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Preservative activities of partially purified bacteriocin extracts of *Lactobacillus mindensis* TMW and *Lactobacillus tucceti* CECT 5920 isolated from Nigerian fermented foods

ABSTRACT

Preservative potentials of lactic acid bacteria (LAB) isolated from traditional fermented foods were evaluated by inoculating 0.1ml aliquots of appropriate dilution of the samples on De Man Rogosa Sharpe (MRS) agar fortified with 50mg of nystatin for the isolation of LAB. The LAB isolates were screened for bacteriocin production by the Agar Well Diffusion assay and two bacteriocin producers identified by (GTG)5-PCR and rDNA sequencing as *Lactobacillus tucceti* CECT 5920 and *Lactobacillus mindensis* TMW. 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 positive effect on bacteriocin activity of *L. tucceti* CECT 5920 against *S. aureus* and *E. coli,* 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 had higher reduction effect (90.03 %) on *S. aureus* in fish sample and 78.07 % against *E. coli* in meat sample while *L mindensis* TMW had highest significant reduction effect (90.12 %) in fish sample against *S. aureus* and 77.80 % against *E. coli* 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.

Key words: Bacteriocins, preservation, fermented foods, LAB, pathogens.

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1.0 Introduction

Traditional food fermentation is a food preservation method intended to extend shelf-life, improve palatability, digestibility and the nutritive value of food by the activities of naturally occurring microorganisms especially Lactic acid bacteria (LAB). Fermentation of various foods by lactic acid bacteria (LAB) is one of the oldest forms of bio-preservation practiced by mankind (Zeuthen, 1995). Traditional fermented foods have been preserved by bioactive substances produced mainly by lactic acid fermentation in which lactic acid bacteria (LAB) predominantly produce lactic acid and other compounds such as bacteriocins. These have antimicrobial activity against other groups of microorganisms.

Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. The antimicrobial activity of bacteriocins produced by LAB has been detected in foods such as dairy products, meats, barley, sourdough, red wine, fermented vegetables (Yildirim and Johnson, 1998; Bromberg *et al.,* 2004). Therefore, the strains of lactic acid bacteria have also potential to act as a bio-preservative or natural food preservative (Klaenhammer, 1998; Luchansky, 1999). The bacteriocins produced inhibited food spoilage and pathogenic bacteria such as S*taphylococcus aureus, Escherichia coli, Bacillus cereus, B. subtilis, Listeria monocytogenes* and *Clostridium perfringens* which are recalcitrant to traditional food preservation method (Bizani and Brandelli, 2002). The interest in the application of LAB and their metabolites in the prevention of the food spoilage and the extension of the shelf life of foods have increased during the last decade (Stiles, 1996).

Currently, different chemical food additives are used in many processed foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as bio-preservatives. The use of bacteriocins or the microorganisms that produce them is attractive to the food industry in the face of increasing consumer demand for natural products and the growing concern about foodborne diseases. It has also necessitated the need to exploit the biologically derived antimicrobial substances produced by LAB which are digested by the human body without any side effect.

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Thus, in this research work, traditional fermented foods were used as good sources of bacteriocins and lactic acid that could be of bio-preservative effects and evaluating their bio-preservative potentials.

RATIONALE

The remedy to the problem presented by chemically based food additives is the use of antimicrobial metabolites of fermentative microorganisms. Many antimicrobial substances have been in use for some time now without any known adverse effect. Many of these organic compounds which have stirred interest are bacterial metabolites used to produce or associated with fermented foods. Hence, it is strongly believed that microbial metabolites will become the next generation of food additives and the current interest in LAB is a step in the right direction.

2.0 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 for analyses. 1g each of the food samples was homogenized in 0.1% peptone water, serially diluted and 0.1ml aliquots of appropriate dilutions inoculated by streaking onto De Man Rogosa and Sharpe (MRS, Oxoid, England) agar medium fortified with 50mg of nystatin (Roissart and Luguet, 1994) for the isolation of lactic acid bacteria (LAB). The plates prepared in triplicates were incubated at 35°C for 48 hrs anaerobically (using anaerobic gas packs) for isolation of mesophilic LAB. The mixed isolates were sub-cultured on MRS agar plates and the pure cultures were on MRS agar slants and stored in refrigerator at 4°C. All the isolates recovered were maintained by ~~by~~ weekly sub-culturing on MRS agar for 48hrs (Cheesbrough, 2004).

