lactobacillus rhamnosus* displayed highest inhibitory effect on all the test organisms. *L. casei* showed the highest zone of inhibition against *E. coli* (3.05±0.2mm).

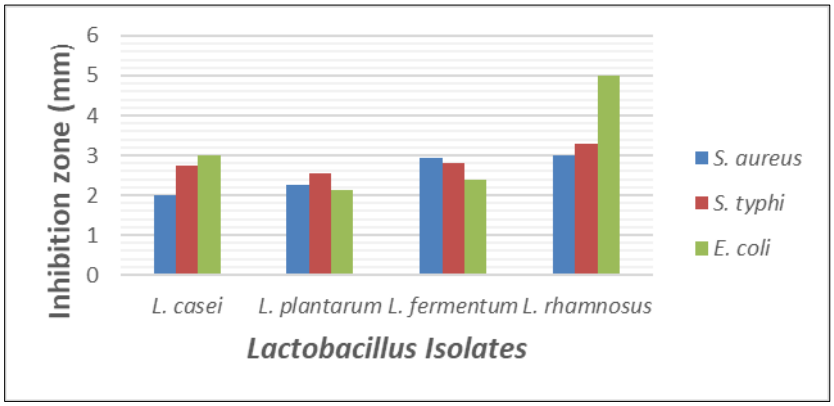

Figure 4 Zones of Inhibition (in mm) by *Lactobacillus species* against the Test Pathogens. Values are mean ± standard deviation of three replicates

3.5. Enzymes

The *Lactobacillus* isolates did not exhibit any antimicrobial activity against the test organisms after treatment with enzymes. Good proteolytic activity was observed from the four isolates. (table 4)

Table 4 Effect of enzyme treatment on *Lactobacillus* isolates

Treatment	**Activity[x]**		
Proteolytic Enzymes	***S. aureus***	***S. typhi***	***E. coli***
Trypsin	-	-	-
Pepsin	-	-	-
Proteinase K	-	-	-
Control	19	27	23

[x] Activity against test organisms in 10 µl of preparation. + = presence of activity after treatment, - = loss of activity after treatment

4. Discussion

The present study identified four *Lactobacillus* species from decaying bananas using morphological, biochemical and molecular characteristics. The result revealed positive growth on MRS agar medium, whitish, circular colonies, the cells were gram-positive, catalase-negative, citrate-negative and oxidase-negative and were confirmed to be *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus rhamnosus* through molecular typing using 16s rDNA sequencing. These identification methods agreed with the previous study by [14, 15,16] on isolating lactic acid bacteria.

To consider bacteria to be a potential probiotic, they must possess several desirable characteristics such as tolerance to Sodium chloride and bile salt, tolerance to acidic pH, and resistance to proteolytic enzymes. The probiotic screening of the isolated *Lactobacillus* strains reveals their tolerance to sodium chloride (NaCl). These is in agreement with the study done by [17, 16]. None of the isolated *Lactobacillus* sp. showed any growth at 10% concentration of NaCl and above. The four *Lactobacillus* strains showed maximum growth when the bile concentration was 0.1%. *Lactobacillus plantarum* showed maximum percentage of growth rate compared to other isolated *Lactobacillus sp*. The growth rate was reduced with an increase in bile concentration. This agreed with the study of Todorov *et al.,* [18] who reported that a reduced growth rate was observed in *L. plantarum* ST19482 and ST441B2 at 0.6% concentration. Bile plays an important role

in guts specific and non-specific defense mechanisms. It is considered an important characteristic of Lactic acid bacteria strains that enable them to survive in gastrointestinal tracts and exert beneficial effects [19]. Probiotic bacteria must possess a good tolerance to acidic pH. From the result obtained, the four isolated *Lactobacillus* species exhibited good tolerance to acidic pH. Maximum growth was observed at pH 5 and 6, similar results were reported by Todorov *et al.*[18] that maximum growth of the *L. plantarum* ST16Pa isolated from papaya was observed at pH 4 and 5. At pH 10, no growth was observed. This was in agreement with study of Padmavathi *et al.* [19] that observed sparse growth of *L. fermentum* at pH 10. These parameters are very important in determining the effectiveness of probiotics since growth and viability during storage and use is one of the important determining factors for the functionality of probiotics [17].

The four *Lactobacillus* species showed an inhibition growth effect on all three test organisms. Different studies have reported the inhibitory effects of various *Lactobacillus* including; *L. fermentum, L. plantarum and L. casei* [20, 13, 21]. The result agreed with the study conducted by Wirawati *et al.* {13] that reported the antibacterial effect and proteolytic activity of *Lactobacillus plantarum* from fermented dadih. The inhibition of both Gram-positive and Gram-negative bacteria was probably an indicative of broad-spectrum antimicrobial substance. These results are in agreement with the findings of [22, 16] who reported that *Lactobacillus* species inhibited the growth of bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Samonella typhimurium, Klebsiella pneumoniae* and *Burkholderia cepacia.* A Similar result was also reported by [13] that *Lactobacilli* had an inhibitory effect on the growth of both Gram-positive and Gram-negative bacteria. Proteolytic enzymes inactivated the antimicrobial activities of the isolated *Lactobacillus* in the present study. This resistance allows them to survive, grow and exert their action in the gastrointestinal tract, an indication that the isolated *Lactobacillus* is proteinaceous in nature and a good probiotic. Our findings agreed with the results of Komkhae *et al.* [23] who treated lactic acid bacteria with pepsin, trypsin and proteinase K and discovered that antimicrobial activity was lost after treatment by those proteolytic enzymes.

5. Conclusion

Four isolates were successfully isolated from and identified as *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus rhamnosus* from decaying banana. The study has shown that the isolated *Lactobacillus* has good probiotic attributes and wide spectrum of antimicrobial activity against three food pathogenic bacteria.

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