Journal of Harbin Engineering University
ISSN: 1006-7043

Vol 46 No. 2
february 2025

Isolation and Characterization of Bacteriocin-Producing Lactic Acid Bacteria from Mbithi: A Kenyan Traditional Fermented Porridge

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Abstract

Introduction: Mbithi is a non-alcoholic, spontaneously fermented product that remains a traditional food cuisine in Kenya, valued for its nutritional properties. Lactic acid bacteria (LAB) are utilized in the food business as a starting culture, with their preservation properties linked to their ability to produce bacteriocins. Due to their eco-friendly properties, LAB's bacteriocins offer a viable solution to the health risks associated with chemical preservatives in the food industry.

Objectives: This study presents results on isolating and characterizing bacteriocin-producing lactic acid bacteria from Mbithi.

Methods: Lactic acid bacteria (LAB) were isolated using MRS and M17 agar. These isolates were then phenotypically characterized, including biochemical and physical tests. The isolates were screened for antimicrobial activity by agar well-diffusion assay, while genotypic characterization was performed based on the 16S rRNA gene sequence.

Results: A total of 50 LAB strains were extracted. Of the fifty (50) isolates, 47 (94%) were selected due to their demonstration of gram-positive characteristics and catalase activity. Furthermore, 12 isolates were heterofermentative for they fermented glucose, whereas (35) isolates did not metabolize glucose (homofermentative). The results on salinity, acidity, and temperature demonstrated that most of the isolates tolerated the salinity with 6.5% of NaCl, acidic at pH 2.0 and pH 2.5 and temperature of 45°C. Nonetheless, none of the isolates withstood a temperature of 10°C. From the inhibition tests, we identified 10 candidate isolates, including three isolates—FP2 24, FP2 28, and FP22—that exhibited antagonism against all evaluated indicator pathogenic microorganisms. The three isolates exhibited inhibitory activity against E. coli, Bacillus subtilis, and Staphylococcus aureus, with inhibition zones of 21±1.41, 26±1.41, and 21±1.41, respectively, and a P-value of less than ($P<0.05$, $P=0.000$). The phylogenetic and Blast analyses indicated that the isolates were categorized into five clusters: *Lactiplantibacillus, Levilactobacillus, Pediococcus, Weisella,* and *Leuconostoc*, with the majority clustering within the *Lactiplantibacillus* genus, with similarities ranging from (95.1%-100%).

Conclusions: The findings revealed potential isolates showing antagonistic activity against pathogenic indicators, suggesting their possible production of secondary metabolites. Despite this research presenting promising outcomes, further characterization is requested to get insights on probiotics properties for the usage of the isolates in preservatives in the food industry domains.

Keywords: Antimicrobial Activity; Lactic Acid Bacteria (LAB); Bacteriocin; Preservatives, Fermented Porridge "Mbithi".

1. Introduction

The use of artificial chemical preservatives has grown over the last few decades, and substantial scientific evidence has surfaced linking food additive sensitivity to a range of health problems. Children's hyperactivity and other

neurophysiological issues have been linked to/caused by consuming chemical preservatives [1]. Many of these preservatives are known to be carcinogenic, while others have been associated with several adverse consequences, as well as heart damage, breathing problems, and other health issues [2]. Pathogen contamination, a common cause related to food-borne illnesses, is among the worries in the food business [3]. One workable substitute for human-use chemical preservatives in food and feed products is the application of certain bacterial peptides serving as antimicrobial agents. This would help to restrict the overuse of these chemicals. Among these, lactic acid bacteria's bacteriocins have drawn interest [3]. LABs are a wide collection of bacteria identified in fermented foods and drinks such as yogurt, cheese, and kefir [4]. LABs have been traditionally isolated in cereal fermentations including Uji, a non-alcoholic fermented cereal beverage made from sorghum, maize, and millet from Kenya, Uganda, and Tanzania [5]; pito, an alcoholic dark brown drink from Nigeria and Ghana made from maize, sorghum, and millet [6]; bushera, a Ugandan non-alcoholic drink made from millet and sorghum [7] and fura, an alcoholic drink made from maize and sorghum from Nigeria [6].

Many bacteriocin-producing LABs have previously been identified in Kenya in fermented foods such as mursik (fermented milk) [8]. These bacteriocins have demonstrated antimicrobial action against a variety of foodborne microbes, which include Salmonella, *Escherichia coli* and *Staphylococcus aureus* [9]. Lactic acid bacteria are an important type of industrial bacteria used within fermented foods production such as sausages and vegetables, nutritional supplements, probiotics, and even cosmetic elements [2]. Lactic acid bacteria include genus *Oenococcus, Streptococcus, Pediococcus, Lactobacillus, Carnobacterium, Aerococcus, Enterococcus, Leuconostoc, Lactococcus, Tetragenococcus, Vagococcus,* and *Weissella*. *Lactobacillus* is the most prominent genus within this group [10]. They are used as starter cultures to improve product texture and flavour. Their capacity to inhibit the growth of spoilage and pathogenic microorganisms contributes to the preservation of product hygiene and quality, as well as the host health [2], [11], [12]. Such inhibitory activity is caused by the antibacterial property of the metabolic product produced by these LABs such as; hydrogen peroxide, organic acids, diacetyl, and bacteriocin [2].

