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Bio-preservative Activities of Partially Purified Bacteriocin Extracts of *Lactobacillus mindensis* TMW and *Lactobacillus tucceti* CECT 5920 Isolated from Nigerian Fermented Foods

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Authors' contributions

This work was carried out in collaboration between all authors. Author CNO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OKA and EN managed the analyses of the study. Author CNO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The preservative potentials of lactic acid bacteria (LAB) recovered from some Nigerian traditional fermented foods were assessed by inoculating 0.1 ml aliquots of suitable dilutions of the food samples on De Man Rogosa Sharpe (MRS) agar fortified with 50mg of nystatin. The isolates were assessed for their ability to produce bacteriocin using Agar Well Diffusion assay method. By using (GTG)5-PCR and 16s rDNA sequencing tools, two bacteriocin-producing LAB namely *Lactobacillus tucceti* CECT 5920 and *Lactobacillus mindensis* TMW were identified. Both LAB isolates had equal level of bacteriocin activity. Studies on inhibitory activity of partially purified bacteriocin extracts showed that temperature of 35°C had maximum effect on bacteriocin antimicrobial activity of *L. tucceti* CECT 5920 against *Staphylococcus aureus* NCTC 8325 and *Escherichia coli* 0157:H7, while pH had same effects on both LAB isolates against the test bacteria. Maximum tolerance of acidic medium was exhibited by both LAB isolates at pH 3-8. Bacteriocin inhibitory activity was best against both test bacteria at 0.2% concentration of NaCl. Combination of ginger with partially purified bacteriocin sample from both LAB isolates had reduction effect on both test pathogens for both Lactic acid bacteria isolates. When stored for 14 days, bacteriocin activity was optimum for the first day against both test pathogens, but decreased progressively with increased in storage time. The two LAB isolates had similar inhibitory activity against both test pathogens. *L. tucceti* CECT 5920 gave greater reduction result (90.03%) on *S.*

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aureus NCTC 8325 in fish sample and 78.07% against *E. coli* 0157:H7 in meat sample while *L mindensis* TMW had highest significant reduction effect (90.12%) in fish sample against *S. aureus* NCTC 8325 and 77.80% against *E. coli* 0157:H7 in fish sample. Both LAB isolates will perform well as preservative agents in food preservation as they showed production of bacteriocin which has antimicrobial activity and rapid acidification in growth medium.

Keywords: Bacteriocins; preservation; fermented foods; LAB; pathogens.

1. INTRODUCTION

Traditional food fermentation has been used as a food preservation method with increased shelf-life of foods, improved palatability, digestibility and enhanced nutritional value of food through the various fermentative activities of resident microorganisms in the foods especially Lactic acid bacteria (LAB) [1]. Traditional fermented foods have been reported to be kept from spoilage due to the biologically active substances produced mostly by LAB's fermentation wherein the LAB predominantly produces lactic acid and other compounds likes bacteriocins which have been reported to possess antimicrobial activity against other microorganisms [2]. The anti-microbial activity of bacteriocins produced by LAB has been reported in foods like dairy products, meats, barley, sourdough, red wine, fermented vegetables [2,3]. Hence, the LAB has also the ability to function as a natural food preservative [4,5]. The bacteriocins produced by LAB were found to inhibit food spoiling and disease-causing bacteria like S*taphylococcus aureus, Escherichia coli, Bacillus cereus, B. subtilis, Listeria monocytogenes* and *Clostridium perfringens* which organisms were found to resist traditional food preservation methods [6]. The desire in using LAB and their metabolic by-products as naturally occurring food preservative against food spoilage and increase the life span of our foods have increased in recent years [7].

Presently, several chemicals are in use as food additives in many processed foods. However, increase in the consumers' knowledge of the possible health challenges associated with some of these substances has led many scientists to examine the likelihood of using bacteriocins produced by LAB as natural food preservative. Using bacteriocins or the microbes that produce them is appealing to our food industries going by the increase in the demands of the consumers for natural products as well as the increased concern towards foodborne diseases. This has brought about the need to attempt using the biologically sourced antimicrobials produced by LAB and which are digested by our bodies without any side effect. This research targeted the use of traditional fermented foods as good sources of bacteriocins and lactic acid bacteria with bio-preservative potentials and evaluating their preservative potentials in foods.

1.1 Rationale

The solution to the health challenges posed by the use of chemical food additives is found in antimicrobial metabolites of fermentative microorganisms. Many antimicrobials have been used for some time now without causing any known health issue. Many of these naturally derived organic compounds which have aroused interest are bacterial metabolites produced in fermented foods. By this, it is strongly believed that microbial metabolites will eventually become the next line of food additives and the present attention in LAB as source of these metabolites is proper.

2. MATERIALS AND METHODS

2.1 Sample Collection and Analyses

Twenty samples each of traditional fermented food: pap (*akamu*), *ugba*, *kunu-zaki* and fermenting cassava were collected from the local producers in universal sterile bottles. The samples were packaged inside a cooler containing ice cubes and quickly transported to the laboratory. 1 g each of the food samples was homogenized in 0.1% peptone water, serially diluted and 0.1 ml aliquots of appropriate dilutions inoculated by streaking onto De Man Rogosa and Sharpe (MRS, Oxoid, England) agar medium fortified with 50mg of nystatin [8] for the isolation LAB. The plates prepared in triplicates were incubated at 35°C for 48 hrs anaerobically using anaerobic gas packs. The isolates were sub-cultured on MRS agar plates and stored in slants at 4°C [9].

2.2 Phenotypic and Biochemical Identification of Isolates

The cultures were identified through colonial, microscopic, biochemical, sugar fermentation

tests [9] and by (GTG)5-PCR and 16s rDNA sequencing for molecular identification.

2.3 Identification of Test Bacteria Pathogens

Cultures of *Staphylococcus aureus* NCTC 8325 and *Escherichia coli* 0157:H7 procured from the Veterinary Department of National Root Crops Research Institute, Umudike, Abia State were used as test isolates. They were sub-cultured onto appropriate media, gram stained and subjected to the necessary biochemical and sugar fermentation test to confirm their identity.

2.4 Screening for Bacteriocin Production and Activity

The ten LAB isolates recovered from the food samples were screened for bacteriocin production and antimicrobial activity by the Agar Well Diffusion (AWD) assay [10]. 10 ml of each of the LAB isolates was inoculated into 500 ml of MRS broth in a 1000 ml glass beaker. The broth was incubated at 37°C for 48 h to determine bacteriocin production and activity. The bacteriocin activity of the LAB was later assayed by aseptically collecting and centrifuging 100 ml of the culture broth at 10,000 rpm for 15 min so as to separate the microbial cells. The pH of the cell free supernatant (CFS) obtained was adjusted to 6.5-7.0 with 1N NaOH to neutralize the organic acids while the inhibitory activity of hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (c-100 bovine liver) [11].

The antimicrobial activity of the partially purified bacteriocin extract was tested by the Agar Well Diffusion (AWD) assay [10]. The CFS was standardized against 0.5 Mcfarland and the antimicrobial activity was tested against *S. aureus* NCTC 8325 and *E. coli* 0157:H7.