2.2 Phenotypic and biochemical identification of isolates

The cultures were identified by observing the colonial morphologies, microscopy, biochemical, sugar fermentation tests (Cheesbrough, 2004) and by (GTG)5-PCR and rDNA sequencing for molecular identification.

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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 LAB isolates recovered from the fermented food samples following the phenotypic characterization were screened for bacteriocin production by growing them in MRS broth for 24 hrs at 35°C. The antimicrobial activity of the partially purified bacteriocin extract was tested by the Agar Well Diffusion (AWD) assay (Lasta *et al.*, 2008). 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) (Daba *et al.*, 1991). 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 A.O.A.C, (1990) by titrating 25ml 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 90.08mg of lactic acid. Total titratable acidity (mg/ml) was determined thus:

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

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

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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 (Daba *et al.*, 1991). 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 (Joshi *et al.,* 2006). The inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (c-100 bovine liver) (Daba *et al.*, 1991). 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 ^{o}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 (Meera and Devi, 2012). 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 (Daba *et al.*, 1991). 200 ml of the CFS of LAB isolates (0.1 %, v/v) were then stored at 37 ^{o}C and 5 ml of the CFS was tested for activity against the test bacteria every 24 h in triplicate for 14 days (Ogunbanwo *et al.,* 2003; Rajaram *et al.,* 2010) 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 (Vaishnavi *et al.,* 2007). Aqueous extracts of the spices were prepared by

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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 and the pH adjusted and the organic acids and the inhibitory activity of hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (Daba *et al.*, 1991). 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) and incubated in triplicates for 24 h at 37 ^oC. 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 ^oC 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:

$$*\text{Reduction of population (\%)} = \frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

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 ^oC 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 seeded aseptically on the surface of Mueller-Hinton Agar (Oxoid, England) previously prepared using sterile swab sticks. Three 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 Lactic acid bacteria isolates to inhibit growth of the test bacteria was indicated by a zone of inhibition along its growth line (Joshi *et al.,* 2006).

2.6.7 Preservative effect of crude bacteriocin extract

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This was determined using a modified method of Udhayashree *et al.* (2012) 5 g 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 were 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 Lactic acid bacteria isolates which was adjusted to 6.5-7.0 with 1N NaOH to neutralize the organic acids and the inhibitory activity of hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (c-100 bovine liver) were added to the meat and fish samples in triplicates and the beakers were incubated at 37 °C for 24 h. The final microbial cell count was determined as earlier described and the values were compared with the control (without crude bacteriocin). The reduction in microbial plate count was determined using the formular:

$$\text{*Reduction of population (\%)} = \frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

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 $P \leq 0.05$ and n=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, Leuconosto*c, *Lactococcus* and *Streptococcus* and ten species.

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3.2 Diversity of lactic acid bacteria isolates

Table 2 shows the diversity of the ten LAB isolates with the highest number of isolates (7) from *ugba* 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 identification of lactic acid bacteria isolates

Plate 1 shows the Gel Electrophoretic rresult 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 Effect of temperature on partially purified bacteriocin activity (mm)

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

3.5 Effect of pH on partially purified bacteriocin activity (mm)

Figure 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 *S. aureus* NCTC 8325 and *E. coli* 0157:H7 respectively. *L. mindensis* TMW had highest antimicrobial activity (12.81 mm) against *S. aureus* NCTC 8325 at pH 8 and 11.02 mm inhibitory activity against *E. coli* 0157:H7 at pH 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)

Figure 3a and b show that NaCl did not inhibit bacteriocin activity between 0.1-0.4 % concentrations in the broth for both LAB. Maximum inhibitory activity was 0.2 % concentration

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for both LAB. *L. tucceti* CECT 5920 had maximum inhibition of 10 mm against *S. aureus* NCTC 8325 and 14 mm against *E. coli* 0157:H7 while *L. mindensis* had maximum inhibitory activity of 11 mm against *S. aureus* NCTC 8325 and 13 mm against *E. coli* 0157:H7. Beyond 0.4 % NaCl concentration, no inhibitory activity was recorded.