Bacteriocins are a broad collection of antimicrobial proteins generated through bacteria that are formed in the ribosomes [2], [3], [13]. They are used in medicine to treat multi-drug-resistant microorganisms and they are regarded as new potential effective control agents against microbial diseases since their action mechanism differs from that of traditional antibiotics [14]. Their alternative uses in both the pharmaceutical and food industries are being researched across the world [4].

Millets are tiny to medium-sized cereals which belong to the family *Gramineae*. They are produced globally for food and fodder, and they are a staple within East, Central, Africa's West, and Great Lakes regions, along with Asia and India. Millets belong to the *Panicoideae* grass subfamily, the same as maize and sorghum [15]. Many African countries prepare fermented millet-based dishes together with beverages for human consumption, including alcoholic and non-alcoholic options [7]. Millets rank as the sixth most significant cereal grain crop globally, following maize, wheat, rice, sorghum, and barley, which are regarded as key economic cereals. Millet grains are gluten-free, do not produce acid, are easy to digest, have a low glycaemic index, and serve as a nutritious food choice for people with celiac disease, a common condition resulting from the intake of cereal proteins [15]. Millet is a vital staple and commercial crop for most people in the eastern region of Kenya.

Mbithi is a traditional non-alcoholic beverage consumed in the eastern part of Kenya and it is made from millet flour, mixed with water, pasteurized to approximately 60℃, allowed to cool to room temperature and left to ferment spontaneously for 12-24 hours in a warm environment as shown in (Figure 1). Mbithi has a particular tangy flavor and somewhat effervescent texture, as well as a sour taste due to the natural yeast and bacteria. It is eaten by both young children and adults. Mbithi is expected to be a promising source of lactic acid bacteria that produce bacteriocins. However, there is a lack of evidence regarding the microbial diversity and

fermentation by-products in Mbithi. The main aim of this study was, therefore, to isolate and characterize bacteriocin-producing lactic acid bacteria from 'Mbithi'.

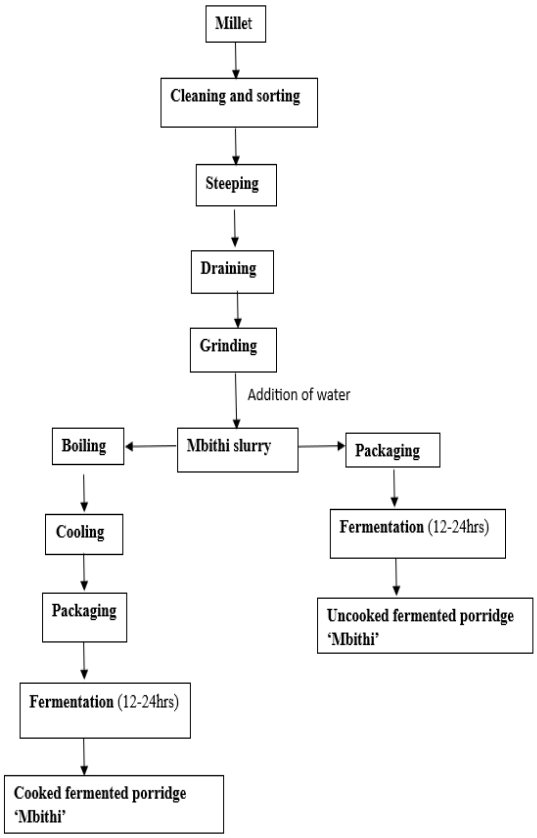

Figure 1: Flow diagram illustrating the production process of Mbithi, a millet-based fermented porridge produced in Kenya.

2. Objectives

2.1 General objective

To isolate and characterize bacteriocin-producing lactic acid bacteria from 'Mbithi' a traditionally fermented porridge, from the Eastern part of Kenya

2.2 Specific objectives

- To determine the phenotypic and biochemical characteristics of isolated lactic acid bacteria from 'Mbithi' a traditionally fermented porridge, from the Eastern part of Kenya.
- To screen the isolated lactic acid bacteria for crude bacteriocins production against standard foodborne pathogens.
- To assess the molecular characteristics of the isolated bacteriocins producing lactic acid bacteria.

3. Methods

3.1 Preparation and fermentation of Mbithi

Mbithi was prepared from millet flour in the Eastern part of Kenya (Maua, Meru County). Millet cereals were sorted to remove stones and other foreign matter. They were then cleaned and soaked in water for 2 hours and drained. The cereals were then wet-ground using a traditional stone grinder. The ground flour slurry was mixed with water to get a thinner consistency followed by pasteurization at approximately 60°C while stirring to prevent lumps in the porridge. The porridge was cooled down before it was transferred to a clean container and covered to allow fermentation for 24 hours.

3.2 Sample collection

The fermented porridge was then transferred into a sterile container (Reagent bottle) and transported in a cool box to the Department of Food Science and Technology, Food Microbiology Laboratory, Jomo Kenyatta University of Agriculture and Technology (JKUAT) for analysis.