2.5 Determination of Titratable Acidity (T.A) of LAB Isolates

MRS broths of the LAB isolates were incubated for 24 hrs at 35°C. The T.A of the broths was determined according to [12] by titrating 25 ml of the MRS broth against 0.1M Sodium hydroxide (NaOH) using phenolphthalein as indicator until a pink colour appeared. Each ml of 0.1M NaOH was equivalent to 90.08 mg of lactic acid. Total titratable acidity (mg/ml) was determined thus:

$$\text{TTA} = \frac{\text{ml NaOH} \times \text{N NaOH} \times \text{M.E}}{\text{Volume of sample used}}$$

Where, ml NaOH = Volume of NaOH used,
N NaOH = Molarity of NaOH used,
M.E = Equivalent factor = 90.08mg

2.6 Effect of Different Parameters on Partially Purified Bacteriocin (PPB) Activity

2.6.1 Effect of temperature

The pH of 5 ml of CFS of the LAB was adjusted and the effect of the organic acids and hydrogen peroxide neutralized as stated earlier [11]. The CFS's in different test tubes were overlaid with paraffin oil to prevent evaporation and then heated for 10 minutes at 25, 30, 35, 40, 45, 50, 55 and 60°C respectively. The heat-treated culture supernatants were then assayed for antimicrobial activity against the test bacteria using well diffusion method as described earlier. The control was assayed without heat treatment.

2.6.2 Effect of pH

A 24 hr old CFS of the LAB isolates was adjusted to pH range of 2, 3, 4, 5, 6, 7, 8, 9 and 10 respectively using diluted 1M HCl and 1M NaOH solutions and was allowed to stand at room temperature for 2 h [13]. The inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (c-100 bovine liver) [11]. The residual bacteriocin activity of the CFS was then determined against the test bacteria by well diffusion method as previously described and then measuring the diameter of zone of inhibition.

2.6.3 Effect of NaCl

Various concentrations of NaCl (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% respectively) were added to MRS broths in different test tubes with distilled water as the control. They were sterilized by autoclaving at 121°C for 15 minutes. The test tubes were later inoculated with 1ml of the overnight broth of the LAB isolates and incubated at 37°C for 24 h [14]. The partially purified bacteriocin activity was later assayed using well diffusion method as described earlier.

2.6.4 Effect of storage time

The pH of 5 ml of CFS of the LAB was adjusted and the effect of the organic acids and hydrogen peroxide neutralized as stated earlier [11]. 200 ml of the CFS of LAB isolates (0.1%, v/v) were

then stored at 37°C and 5 ml of the CFS was tested for activity against the test bacteria every 24 h in triplicate for 14 days [15,16] using well diffusion as described earlier.

2.6.5 Effect of spices

The possible effects of three spices namely ginger, onion and garlic used as food preservatives on the PPB were tested [17]. Aqueous extracts of the spices were prepared by crushing coarsely 5 g each of the spices with mortar and pestle and boiling in 30 ml of distilled water for 10 min till 25 ml of the extract was obtained. All the extracts were individually passed through Whatman No 1 filter paper and then filtered through 0.45 µm Milipore filter (Milex, USA). The CFS of LAB culture was filter sterilized [11] and 0.1% vol of the spice extracts was added independently to the CFS's to give a final concentration of 1% (v/v) respectively and the medium was then inoculated with 1.0 x 10^6 CFU/ml (0.5 Mcfarland Turbidity Standard) of the test bacteria and in control (CFSs excluding the spices). The setup was incubated in triplicates for 24 h at 37°C. After the incubation, 1 ml of the medium was serially diluted and 0.1 ml aliquot of suitable dilution was inoculated on Mannitol salt Agar (for *S. aureus*) and McConkey Agar (for *E. coli*) for 24 h at 37°C and the mean microbial plate count in CFU/ML was determined. The mean reduction in microbial plate count of the test bacteria was calculated using the formula:

*Reduction of population (%) =

$$\frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

2.6.6 Antimicrobial activity testing of partially purified bacteriocin

The LAB isolates (1% v/v) were inoculated into MRS broth (Hi-Media, India), incubated for 18 h and the turbidity was matched with a 0.5 McFarland standard to give a concentration of 1.0 x 10^6 CFU/ml. The pH of the broth, the organic acids and the inhibitory activity of hydrogen peroxide were adjusted as stated earlier. The test bacteria were inoculated in 100 ml of peptone water and incubated for 18 h at 37°C and the turbidity was then matched against 0.5 Mcfarland Standard to obtain a concentration of 1.0 x10^6 CFU/ml. The test bacteria were then streaked evenly on the surface of Mueller-Hinton Agar (Oxoid, England) previously prepared using sterile swab sticks. 3 mm deep wells were made on the Mueller-Hinton agar using a sterile cork borer (4 mm wide) and the 10 µl of the lactic acid bacteria's CFS was aseptically placed into each agar well using sterile micropipette. The inoculated plates were kept at room temperature for 2 h and then incubated at 37°C for 24 h. The ability of the LAB to inhibit growth of the test bacteria was indicated by a zone of inhibition along its growth line [13].

2.6.7 Preservative effect of crude bacteriocin extract

This was determined using a modified method of [18]. 5g each of meat and fish samples collected from retailers were aseptically weighed and placed in five different sterile 100 ml beakers for the meat and another five sterile beakers for the fish samples. To each of the samples in separate beakers was added 10 ml (1% v/v) of *S. aureus* NCTC 8325 and *E. coli* 0157:H7 at 10^6 CFU/ml (determined with 0.5 McFarland Standard after 18 h incubation) and the initial counts of the test bacteria were recorded. Then, 5, 10, 20, 30, 40 and 50 ml of CFS of LAB isolates were added to the meat and fish samples in triplicates and the beakers incubated at 37°C for 24 h. The final microbial count was determined by spread plating on MRS agar after serial dilution and the values were compared with the control (without crude bacteriocin). The reduction in microbial plate count was determined using the formular:

*Reduction of population (%) =

$$\frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

2.7 Statistical Analyses

Data were expressed as mean± Standard deviation of triplicate means. Mean separation was done using Duncan Multiple range test using Statistical Package for Social Sciences (SPSS) version 20. Differences in statistical significance were considered at P =.05 and n=3.

3. RESULTS

3.1 Phenotypic and Biochemical Characterization of the Isolates from Various Fermented Foods

In Table 1, 65 LAB were isolated from twenty fermented food samples. They were considered

as LAB because they were Gram positive, catatlase negative, non-spore forming, non-motile and by sugar fermentation and they were grouped into five genera namely *Lactobacillus, Pseudochrobactrum, Leuconostoc*, *Lactococcus* and *Streptococcus* and ten species.

3.2 Diversities of Lactic Acid Bacteria Isolates

Table 2 shows the diversity of the ten LAB isolates with *ugba* giving seven isolates which is the highest while the lowest number of isolates (3) was from fermenting cassava. *L. mindensis* TMW was the most isolated LAB (38%) while *L. plantarum* occurred least (6.4%) in the four food samples.