3.7 Effect of storage time on partially purified bacteriocin activity (mm)

Figures 3a and b show a general decrease pattern in inhibitory activity 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 14th day of storage. *L. tucceti* CECT 5920 also had highest inhibition against *E. coli* 0157:H7 (18.05) for the first 3 days of storage, but later dropped to 6.01 mm on the 14th day. *L. mindensis* TMW had highest inhibition (18.02) for the first three days on both test pathogens, but dropped to 3.01 mm and 3.07 mm for *S. aureus* NCTC 8325 and *E. coli* 0157:H7 respectively on the 14th day. The inhibitory activity of *L. tucceti* CECT 5920 on the 14th day of storage was about was one-sixth compared with the value on the first day of storage, while the inhibitory potential of *L. mindensis* TMW on the 14th 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 against *S. aureus* NCTC 8325 and *E. coli* 0157:H7. The similar result was observed for PPB of *L. mindensis* TMW.

3.9 Antimicrobial activity 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 rrspectively for *S. aureus* NCTC 8325 and *E. coli* 0157:H7 while *L. mindensis* TMW gave 18.00 and 17.00 mm of inhibition respectively against *S. aureus* NCTC 8325 and *E. coli* 0157:H7.

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3.10 Preservative activities of partially purified bacteriocins (%)

Table 5 shows that *L. tucceti* CECT 5920 had more inhibitory effect against *S. aureus* NCTC 8325 from meat (78.07 %) than fish at 0.5 % concentration while it has more inhibitory effect against *E. coli* 0157:H7 from meat (90.03 %). *L. mindensis* TMW had more preservative activity against *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.

280 **Table 1: Phenotypic and biochemical identification of lactic acid bacteria isolates**

S/N	Colonial Morphology	Gram reaction	Catalase	Motility	Coagulase	Growth at 37°C	Growth at 45°C	4% NaCl	6.5% NaCl	Glucose	Sucrose	Maltose	Fructose	Lactose	Mannitol	Galactose	Saccharose	Oxidase	Methyl red	Gas production	V. P	Isolate
1	Flat, creamy Mucoid	+rods	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	-	+	-	-	*Lactobacillus casei*
2	Circular, milky, Mucoid	+rods	-	-	-	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	-	*Pseudochrobactrum asaccharolyticum*
3	Creamy, Mucoid	+cocci	-	-	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	-	-	*Pseudochrobactrum saccharolyticum*
4	Circular, milky, 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

281 Positive reaction (+), Negative reaction (-)

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Table 2: Diversity 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 tucceti CECT 5920	12(4.8)	+++
		Lactobacillus mindensis TMW	20(8.0)	+++
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.

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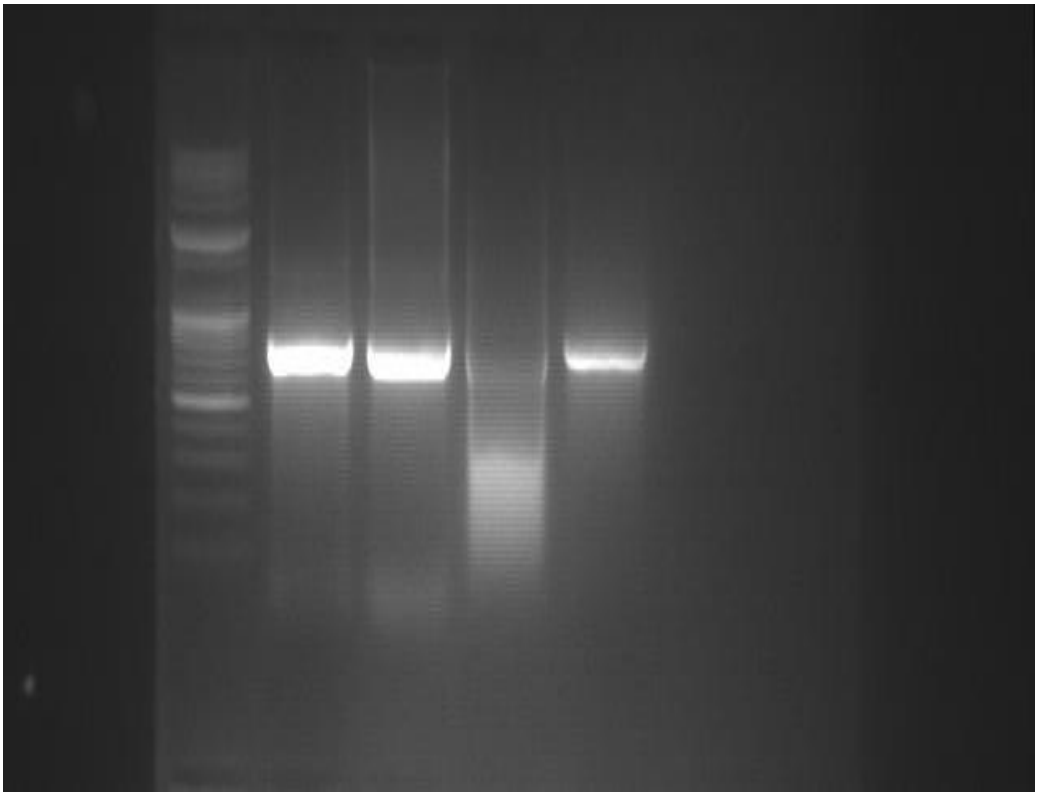