3.3 Isolation of lactic acid bacteria from Mbithi

Mbithi was properly mixed followed by transferring 1 mL of the mixture to a 9 mL test tube holding aseptic quarter-strength ringer's solution to create a10-fold serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. These preparations were then mixed by vortexing. A volume of 100 µL from each preparation was spread plated onto MRS agar (De Man, Rogosa, and Sharp) and M17 agar (Himedia, Mumbai, India). Plated MRS agar and M17 agar plates were incubated in aerobic conditions at 30°C for 24 to 48 hours. The distinct colonies were selected from the plates inoculated with the higher dilutions. The selected isolates were transferred and grown aerobically in MRS broth at 30°C. The isolates were streaked for three cycles to ensure their purity. The purified isolates were cryopreserved in MRS broth containing 20% of glycerol and stored at -75°C.

3.4 Phenotypic characterization

Presumptive lactic acid bacteria (LAB) isolated from Mbithi were subjected to additional characterization. Cell morphology was assessed microscopically using a phase contrast microscope (Shimadzu CX41, Tokyo, Japan), Gram reaction,

catalase test, production of gas (CO_2) from glucose metabolism in MRS broth, and growth at a 6.5% NaCl concentration were assessed as described by Wafula [42]. Growth at various temperatures (between 10℃ and 45℃) was also determined as described by Somashekaraiah [36] and Sridharan [37], and growth at pH 2.0 and 2.5 was also determined as described by Mulaw [27]. The isolates were categorized into four groups according to their phenotypic traits: heterofermentative rods, heterofermentative cocci, homofermentative rods, and homofermentative cocci.

3.5 Screening isolates for bacteriocin production

Antibacterial activity was established by the agar well diffusion method as recommended by Tagg & McGiven [16] and improved by Kormin [17] with minor modifications. This required culturing indicator strains (*Escherichia coli*, *Bacillus subtilis,* and *Staphylococcus aureus*) on nutrient agar (Himedia, Mumbai, India) at 37℃ overnight. The LAB isolates were cultured aseptically in MRS broth and incubated at 30°C for 24 hours. After 24 hours, the cultures were centrifuged at 15,000 rpm for 5 minutes and filtered by 0.22 μm microfilter to get cell-free supernatant (CFS). CFS was neutralized to a pH 6.5 using 1 N NaOH and catalase was used to counteract organic acid and hydrogen peroxide generated by LAB. This was followed by adding 100 µl of the cell-free supernatants to the wells. The antimicrobial activity was compared to that of commercial antibiotic disks containing Kanamycin (30 mcg), Gentamicin (10 mcg), Sulphamethoxazole (200 mcg), Chloremphenical (30 mcg), Ampicillin (25 mcg), Tetracycline (25 mcg), Co-Trimoxazole/Sulpha/Trimethoprim (25 mcg), Streptomycin (10 mcg), 0D215R-100N0, HiMedia Laboratories Pvt. Ltd. (Mumbai, India) acting as positive control and negative control (blank broth). The indicator strains were obtained from the Laboratory of Molecular Biology and Biotechnology of PAUSTI (Juja, Nairobi, Kenya). The inhibition zones were computed in millimeters after 24 hours of incubation at 37°C. The experiment was carried out in duplicates.

3.6 Genotypic Characterization

DNA was extracted from bacterial cells of isolates selected with biopreservative potential for 16S rRNA gene sequence analysis using Quick-DNA Fungal/Bacterial Miniprep kit, D6005 (Zymo research, city, USA) according to the manufacturer's instructions. The genomic DNA was used as a template for amplifying the 16S rRNA genes of the isolates. This was carried out by a pair combination of primers 27F (5'-AGAGTTTGATCCTGGCTCAG3') and 1492R ((5'-ACGGCTACCTTGTTACGACTT-3') bacterial universal primers. PCR was performed in a 50 µL mixture containing 25 µL OneTaq® 2XMasterMix, RC101 (Accuris PCR reagents, city, USA) 1 µL of each primer, 1 µL of DNA template (10 ng), and 22 µL Nuclease-free water. The PCR conditions were set as follows: Initial denaturation at 95°C for 5 minutes, Denaturation at 95°C for 30 seconds, primer annealing at 57°C for 30 seconds, extension at 72°C for 1 minute and 30 seconds, and a final extension at 72°C for 5 minutes. Amplification products (1500 bp) were separated on a 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining. They were purified and sequenced by Macrogen Asia (Biopolis, Singapore).

3.7 Phylogenetic Analysis

The 16S rRNA gene sequences of the bacteria isolates were viewed for quality determination and edited using the latest ChromasPro software package 2.8.1 (https://chromaspro.software.informer.com, accessed on 2nd October 2024). The sequences were then compared with available standard sequences of bacteria lineages in the public databases in the National Center for Biotechnology Information (NCBI) gene bank using nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 2nd October 2024) to find closely related bacterial 16S rRNA gene sequences. The parameters such as percentage similarities, query coverage, and E-values were used. This was followed by aligning the sequence using Clustal W software. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Kimura 2-parameter model [18] with the MEGA version 7.0 software package [19]. The trees'

topologies were evaluated using the bootstrap resampling method [20] based on 1000 replicates.

3.8 Data Analysis

All results were interpreted as mean ± standard deviation (SD). The statistical package (Minitab, Pennsylvania, USA) program version Minitab® 21.2 (64-bit) was used to analyze the data using one-way analysis of variance (one-way ANOVA). Tukey's honest significant difference (HSD) test was employed to determine the significance of the difference between means at a probability level of ($P<0.05$).