3.3 Molecular Characterization of LAB

Plate 1 shows the Gel Electrophoretic result of the molecular weights of the four presumptive LAB. The samples are arranged from left to right: Lane 1 on the extreme left is the molecular maker (1000 bp) while L2, L3, L4 and L5 following are for *Pseudochrobactrum asaccharolyticum*, *Pseudochrobactrum saccharolyticum*, *Lactobacillus tucceti* CECT 5920 and *Lactobacillus mindensis* TMW.

3.4 Effects of Temperature on Partially Purified Bacteriocin Activity (mm)

Fig. 1a and b show the effect of temperature on partially purified bacteriocin activity. Highest effect was recorded at 35°C for both LAB isolates. There were losses in inhibitory activity for both LAB from 40°C. Beyond 45°C, inhibitory activity was completely lost by the two LAB isolates.

3.5 Effect of pH on Partially Purified Bacteriocin Activity (mm)

Fig. 2a and b show the effect of pH on partially purified bacteriocin activity. *L. tucceti* CECT 5920 had optimum inhibitory activity (12.06 and 11.81 mm) at pH 7 against *Escherichia coli* 0157:H7 and *Staphylococcus. aureus* NCTC 8325 individually. *L. mindensis* TMW had highest antimicrobial activity (12.81 mm) against *S. aureus* NCTC 8325 at pH 8 and 11.02 mm inhibitory activity on *E. coli* at pH of 7. Beyond pH 10, the two LAB did not exhibit any inhibitory activity against the test pathogens.

3.6 Effect of NaCl on Partially Purified Bacteriocin Activity (mm)

Fig. 3a shows that the inhibitory activity of the PPB was effective between 0.1-0.4% NaCl concentrations for both LAB. Beyond 0.4% NaCl concentration, no inhibitory activity was recorded. Maximum inhibitory activity of *L. tucceti* CECT 5920 and *L. mindensis* TMW against *S. aureus* NCTC 8325 was 11 mm and 10 mm respectively at 0.2% NaCl. In Fig. 3b, *L. tucceti* CECT 5920 and *L. mindensis* TMW had maximum inhibition (14 and 13 mm) respectively against *E. coli* 0157:H7 at 0.2% NaCl.

3.7 Effect of Storage Time on Partially Purified Bacteriocin Activity (mm)

Fig. 4a and b indicate a decrease in inhibitory activity pattern with increase in storage time of PPB of both LAB. The inhibitory activity of *L. tucceti* CECT 5920 was highest (19.01 mm) against *S. aureus* NCTC 8325 for the first three days of storage but dropped to 6.02 mm on the 14^{th} day of storage. *L. tucceti* CECT 5920 also had highest inhibition against *E. coli* 0157:H7 (18.05 mm) for the first 3 days of storage, but later dropped to 6.01 mm on the 14^{th} day. *L. mindensis* TMW had highest inhibition (18.02) for the first three days on both test pathogens, but dropped to 3.01 mm for *S. aureus* NCTC 8325 and 3.07 mm for *E. coli* 0157:H7 on the 14^{th} day. On the 14^{th} day of storage, the activity of *L. tucceti* CECT 5920 was about was one-sixth of the value on the first day of storage, while the inhibitory potential of *L. mindensis* TMW on the 14^{th} day was almost one-eight of its value on the first day of storage.

3.8 Effect of Spices on Partially Purified Bacteriocin Activity (%)

Table 3 shows that a combination of PPB of *L. tucceti* CECT 5920 and ginger spice at 0.1% concentration gave the best inhibitory activity result on *E. coli* 0157:H7 and *S. aureus* NCTC 8325. Similar result was observed for PPB of *L. mindensis* TMW.

3.9 Antimicrobial Activities of Partially Purified Bacteriocin (mm)

Table 4 shows that the antimicrobial activity of PPB of *L. tucceti* CECT 5920 was 19.00 and 18.00 mm respectively for *S. aureus* NCTC 8325 and *E. coli* 0157:H7 while *L. mindensis* TMW gave 18.00 mm against *S. aureus* NCTC 8325 and 17.00 mm against *E. coli* 0157:H7.

Table 1. Phenotypic and biochemical identification of lactic acid bacteria isolates

S/N	Colonial morphology	Gram reaction	Motility	Coagulase	Growth at 37°C	Growth at 45°C	4% NaCl	Fructose	Oxidase	Catalase	6.5% NaCl	Glucose	Sucrose	Maltose	Lactose	Mannitol	Galactose	Saccharose	Methyl red	Gas production	V. P	Isolate
1	Creamy-colored, flat, mucos-like	+rods	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	-	+	-	-	*Lactobacillus casei*
2	Round, milkish and mucoid	+rods	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	-	-	*Pseudochrobactrum asaccharolyticum*
3	Cream-like, mucoid	+cocci	-	-	+	-	-	+	-	-	-	+	+	+	+	+	+	-	+	-	-	*Pseudochrobactrum saccharolyticum*
4	Round, milkish, slimy	+cocci	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesenteroides*
5	Circular, creamy, convex	+rod	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	-	*Lactococcus plantarum*
6	Circular, milky, slimy	+cocci	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	*Streptococcus thermophilus*
7	Circular, milky, Mucoid	+rod	-	-	+	+	-	+	-	-	-	+	-	+	+	+	+	-	+	-	-	*Lactobacillus acidophilus*
8	Flat, milky, slimy	+rod	-	-	+	+	+	-	-	-	+	+	-	+	+	+	+	-	+	-	-	*Lactobacillus helveticus*
9	Circular, creamy, convex	+rod	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	-	-	*Lactobacillus tucceti* CECT 5920
10	Flat, membranous	+rod	-	-	+	+	-	+	-	-	-	+	+	-	+	+	+	-	+	-	-	*Lactobacillus mindensis* TMW

Positive reaction (+), Negative reaction (-)

Table 2. Diversities of lactic acid bacteria isolated from fermented foods

S/No	Fermented food	Lactic acid bacteria isolate	Number of species/percentage	Bacteriocin activity
1	Cassava	*Pseudochrobactrum saccharolyticum*	10(4.0)	+
		Lactobacillus acidophilus	5(2.0)	-
		Lactobacillus helveticus	8(3.2)	-
2	*Ugba*	*Lactobacillus casei*	18(7.2)	-
		Pseudochrobactrum asaccharolyticum	17(6.8)	+
		Leuconostoc mesenteroides	16(6.4)	-
		Lactococcus plantarum	8(3.2)	-
		Lactobacillus acidophilus	10(4)	-
		Lactobacillus mindensis TMW	20(8.0)	+++
		Lactobacillus tucceti CECT 5920	12(4.8)	+++
3	*Ogi*	*Pseudochrobactrum asaccharolyticum*	17(6.8)	+
		Lactococcus plantarum	8(3.2)	-
		Streptococcus thermophilus	9(3.6)	-
		Lactobacillus acidophilus	10(4.0)	-
		Lactobacillus tucceti CECT 5920	10(4.0)	+++
		Lactobacillus mindensis TMW	18(7.2)	+++
4	*Kunu-zaki*	*Pseudochrobactrum saccharolyticum*	10(4.0)	+
		Leuconostoc mesenteroides	16(6.4)	-
		Streptococcus thermophilus	9(3.6)	-
		Lactobacillus helveticus	20(8.0)	-