Plate 1**:** Electrophoretic bands of the four bacteriocin-producing LAB.

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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

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GCGTCAGTAATGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCCTGC
GAATATCTACGAATTTCACCTCTACACTCGCAATTCCACTTACCTCT
TCCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC
CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACG
CCCAGTAAATCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTG
CTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCGTCATTATC
TTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACG
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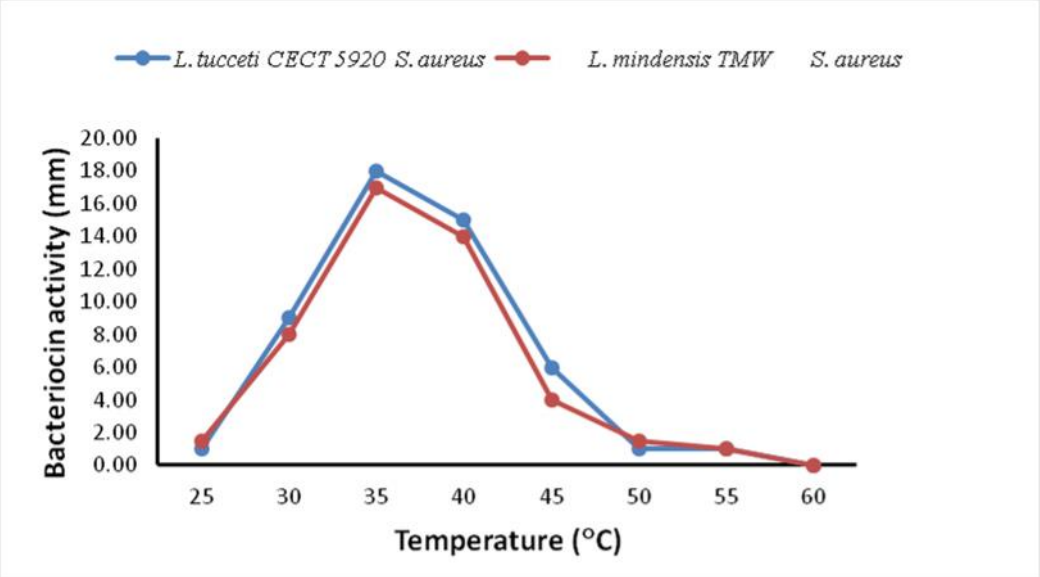

Fig. 1a: Effect of temperature on partially purified bacteriocin activity (mm)

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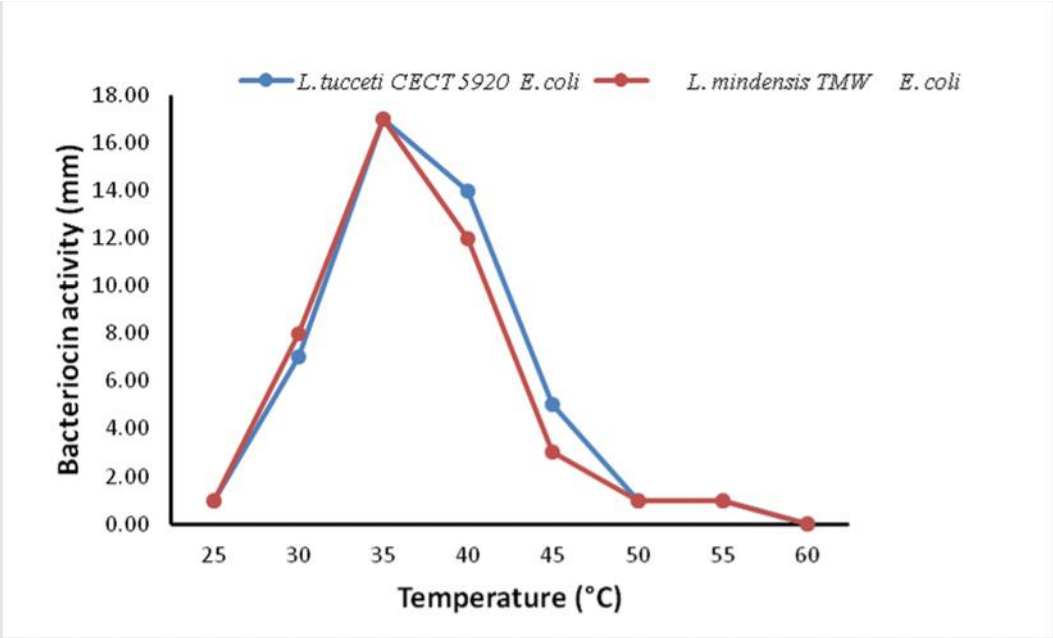