4. Results

4.1 Isolation and enumeration of LAB from Mbithi

The average count of lactic acid bacteria in traditional fermented porridge (Mbithi) was approximately log 7 after 24 hours. It was increased approximately to log 8 after 48 hours in both samples, cooked fermented porridge and uncooked fermented porridge as shown in (Figure 2).

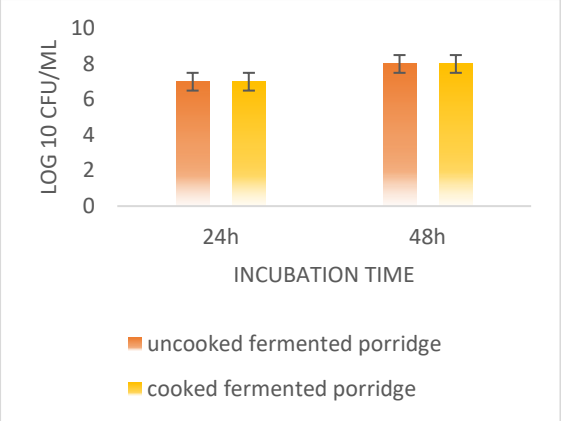

Figure 2: Lactic acid bacteria colony counts from fermented Mbithi.

4.2 Phenotypic characterization

A total of 50 isolates were obtained from mbithi (fermented porridge). The isolates were identified as Gram-positive and catalase-negative with rod or cocci shapes. Out of 50 isolates, 47 were Gram-positive and catalase-negative (Table 1) while 3 were catalase-positive and thus discarded [21], [22]. Of the retained isolates, 38 were rod-shaped while 9 were cocci shaped. Most of the isolates grew in 6.5% NaCl and at 45°C, but none grew at 10°C. Out of 47 isolates, 12 isolates produced gas from glucose metabolism and 35 isolates were homofermentative or did not produce gas from glucose metabolism [23]. Out of 12 heterofermentative isolates, 8 were rod-shaped, produced gas from glucose fermentation and thus were considered either heterofermentative rods belonging to the genus *Lactobacillus* or *Weissella*. Whereas 4 isolates were coccus-shaped and produced gas from glucose fermentation and thus were identified as heterofermentative cocci which belonged to the genera *Leuconostoc* or *Weissella.*

Out of 35 isolates, 30 were Gram-positive, rod-shaped, catalase-negative, and produced no gas from glucose metabolism Therefore, they were classified as homofermentative rods belonging to *Lb*. *plantarum* and 5 isolates were Gram-positive, cocci-shaped, and catalase-negative, they did not produce gas from glucose metabolism and thus were classified as homofermentative cocci which include *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*) [23].

Table 1. Phenotypic and molecular identification of lactic acid bacteria from Mbithi

	Phenotypic Characterization									Molecular Identification		
Sample ID	Cell shape	Gram status	Catalase	CO2	6.5% NaCl	Growth at 10°C	Growth at 45°C	Growth at pH	Growth at pH	Closest Relatives	% Identity	Accession No.
FP1 02	Rods	+	–	+	+	–	+	–	–	Weissella cibaria	99.63	MN598068
FP1 03	Cocci	+	–	+	+	–	–	–	–	leuconostoc citreum	99.69	MG754638
FP1 04	Cocci	+	–	+	–	–	+	–	–	leuconostoc lactis	98.78	MK855341
FP1 05	Cocci	+	–	+	+	–	+	+	+	leuconostoc citreum	99.74	PP916849
FP2 05	Rods	+	–	+	+	–	+	+	+	lactobacillus	99.54	MH924297
FP2 07	Rods	+	–	–	–	–	–	–	–	lactiplantibacillus plantarum	100	MW674080.1
FP2 08	Rods	+	–	–	+	–	+	+	+	lactiplantibacillus	99.71	MW674306.1

MN658812	OQ692119.1	MW674080.1	KJ690745.1		**Accession No.**	MG551203.1	MT464412.1	PP916881.1		MF992228.1	MW674080.1	MH899317.	MG822862	PP917535.	OM866164.	OK189684.1
95.13	99.88	99.48	99.9		**% Identity**	99.6	99.76	99.61		99.56	98.94	97.81	99.7	100	99.69	100
pediococcus	levilactobacillus brevis	lactiplantibacillus plantarum	lactobacillus plantarum	**Molecular Identification**	**Closest Relatives**	levilactobacillus brevis	lactobacillus plantarum	pediococcus pentasaceus		lactiplantibacillus plantarum	lactiplantibacillus plantarum	lactobacillus plantarum	lactobacillus	pediococcus	lactobacillus plantarum	Lactiplantibacillus plantarum
–	–	+	+		**Growth at pH**	–	+	+		+	+	+	–	–	+	+
–	–	–	+		**Growth at pH**	–	+	+		–	+	+	–	–	+	+
+	–	+	+		**Growth at**	+	+	+		–	–	–	+	+	–	+
–	–	–	–		**Growth at**	–	–	–		–	–	–	–	–	–	–
–	–	+	+		**6.5% NaCl**	+	+	+		–	–	+	+	+	+	+
–	+	–	–		**CO2**	+	+	–		–	–	–	–	–	–	–
–	–	–	–		**Catalase**	–	–	–		–	–	–	–	–	–	–
+	+	+	+	**Phenotypic Characterization**	**Gram status**	+	+	+		+	+	+	+	+	+	+
Cocci	Rods	Rods	Rods		**Cell shape**	Rods	Rods	Cocci		Rods	Rods	Rods	Rods	Cocci	Rods	Rods
FP2 10	**FP2 11A**	**FP2 14**	**FP2 16**		**Sample ID**	**FP2 18**	**FP2 20A**	**FP2 20B**		**FP2 22**	**FP2 23**	**FP2 24**	**FP2 26**	**FP2 27**	**FP2 28**	**MFP2 05**