Key: (-) No inhibition; (+) Low inhibition; (+++) High inhibition

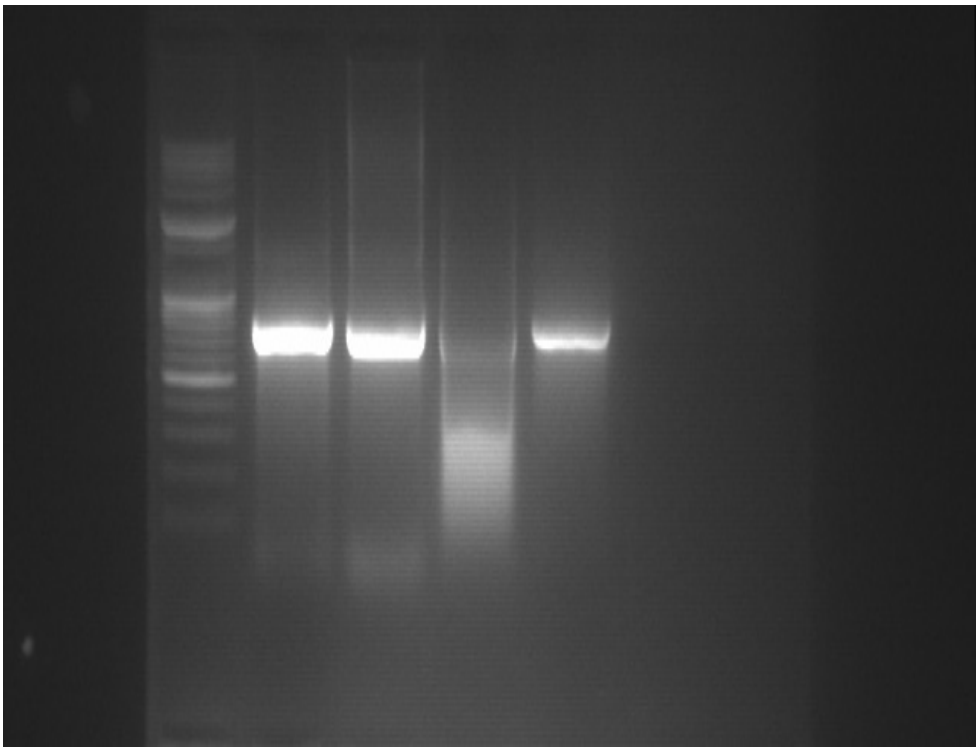

Plate 1. Electrophoretic bands of the four bacteriocin-producing LAB: L2, L3, L4 and L5

L2: *Pseudochrobactrum asaccharolyticum*

GCGTCATAACAGAACAGACACCCGCC
TTCGCCACTGGTGATCCTGCTATCTACGAATTTCACCGCTACACAG
GAATTCTACTTACCTCTATATTACTCAAGCTCTGCAGTATCCAAGGCACT
TTCCCGGTTGAGCTAGGAATTTCACTCTGACTTAAAAAACCGCCTACG
AACGCTTTACACCCAATAAATCCGGACAACGCTCGCATCCTACGTATTAC
CGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTTTTCGTAGAGTACCGTCA
AGACCCTAACCGTAGGGAGGATTCTTCTTGTACAAAAACAACTTAAATTC
CATAGCACGAACCCCTTGCGCGCGGCACGGCTGGGCCACAGTCGCCTCTG
TTGCCTAGTATAAGATTCTGCAGCGTCGCCTACGAGTCGGGTGCGGGTCT
CGTCACCAGCTGGGGGATCTAACTCCCCTGACCCGTAAGCATCGTTGCC
TTGGTATGGCGAGACCACCCCCGCTAATGATAAACATGCCGTC
ATACCGAGAAATGATTACATATATGCCATATCGATAAACCATGG
AGCATTAATACGAATTTCTTCAGGCTATTCCCCTGTATAAGGCAAGTTGC
AGACCCGTTACTCACCCGTGCGCCGGTCTCCAACAGCATGCTCATG

L3: *Pseudochrobactrum saccharolyticum*

TTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTAA
GCCGCCTTCGCCACTGGTGTTCCTGCGAATATCTACGAATTTCACCTCTA
CACTCGCAATTCCACTTACCTCTTCCATACTCAAGACTTCCAGTATCAAA
GGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGACTTAAAAGTCCG
CCTACGTGCGCTTTACGCCCAGTAAATCCGAACAACGCTAGCCCCCTTCG
TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTAC
CGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCA
CTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCA
CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGSTGA
TCATCCTCTCAGACCAGSTATGGATCGTCGCCTTGGTAGGCCTTTACCCTA
CCAACTAGGTAATCCAACATGGGCTCATCATTCTCCGATAAATCTTTCCCC
AAAAGGGCGTATACGGTATTAGCACAA

L4: *Lactobacillus tucceti* cect 5920

GCGTCAGTTACAGACCAGAAAGCCGCCTTCGCCACTGGTGTTCT
TCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTT
TCCTCTTCTGCACTCAAGTTTACCAGTTTCCGAAGCACTTCCTC
GGTTGAGCCGAGGGCTTTCACTTCAGACTTAAAAAACCGCCTAC
GTTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCCCTACG
TATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTGGT
TGAATACCGTCAATACGTGAACAGTTACTCTCACACATGTTCTT
CTTCAACAACAGAGTTTTACGAGCCGAAAACCTTCTTCACTCAC
GCGGCTGTGCTCCATCAGGCTTTCGTCCATTGTGGAAGATTCCG
TACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAA
TGTGGCCGATTACCCTCTCAGGTCGGCTACGTATCATTGCCTTG
GTGAGCCGTTACCTCACCAACTAGCTAATACNCCGCGGGTCCAT
CCNAAAGCGATAGCAGAACCATCT.

L5: *Lactobacillus mindensis* TMW

ACTACAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCA
GCGTCAGTAATGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCCTGC
GAATATCTACGAATTTCACCTCTACACTCGCAATTCCACTTACCTCT
TCCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC
CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACG
CCCAGTAAATCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTG
CTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCGTCATTATC

TTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACG
CGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCCCT

3.10 Preservative Activities of Partially Purified Bacteriocins (%)

Table 5 shows that *L. tucceti* CECT 5920 had more inhibition on *S. aureus* NCTC 8325 from meat (78.07%) than fish at 0.5% concentration while it had more inhibitory effect against *E. coli* 0157:H7 from meat (90.03%). *L. mindensis* TMW had more preservative effect on *S. aureus* NCTC 8325 in meat (77.80%) as well as against *E. coli* 0157:H7 in fish (90.12%). Comparatively, *L. tucceti* CECT 5920 had more inhibitory effect against *S. aureus* NCTC 8325 in fish (77.82%) than *L. mindensis* TMW and also against the same pathogen in meat (78.07%). The two LAB showed same level of antimicrobial activities on meat and fish samples against *E. coli* 0157:H7.