Fig. 1b: Effect of temperature on partially purified bacteriocin activity (mm)

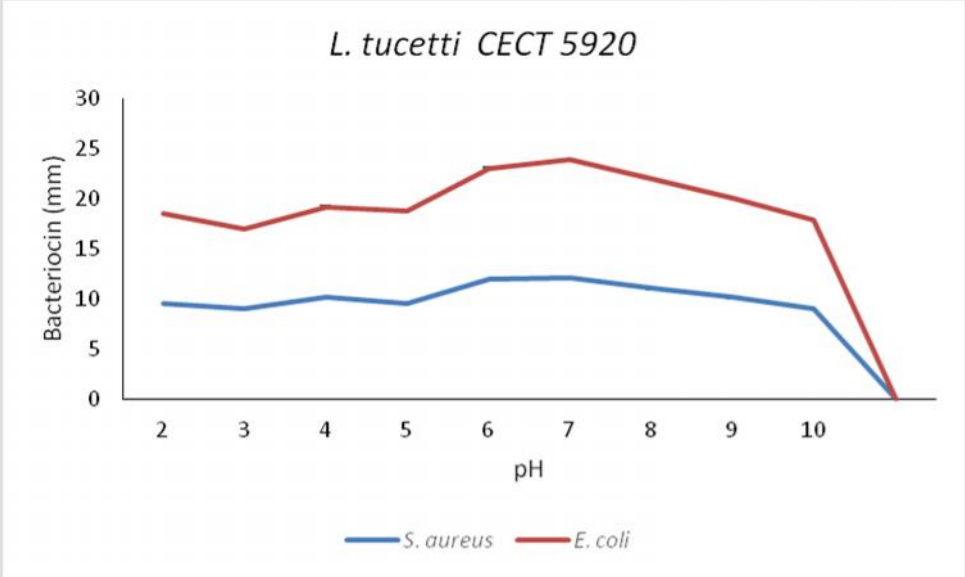

Fig. 2a: Effect of pH on partially purified bacteriocin activity (mm)

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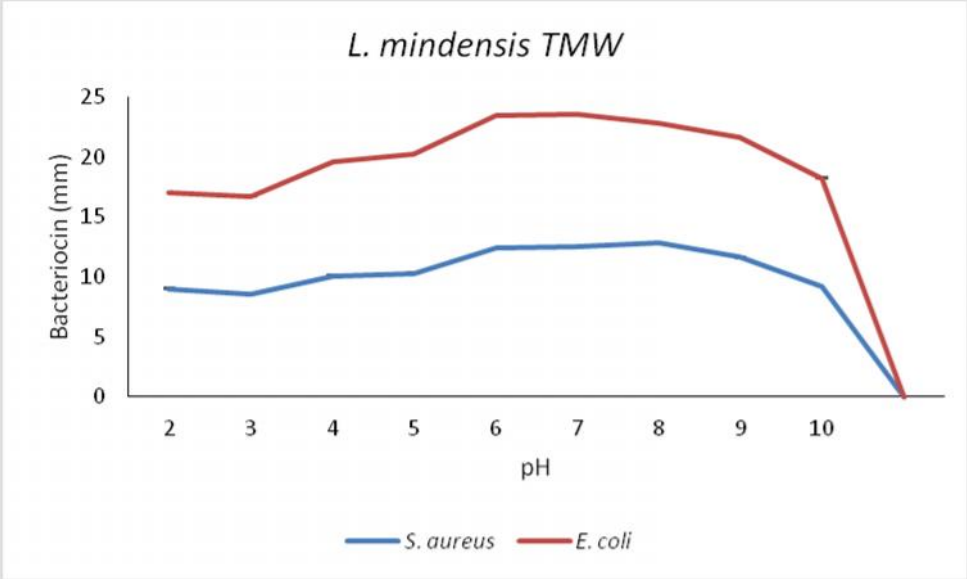