+: growth, –: no growth

4.3 Antimicrobial activity of bacteriocins

The antimicrobial effects of the cell-free supernatant from the lactic acid bacteria strains were assessed against three indicator pathogens such as *Escherichia coli* (Gram-negative), *Bacillus subtilis* (Gram-positive), and *Staphylococcus aureus* (Gram-positive), and the results were presented as the diameters of zones of inhibition. Not all LAB strains were able to inhibit all tested bacteria as shown in (Table 2 and Figure 3). The antibiotic disk was used as a positive control (Table 3).

Table 2: antimicrobial activity of the lactic acid bacteria isolates

Isolates	***E. coli*** **D(mm)**	***B. subtilis*** **D(mm)**	***S. aureus*** **D(mm)**
FP1 02	–	–	14.5±0.70cde
FP1 03	–	18.5±0.70bcde	11±1.41fgh
FP1 04	–	19.5±0.70bcd	14.5±0.70cde
FP1 05	–	15.5±0.70ef	13.5±0.70defg
FP2 05	14.5±0.70bcd	21.5±0.70b	–
FP2 07	–	21±1.41b	18.5±0.70ab
FP2 08	16.5±0.70b	20.5±0.70b	19±00ab
FP2 10	–	20.5±0.70b	14.5±0.70cde
FP2 11A	10.5±0.70ef	14.5±0.70fg	16.5±0.70bcd
FP2 14	15.5±0.70bc	21±1.41b	10.5±0.70gh
FP2 16	14.5±0.70bcd	25.5±0.70a	13.5±0.70defg
FP2 18	–	11.5±0.70gh	–
FP2 20A	–	21±1.41b	14.5±0.70cde
FP2 20B	–	16.5±0.70def	13±1.41efg
FP2 22	13±1.41cde	20±00bc	21±1.41a
FP2 23	12.5±0.70de	21.25±0.35b	17±1.41bc
FP2 24	21±1.41a	20.75±1.06b	15±00cde
FP2 26	14.5±0.70bcd	25.5±0.70a	15±00cde
FP2 27	15.5±0.70bc	17.1±0.14cdef	14±00cdef

FP228	13.5±0.70cd	26±1.41a	16.5±0.70bcd
MFP205	12.5±0.70de	–	15±00cde
p-value	0	0	0
F-value	53.92	65.96	30.17

Means were computed from replications (n=2) ± std. Means that do not share a letter are significantly different (p<0.05). No inhibition is denoted by –, D (mm): diameter of inhibition zone in millimeters.

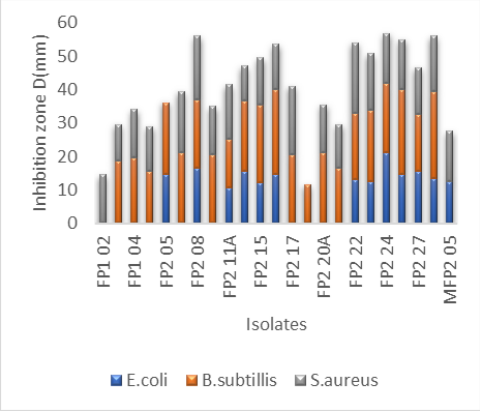

Figure 3: Antimicrobial activity of crude bacteriocin from Mbithi's lactic acid bacteria against foodborne pathogens.

Table 3: Antibiotic activity test (positive control) against indicator strains

Antibiotics	***E. coli* D(mm)**	***B. subtilis* D(mm)**	***S. aureus* D(mm)**
K-30	18±00c	15.5±0.70c	12±2.82b
GEN-10	16.5±0.70d	21±1.41b	12.5±2.12b
SX-200	–	18.5±2.12bc	22.5±3.53a
C-30	26.5±0.70a	20±00b	10±00b
AMP-25	–	–	–
TE-25	21±00b	25±0.70a	12±2.82b
COT-25	–	–	–
S-10	10±00e	15±00c	–
p-value	0	0	0
F-value	1786.29	192.35	31.54

Means were computed from replications (n=2) ± std. Means that do not share a letter are significantly different (p<0.05). No inhibition is denoted by –, D (mm): diameter of inhibition zone in millimeters. K-30: Kanamycin (30mcg), GEN-10: Gentamicin (10mcg), SX-200: Sulphamethoxazole (200mcg), C-30: Chloremphenical (30mcg), AMP-25: Ampicillin (25mcg), TE-25: Tetracycline (25mcg), COT-25: Co-Trimoxazole/Sulpha/Trimethoprim (25mcg), S-10: Streptomycin (10mcg).