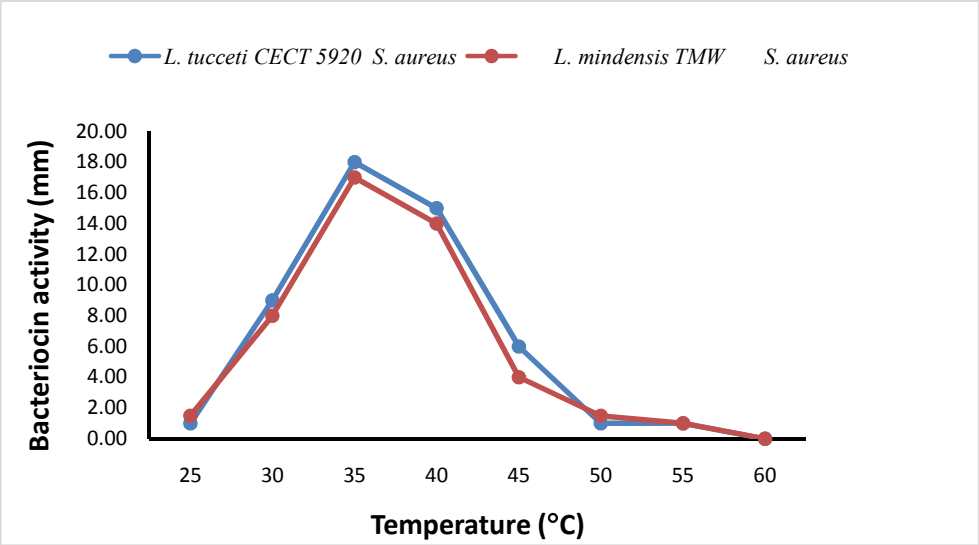

Fig. 1a. Effects of temperature on partially purified bacteriocin activity (mm)

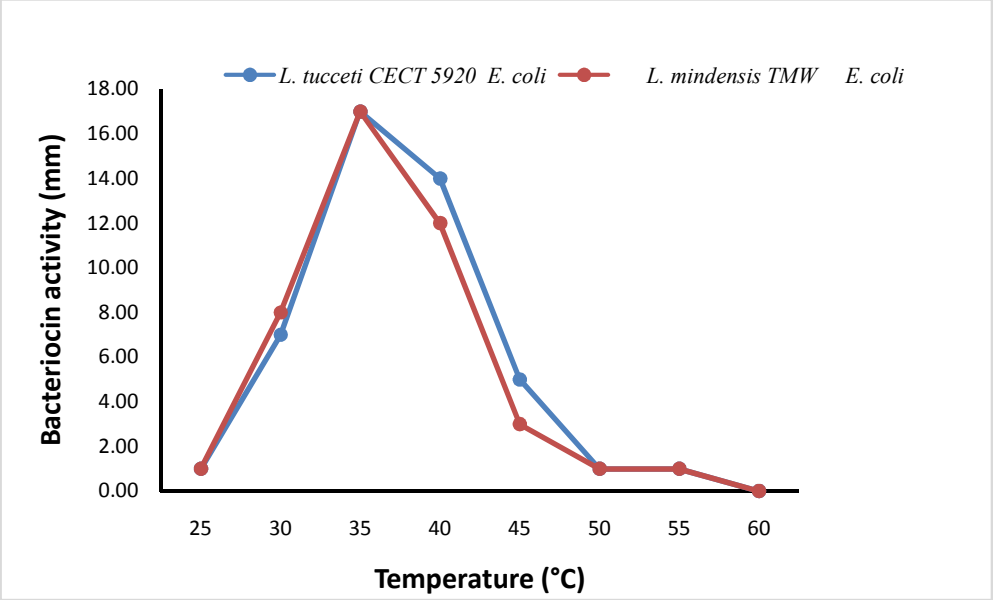

Fig. 1b. Effects of temperature on partially purified bacteriocin activity (mm)

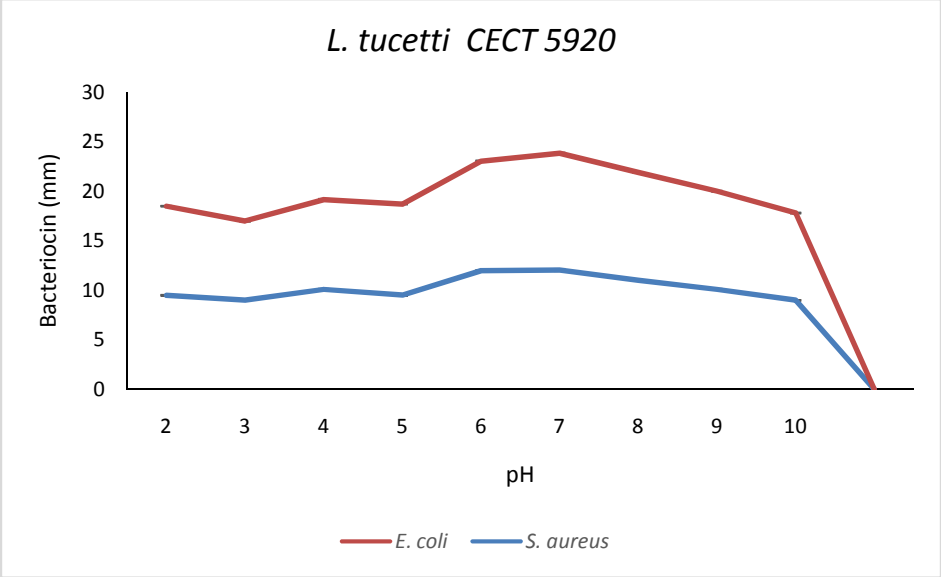

Fig. 2a. Effect of pH on partially purified bacteriocin activity (mm)