Fig. 2b: Effect of pH on partially purified bacteriocin activity (mm)

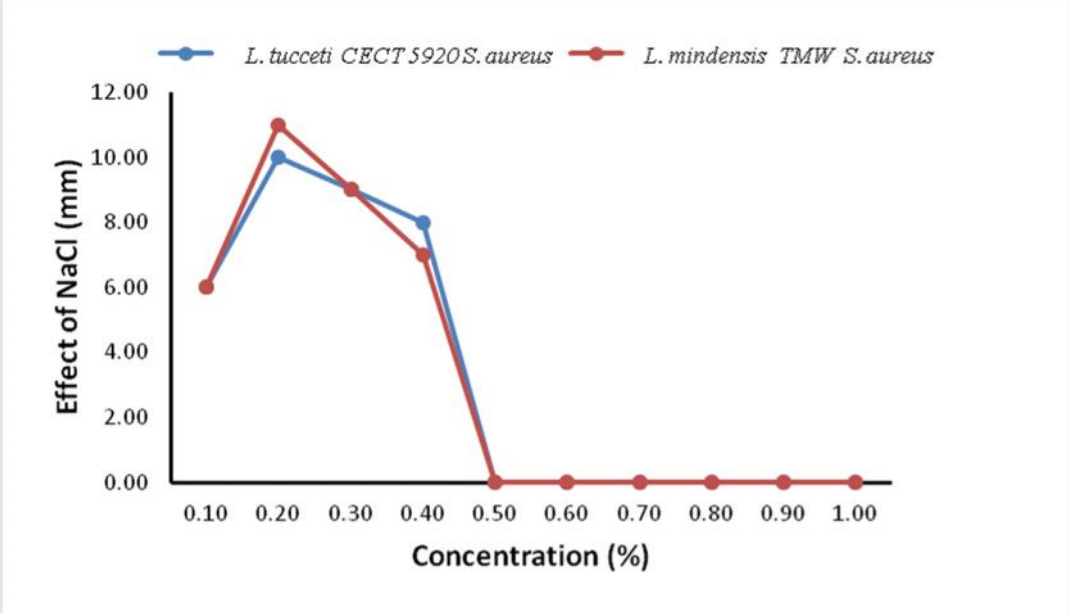

Fig. 3a: Effect of NaCl on partially purified bacteriocin activity (mm)

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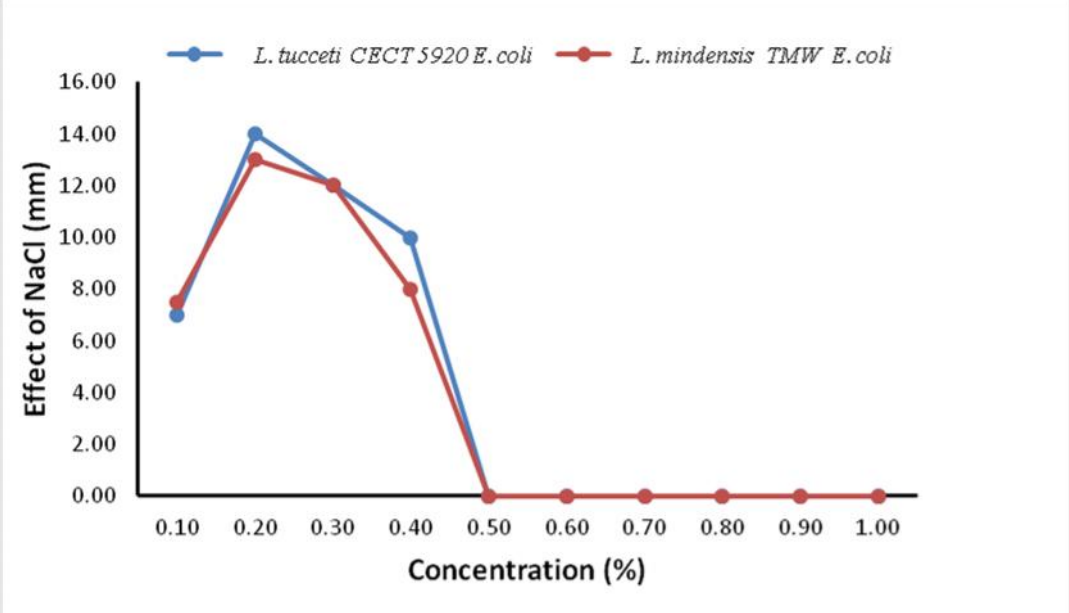