4.4 Molecular characterization

A total of 21 isolates with bio-preservative and starter culture potential were selected for 16S rRNA gene sequencing. Blast analysis of the sequence showed that 57% were from the genus *Lactobacillus* (12 strains) with similarity between 97.81% and 100%. 14 % belonged to the genus *Pediococcus* (3 strains) with similarities of 95.13 % to 100 %. 14% belonged to *Leuconostoc* (3 strains) with a similarity of 99.69 % to 99.78%. 10 % belonged to the genus *Levilactobacillus* (2 strains) with a similarity of 100 %. 5% constituted by genus *Weissella* (1 strain) with a similarity of 100 % as shown in (Table 1, and Figure 4). Further, phylogenetic analysis showed that 12 isolates clustered into genus *Lactobacillus sp* with FP205, FP207, FP208, FP214, FP216, FP220A, FP222, FP223, FP224, FP226, FP228 and MFP205 being closely related to *Lactobacillus plantarum* (KJ690745.1). The analysis showed that isolates FP220A, FP210, and FP227 were closely related to *Pediococcus pentosaceus* (PP917535.1), FP211A and FP218 were closely related to *Levilactobacillus brevis* (OQ692119.1), *and* FP102 was closely related to *Weissella cibaria* (MN598068.1). Other isolates were clustered into genus *Leuconostoc sp* with FP103 and FP105 being closely related to *Leuconostoc citreum* (PP916849.1), while FP104 was closely related to *Leuconostoc lactis* (MK855341.1) as shown in (Figure 5).

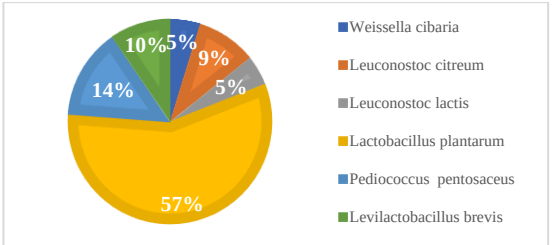

Figure 4: Distribution of Lactic acid bacteria species isolated from fermented Mbithi different species

Five main clusters were found in the phylogenetic grouping of lactic acid bacteria that were isolated from fermented porridge 'Mbithi' and represent the genera *Lactobacillus*, *Levilactobacillus*, *Pediococcus*, *Weissella*, and *Leuconostoc*. In the cluster of *Lactobacillus Plantarum, twelve strains were found, for instance* strains FP205, FP207, FP208, FP214, FP216, FP220A, FP222, FP223, FP224, FP226, FP228, MFP205 (MH924302.1, MW674080.1, MW674306.1, MW674080.1, KJ690745.1, MT464412.1, MF992228.1, MW674080.1, MH899317.1, MG822862.1, OM866164.1, and OK189684.1), in the cluster associate with *Levilactobacillus brevis,* two strains were found, for instance, FP211A, and FP218 (OQ692119.1 and MG551203.1), strains FP210, FP220B, and FP227 clustered with *Pediococcus pentosaceus* (MN658812.1, PP916881.1, and PP917535.1). Strains FP102 constructed a separate cluster associated with *Weissella cibaria* (MN598068.1). The final cluster included strains such as FP103, and FP105 clustered with *Leuconostoc citreum* (MG754638.1 and PP916849.1) and the sub-cluster included strain FP104 clustered with *Leuconostoc lactis* (MK855341.1) (Figure 5).