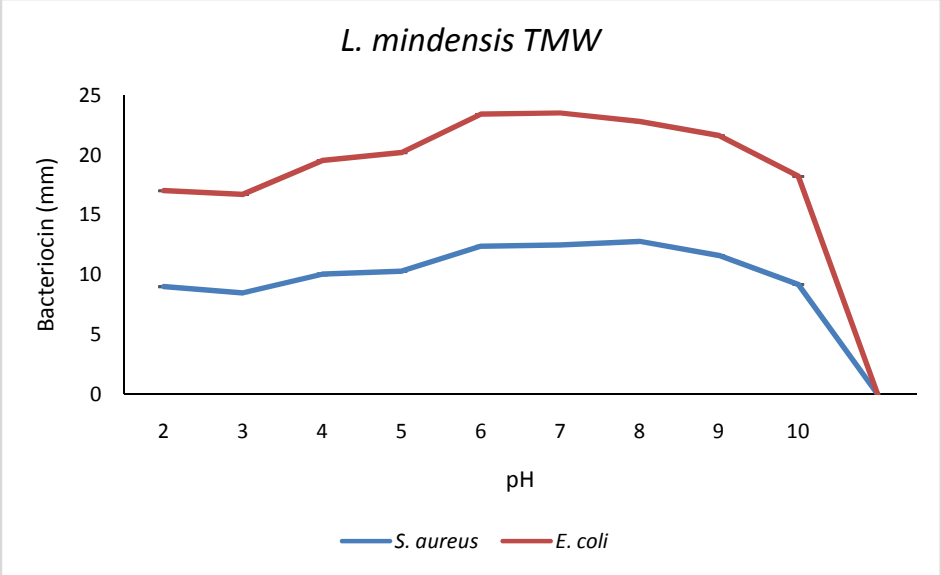

Fig. 2b. Effect of pH on partially purified bacteriocin activity (mm)

Table 3. Effect of spices on partially purified bacteriocin activity (%)

Spice	*L. tucceti* CECT 5920		*L. mindensis* TMW	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
Bacteriocin + Ginger	42.61±0.01	36.61±0.01	39.61±0.01	32.61±0.01
Bacteriocin + Onion	30.61±0.01	26.61±0.02	29.61±0.02	28.61±0.02
Bacteriocin + Garlic	9.80±0.00	30.61±0.01	13.80±0.01	20.61±0.01

Values are means of three replicates ± standard deviation (SD)

Table 4. Antimicrobial activities of partially purified bacteriocin (mm)

L. tucceti CECT 5920		*L. mindensis* TMW	
S. aureus	***E. coli***	***S. aureus***	***E. coli***
19.00±0.02	18.00±0.02	18.00±0.02	17.00±0.01
*Control	0	0	0

Values are means of three replicates ± standard deviation (SD).
**Control: MRS broths without Lactic acid bacteria isolates*

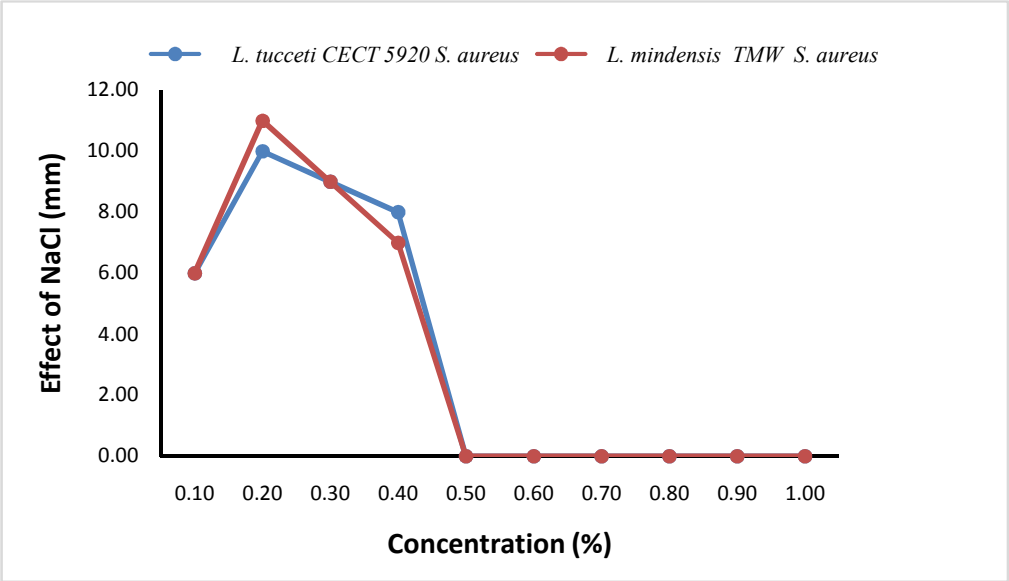

Fig. 3a. Effect of NaCl on partially purified bacteriocin activity (mm)

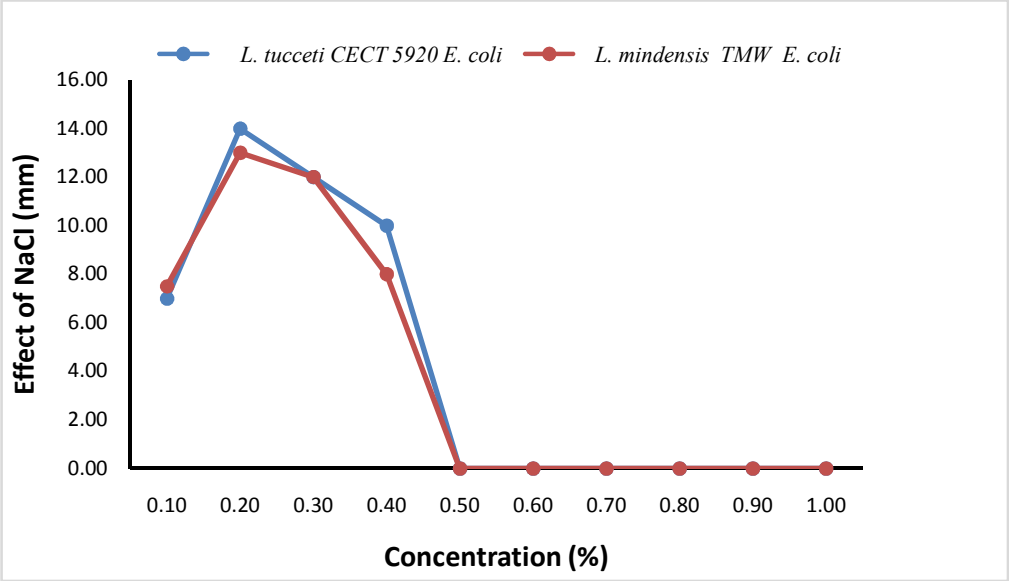

Fig. 3b. Effect of NaCl on partially purified bacteriocin activity (mm)

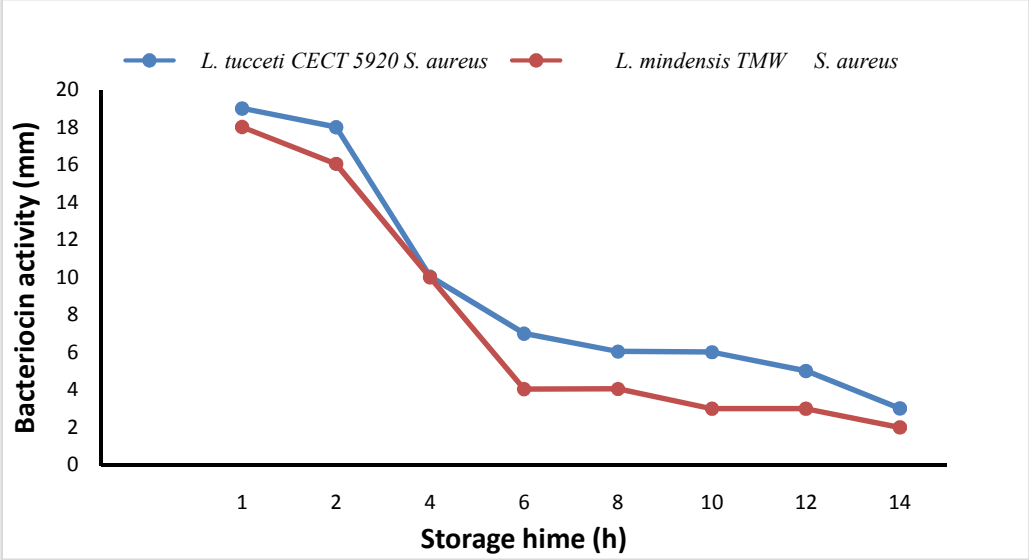

Fig. 4a. Effect of storage time on partially purified bacteriocin activity (mm)