Fig. 3b: Effect of NaCl on partially purified bacteriocin activity (mm)

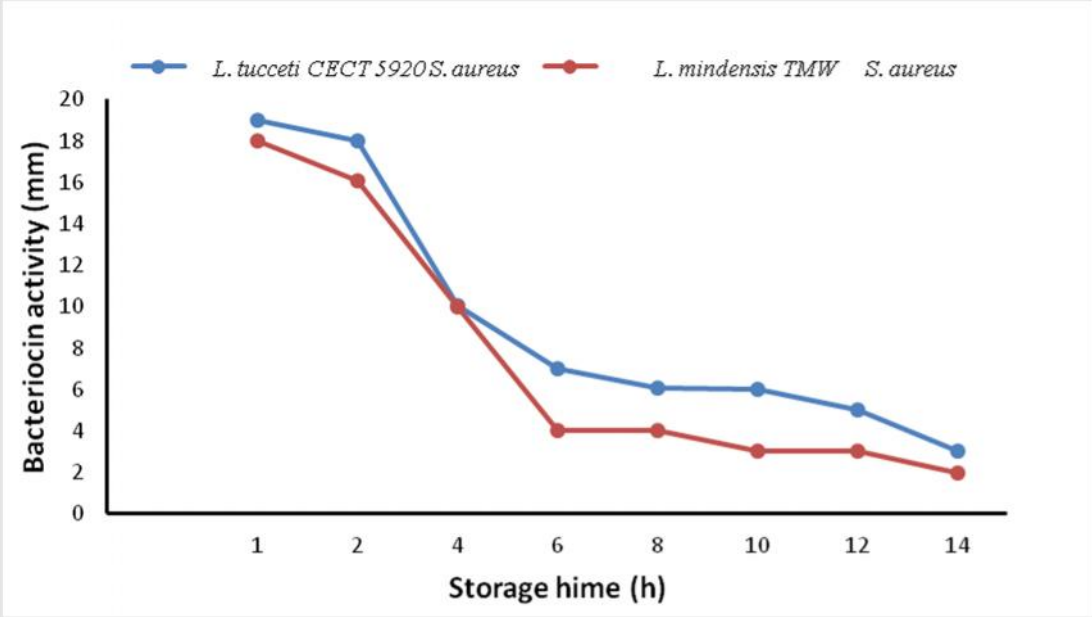

Fig. 4a: Effect of storage time on partially purified bacteriocin activity (mm)

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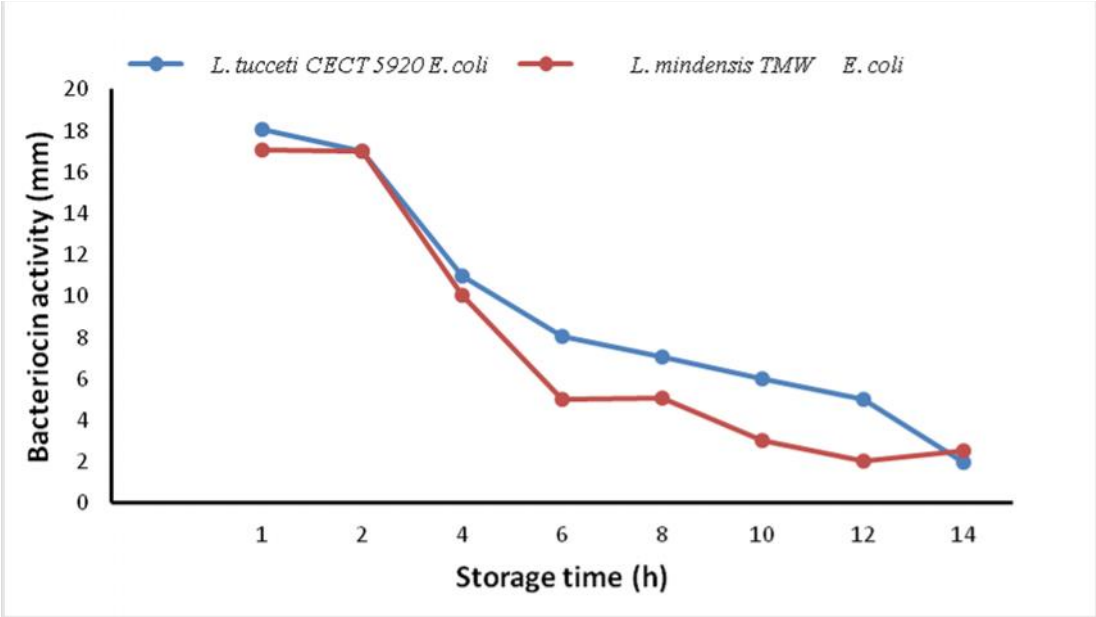