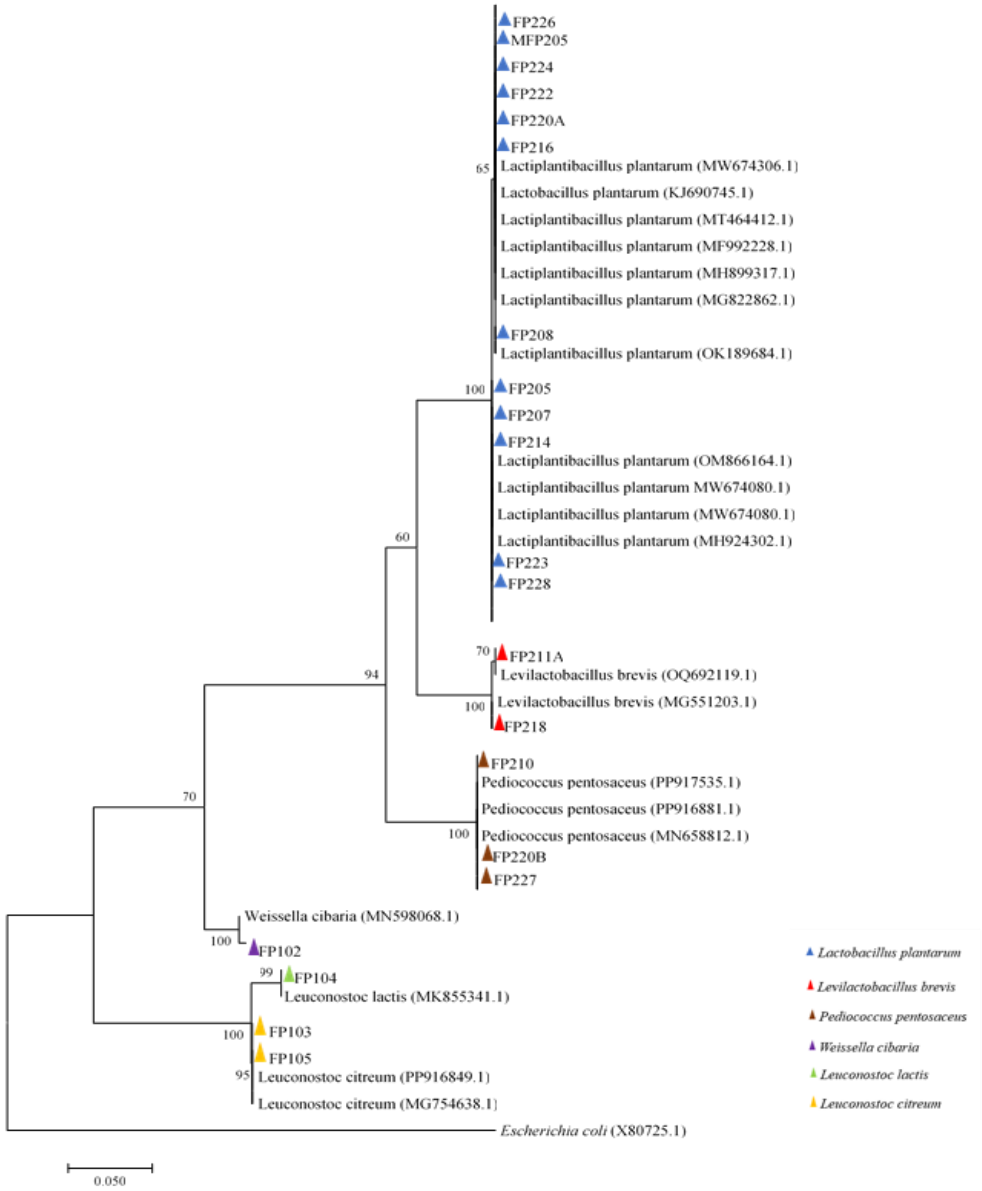

Figure 5: A phylogenetic tree based on 16S rRNA gene sequences shows the relationship between lactic acid bacteria isolated from Mbithi and representatives of other related taxa. The scale bar indicates 0.05 substitutions per nucleotide position.

The number beside the node is the statistical bootstrap value. In brackets are the GenBank accession numbers. The gene sequence of *E. coli* (X80725.1) was used as an out-group/control.

5. Discussion

Lactic acid bacteria (LAB) have been ingested by humans in traditional fermented food products for many decades due to their good health effects. LAB are Gram-positive, catalase-negative, non-motile bacteria that exhibit the production of antimicrobial peptides called bacteriocins as their defense mode of action [4]. Bacteriocins, a type of antimicrobial peptide, are gaining attention for their potential utility in food security by eliminating or inhibiting bacterial growth [3]. The use of metabolic products from LAB is widely acknowledged as safe [24]. In this study, LAB were isolated from Kenyan fermented porridge 'Mbithi', cell free supernatant CFS was extracted to investigate its activity against three foodborne pathogens *E. coli* (Gram-negative), *B. subtilis* (Gram-positive), *S. aureus* (Gram-positive) bacteria. 47 isolates were identified as LAB based on their morphological, biochemical, and physiological properties, which were classified as homofermentative and heterofermentative kinds in line with Mulaw [25]. The LAB isolates were tested for their growth under different conditions like temperature (10℃ and 45℃), pH (2.0 and 2.5), and their ability to grow in 6.5% of sodium chloride. Some of the LAB isolates were able to grow at 45℃ while no growth was observed at 10℃, and some of the LAB isolates grew to 6.5% NaCl. Similar results were reported by Sridharan [26], Com [27], and Abdelbasset & Djamila [28]. Some of the isolates were also able to grow at pH 2.0 and pH 2.5 similar to the findings reported by Mulaw [25].

The results showed that the household fermented porridge 'Mbithi' was controlled by four distinct groups of lactic acid bacteria (LAB) genera: *Lactobacillus* (*Lactiplantibacillus* and *Levilactobacillus*), *Pediococcus*, *Weissella* and *Leuconostoc* (Figure 2). Some previous studies have shown that *L. paracasei* has been isolated from Uji a non-alcoholic fermented porridge made from maize, sorghum, and millet from Kenya, Uganda, and Tanzania [7], [29], [30]. *Leuconostoc mesenteroides* has been isolated from Pito an alcoholic dark brown drink prepared from maize, sorghum, and millet made from Nigeria and Ghana, *L. plantarum*, and *Lb. brevis* have been isolated from Ogi an alcoholic beverage made from maize, millet, and sorghum made in Nigeria [6], and from Koko a fermented porridge prepared from maize from Ghana. *Lc. lactis subsp. Lactis* has been isolated from Mahewu prepared from maize from Zimbabwe [31]. *Pediococcus* and *Lb. casei* have been isolated from Busaa made from rice, millet, and maize from Nigeria and Ghana. *L. fermentum* has been isolated from Kenkey, a fermented mush prepared from maize from Ghana [30]. *Enterococcus* and *Weissella sp* have been isolated from Bushera, a Ugandan traditional fermented beverage made from sorghum and millet [7]. Among the genera isolated from household Mbithi, *Lactobacillus plantarum* is the dominant organism in the cereal-fermented products (Figure 4) which is similar to the findings of Muyanja [7] who reported similar results.