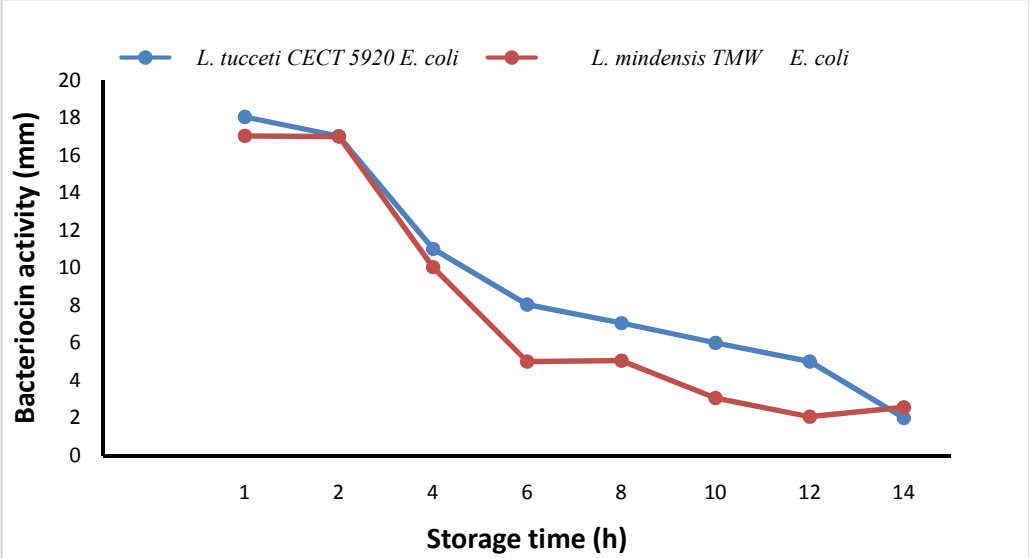

Fig. 4b. Effect of storage time on partially purified bacteriocin activity (mm)

4. DISCUSSION

In the present study, isolation and identification lactic acid bacteria that produce bacteriocin isolated from traditional fermented foods is aimed at developing a bio-preservative culture. The preliminary identification of isolated lactic acid bacteria was in agreement with previous studies on lactic acid bacteria preliminary identification [19]. Non-production of gas by the isolates suggests that they are homo-fermentative lactic acid bacteria.

Four of the five genera identified (*Lactobacillus, Leuconostoc*, *Lactococcus* and *Streptococcus)* have been isolated from traditional fermented foods in the past [20,21] by morphological and biochemical characterization. However, *Lactobacillus tucceti* CECT 5920, *L. mindensis* TMW, *Pseudochrobactrum asaccharolyticum* and *P. saccharolyticum* (Table 1) to the best of our knowledge have not been reported before now as LAB isolated either from Nigerian traditional fermented foods or fermented foods from any other part of the world. This result

might be due to the type of fermented foods used.

The highest diversity of isolates showed by *L. mindensis* TMW in all the traditional fermented food samples compared with other lactic acid bacteria isolates indicates that these isolates have become ecologically and physiologically adapted to the fermentation of these food samples. *Ugba* having the highest diversity of lactic acid bacteria could be due to its high proteinous content which supported the greatest number of lactic acid bacteria compared with the other traditional fermented foods sampled. However, contrary to this finding is the report of [22] which showed that *Pediococcus acidilactici, L. plantarum, L. fermentum, L. lactis* and *Leuconostoc mesenteroides* as the most diverse LAB to be isolated from traditionally fermented maize gruels from five different western states of Nigeria respectively.

Some factors might have influenced the low number of bacteriocin-producing LAB isolated such as the culture medium, incubation conditions and genetic make-up of the isolate, or the sensitivity methods used in determining the antimicrobial activity of the isolates. Wang et al. [23] reported that cell aggregation and medium composition can affect bacteriocin production.

The presence of one band on each of the four ladders indicates that the DNA strands of the four isolates were cut once with suitable restriction endonucleases. Agaliya and Jeevaratnam [24] reported the isolation of *L. pentosus* having 260 bp using 16S rRNA analysis and this result is close to *L. tucceti* CECT 5920 isolate that has 250 bp. Their result also showed that *L. plantarum* with subspecies unidentified, *L. plantarum* subsp*. argentoratensis. L. plantarum* with subspecies unidentified and *Lactobacillus plantarum* subsp*. plantarum* had about the same molecular weight of 550 bp and this result is close to *P. asaccharolyticum*, *P. saccharolyticum* and *L.mindensis* TMW that have molecular weight of about 520 bp. *L. mindensis* TMW, *P. saccharolyticum, L. tucceti* CECT 5920 and *P. asaccharolyticum* have not been reported in all the literatures within my reach as part of lactic acid bacteria flora encountered in traditional fermented foods.

Bacteriocins which are small peptides have antimicrobial effects against bacteria of the same or closely related species and sometimes against a wide spectrum of species [25]. Bacteriocin-producing microorganisms have immunity mechanisms to present self-protection [26]. Bacteriocin production is an important attribute of LAB as suitable bio-preservative as bacteriocin production in traditional fermented foods will confer preservative status on the fermented foods. By this, pathogenic and food spoilage organisms will be eliminated from the food thus increasing the shelf life and safety of the food [27]. Generally, the production of bacteriocin is affected greatly influenced by temperature, medium constituents, pH, incubation time and other environmental factors [28].

The LAB isolates exhibited mesophilic inhibitory activities and such temperature of activity of the isolates indicated that they could only be useful in food preservation at temperature range that the isolates can tolerate. This however will be a demerit in food industries that operate at higher temperatures. A doubling in activity was observed between 30-35^{o}C suggesting that increase in temperature positively affected the bacteriocin activity. However, temperature above 35^{o}C could have denatured the 3-Dimensional pattern of the bacteriocins since they are proteins. Two bacteriocins that were thermally stable up to 100°C for 15 mins have been reported Djadouni and Kihal [29]. The ecological conditions prevailing in traditional fermented foods where the two LAB were isolated could have contributed to this finding. The isolates may have adapted to the mesophilic temperature range found in fermented foods. Meera and Devi [30] reported significant decrease in bacteriocin production with increase in temperature and this agrees with the findings in this study. Growth temperature plays important role in bacteriocin activity.