Fermentative lactic acid bacteria play an important role in the inherent qualities of fermented food products [32]. LAB natural isolates are an excellent source of novel antimicrobial molecules [33]. All the 21 lactic acid bacteria isolated from "Mbithi" fermented porridge were identified and investigated for their antimicrobial activity against indicator bacteria through agar well diffusion assay. All 21 isolates of LAB exhibited antimicrobial activity with varying diameters of the inhibition zone on selected indicator strains. Although LAB produces bacteriocin molecules, they have varying inhibitory effects on indicator bacteria. The actual antimicrobial activity of cell-free supernatant (CFS) from Mbithi's LAB was investigated after eliminating the effect that can be caused by organic acid and H_2O_2 produced by LAB. The activity of the organic acid of CFS was neutralized by the addition of 6.5% of sodium hydroxide as recommended by Amarantini [34] and catalase was used to break down hydrogen peroxide into water and oxygen as recommended by Narendranath [35]. One of the most relevant attributes of the LAB as a probiotic or starter culture is its antimicrobial activity [32]. Isolated LAB strains were screened for antimicrobial activities, inhibition zones ranged between 10.5 - 25.5 mm. LAB isolate FP224 exhibited strong antagonistic activity against *E. coli*

with an average inhibition zone of 21±1.41 mm, whereas FP216 exhibited strong antagonistic activity against *B. subtilis* with an average inhibition zone of 25.5±0.7 mm, while FP222 exhibited strong antagonistic activity against *S. aureus* with an average inhibition zone of 21±1.41 mm. As observed here, these results showed a good inhibition zone against *E. coli* and *S. aureus* which is higher than the study results from Jimma town, Southern Ethiopia by Goa [32] on fermented milk, who reported 12 ± 1.8 mm and 13.6±3.1 mm respectively as the maximum inhibitory zones, and compared to the study findings performed in Turkey by Erdoğmuş [36] who reported 16±0.9 mm, 19±1.2 mm and 18±0.9 mm as maximum inhibition zone of cell-free supernatant against *E. coli, B. subtilis,* and *S. aureus* respectively. Based on the mean inhibition zone produced by the LAB isolates, *Lactobacillus* (FP205, FP207, FP216, FP220A, FP222, FP223, FP224, FP226, FP228) exhibited the highest inhibition to the test indicator strains compared to *Pediococcus*, *Levilactobacillus*, *Leuconostoc,* and *Weissella* as shown in (Table 2). These results are similar to the study conducted in Ethiopia by Amenu [37] and Goa [32], who reported that *Lactobacillus* exhibited the highest inhibition zones against tested bacteria.

In the group of indicator strains tested, *Bacillus subtilis* was highly susceptible to the LAB isolates followed by *Staphylococcus aureus* and *Escherichia coli.* It was indicated that *B. subtilis* has a high sensitivity to LAB's antibacterial action, likely due to its effectiveness against related genera as indicated by Abdelbasset & Djamila [28] and Timothy [38].

Lactobacillus plantarum contributes to the flavor, texture, and preservation of natural fermentation processes of various food products. It also produces the functional properties of fermented foods by producing a variety of bioactive components, including exopolysaccharides, γ-aminobutyric acid, riboflavin, folic acid, and vitamin B12. Besides, *Lb. plantarum* is one of the most used LAB strains in food processing and preservation as a food preservative through the production of organic acid and various dominant bacteriocins (class I and II) [39]. It is also investigated to produce plantaricin [40]. *Pediococcus pentosaceus* not only produces lactic acid but also can contribute to other flavor compounds that increase the general sensory profile of fermented products, providing their properties of taste and aroma. *P. pentosaceus* known to produce pediocin as bacteriocin [41]. *Leuconostoc lactis*, *Leuconostoc citreum*, *Levilactobacillus brevis,* and *Weissella cibaria* also contribute to the flavor development of food products, and preservation through acidification [42], [43]. *L. brevis* is known for its contribution to the fermentation of beer [43]. The tested indicator bacteria, *E. coli*, *B. subtilis,* and *S. aureus*, were statistically different from each other in response to the antimicrobial effect by some LAB isolates with $P < 0.05$ based on the zones of inhibition.

6. Conclusion

Lactic acid bacteria (LAB) were isolated from traditional fermented porridge 'Mbithi'and identified. The isolates belonged to *Lactobacillus plantarum* followed by *Pediococcus pentosaceus*, *Levilactobacillus brevis*, *Leuconostoc citreum*, *Leuconostoc lactis*, and *Weissella cibaria* respectively. The LAB isolates showed antimicrobial effects against indicator strains such as *E. coli*, *B. subtilis,* and *S. aureus*. Lactobacillus isolates exhibited the highest antimicrobial activity against indicator bacteria. The LAB isolates showed potential for starter culture, probiotics, and bio-preservation. However, additional studies are suggested to ascertain the applicability of these isolates in the food industry as probiotics, starter cultures, and bio-preservative agents. Further analysis is needed to test the lactic acid bacteria isolates in controlled fermentation of Mbithi.

ACKNOWLEDGMENTS: The authors appreciate the Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI) and Jomo Kenyatta University of Agriculture and Technology Department of Food Science and Technology from where this work was done. The authors also appreciate Jennifer Wambugu for the laboratory assistance.

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