Both isolates had wide range of pH tolerance and activity since the isolates inhibited the test pathogens at pH of 3-10. This shows that the isolates possess acidic and slightly alkaline activities. Both isolates showed optimum inhibitions at or near neutral pH and will probably be well suited in traditional fermentation of several foods as starter cultures. Djadouni and Kihal [29] had earlier been reported that some bacteriocins remained active at pH 2-6 and this agrees with the result here. Djadouni and Kihal [29] also reported that microbial cells are affected significantly by the pH of their growth environment as they apparently lack mechanisms to adjust their cellular pH.

Table 5. Preservative effects of partially purified bacteriocin (%)

Bacteriocin (%)	*L. tucceti* CECT 5920				*L. mindensis* TMW			
	S. aureus		*E. coli*		*S. aureus*		*E. coli*	
	Meat	**Fish**	**Meat**	**Fish**	**Meat**	**Fish**	**Meat**	**Fish**
5	10.61±0.01	11.26±0.09	14.04±0.05	9.51±0.01	9.80±0.00	10.02±0.02	14.06±0.09	9.21±0.01
10	33.22±0.02	31.28±0.11	37.05±0.06	15.06±0.08	30.61±0.01	30.05±0.07	37.03±0.04	15.61±0.01
20	46.81±0.01	48.85±0.06	50.62±0.02	27.31±0.01	46.61±0.01	44.05±0.06	52.05±0.07	27.02±0.03
30	64.56±0.08	64.09±0.12	71.92±0.03	54.05±0.07	65.02±0.03	65.62±0.02	73.00±0.01	54.51±0.01
40	72.02±0.03	68.12±0.16	84.47±0.09	59.09±0.12	72.81±0.01	71.11±0.02	83.06±0.08	59.81±0.01
50	77.82±0.03	78.07±0.09	90.03±0.04	61.05±0.06	69.12±0.02	77.80±0.01	90.12±0.03	61.22±0.02

Values are means of three replicates
± Standard deviation (SD)

$$\text{Preservative effects (\%)} = \frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

It has been suggested that inorganic salts have a synergistic effect on bacteriocin efficacy when added with specific concentrations in foods [29]. Also, Bacterial metabolism is sensitive to salt, because salt exhibits specific ionic and water binding properties [31]. Mahrous et al. [32] reported that in MRS broth, 2% NaCl increased the activity of bacteriocins isolated from *Lactobacillus pentosus* CH2 against *E. coli* ATCC 25922 (14 mm zone of inhibition) while *L. fermentum* M1 had 15 mm zone of inhibition against *Bacillus subtilis* NCIB3610.

The higher activity shown against *E. coli* 0157:H7 (a gram negative bacterium) by the two Lactic acid bacteria isolates could be attributed to the nature of Gram negative cell wall which seems to be more susceptible to salt than gram positive cell wall possibly due to the thicker peptidoglycan nature of the later. Detrimental effects of high concentrations of NaCl have been reported by Verluyten et al. [33].

Storage time affected bacteriocin's inhibitory activity with highest activity shown on the first and second days of storage. After this, there was gradual reduction in the antimicrobial activity as storage time increased for both Lactic acid bacteria isolates. However, bacteriocin activity was recorded over the 14 days of storage against the two test isolates. So, this attribute can prolong the shelf life of the fermented food. The inhibitory activity of *L. tucceti* CECT 5920 on the 14^{th} day of storage was about was one-sixth when compared with the first day of storage, but it was almost one-eight while for *L. mindensis* TMW. This indicated that there was a breakdown in the chemical structure of the bacteriocins with time, thus, traditional fermented foods will not stay long without spoilage unless they are preserved in the refrigerator.

Assessment of effect of spices showed that ginger had the highest percentage reduction of *S. aureus* NCTC 8325 against *S. aureus* NCTC 8325 by *L. tucceti* CECT 5920 at 0.1% spice concentration. With the bacteriocin of *L. mindensis* TMW at 0.1% concentration of the spices, the highest percentage reduction of *S. aureus* NCTC 8325 (39.61) was also from ginger. The same spice also reduced the concentration of *E. coli* 0157:H7 (32.61). Ginger compounds are active against specific type of bacteria that cause diarrhoea which is a leading cause of death in infant in developing countries. It has been reported that the main ingredients of ginger like volatile oil, gingerol, shogaol and diarylheptanoids work as antioxidant, anti-inflammatory, anti-lipid, anti-diabetic, analgesic, antipyretic and anti-tumor [34]. Azu and Onyeagba [35] reported that ginger has antimicrobial activity against *E coli*, *Salmonella typhi* and *Bacillus subtilis* and its ethanolic extract showed widest zone of inhibition against *Salmonella typhi* and this is similar to our result.

Antimicrobial activities indicated that the bacteriocins produced by the isolates gave wide range of activity against gram positive and gram negative bacteria. Adesokan et al. [36] reported similar results against *S. aureus, Pseudomonas aeruginosa* and *E. coli.* The inhibitory effect recorded is assumed to be due to bacteriocin and not H_2O_2 since there was no oxidizing effect on the lactic acid bacterial cells which will destroy the basic molecular structure of the lactic acid bacteria cell proteins because the CFS tested was treated with NaOH and catalase to neutralize the possible oxidizing effects of organic acids and H_2O_2 respectively. Lactic acid bacteria can stop the growths of pathogenic and spoilage bacteria and therefore can be used to extend the shelf life of foods. According to Ten et al. [37] their inhibitory abilities could be due to the low pH, organic acids and other primary and secondary inhibitory metabolites.

The removal of the test pathogens from the samples was in linear pattern with the concentrations of the bacteriocins (5-50%). At 50% concentration, both LAB isolates showed the highest preservative effect from both meat and fish against both test bacteria. This was not so with the control. This result showed that the lactic acid bacteria isolates have the potential of being used as starter culture as their metabolite will confer preservative status on traditional fermented foods. Joshi et al. [13] found that partially purified bacteriocin inhibited *B. cereus* while the inhibitory effects in wine, juice and pulp was higher when the concentration of the bacteriocin increased. Result showed that bacteriocins have the antimicrobial potential suitable for a bio-preservative agent.

5. CONCLUSION AND RECOMMENDA-TION

The studies on the bio-preservative abilities of the two LAB isolated from traditional fermented foods showed that the two isolates produced bacteriocins that gave inhibitory effects against test pathogens as well as rapid acidification in

growth medium. The interest in bacteriocin production by LAB is motivated by their potential applications as substitutes to chemicals as food preservatives. The isolates performed well in inhibition of pathogenic strains, stability over wide range of pH, heat and salt tolerance. By this, they possess promising agents in food preservation. Both isolates would make good candidates in future works as regards the use of LAB metabolites as better substitutes in food preservation.

The authors recommends that the government and cooperate bodies put interests in researches involving the preservative abilities of LAB by providing financial aids to the researchers.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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