Fig. 4b: Effect of storage time on partially purified bacteriocin activity (mm)

	L. tucceti CECT 5920		*L. mindensis* TMW	
Spice	***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

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

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

Table 4: Antimicrobial activity of partially purified bacteriocin (mm)

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

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

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

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

Total count in control

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

Preservative effects (%) = Reduction in microbial count x100

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Discussion

In the present study ~~aimed at the~~ isolation and characterization of bacteriocin-producing lactic acid bacteria isolated from traditional fermented foods ~~for use as~~ starter culture. The preliminary identification of isolated lactic acid bacteria were in agreement with ~~almost each and every~~ study on lactic acid bacteria preliminary identification (Sudi *et al.,* 2008). Non-production of gas by the isolates suggests that they are homo-fermentative lactic acid bacteria. Thus, they produced only organic acids in the growth medium.

Four of the five genera identified (*Lactobacillus, Leuconostoc*, *Lactococcus* and *Streptococcus)* have been isolated from traditional fermented foods in the past (Oyedeji *et al.,* 2013; Oyewole and Isah, 2012) by morphological and biochemical characterization. However, *Lactobacillus tucceti* CECT 5920, *L. mindensis* TMW, *Pseudochrobactrum asaccharolyticum* and *P. saccharolyticum* have not been reported before now as lactic acid bacteria isolated either from Nigerian traditional fermented foods or fermented foods from any other part of the world. This could be due to the type of fermented foods used or the environment of the study.

The highest diversity of isolates shown 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 Muller (2000) which showed that *Pediococcus acidilactici, L. plantarum, L. fermentum, L. lactis* and *Leuconostoc mesenteroides* as the most diverse lactic acid bacteria isolated from traditionally fermented maize gruels from five different western states of Nigeria respectively

Some factors might have influenced the low number of isolation of bacteriocin-producing lactic acid bacteria 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.

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Wang *et al.* (2010) 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 (2013) 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. *P. asaccharolyticum*, *P. saccharolyticum, L. tucceti* CECT 5920 and *L. mindensis* TMW ~~have~~ not ~~been~~ reported ~~in any other literature~~ as part of lactic acid bacteria flora encountered in traditional fermented foods.

Bacteriocins are small peptides contains 30-60 amino acid with antimicrobial properties against bacteria usually of the same or closely related species (narrow spectrum), and sometimes against a wide spectrum of species (Rantsiou *et al.,* 2006). Microorganisms that produce bacteriocins also have immunity mechanisms to present self-protection from committing "suicide" (Draper *et al.,* 2008). Bacteriocin production is considered a very important attribute of LAB as suitable starter cultures 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 (Foegeding *et al.,* 1992). Generally, production of bacteriocin can be significantly influenced by pH, temperature, incubation time other environmental factors (Annamalai *et al.,* 2009).

The LAB isolates exhibited mesophilic inhibitory activities and the mesophilic temperature of activity of the isolates showed that they could only be useful in food preservation and as starter cultures in traditional food fermentation where the temperature is within that which the isolates can tolerate. However, this narrow range of temperature tolerance will be a disadvantage in modern food industries where higher temperatures are encountered. A doubling

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488 in activity was observed from 30-35°C suggesting that increase in temperature positively affected
489 the bacteriocin activity. However, increase in temperature beyond 35 ^{o}C could have possibly
490 destabilized the 3-Dimensional structure of the bacteriocins (which are proteins) resulting in their
491 denaturation. Djadouni and Kihal (2013) reported two bacteriocins that were thermally stable
492 over a wide temperature range up to 100 °C for 15 mins. This could be due to the ecological
493 conditions prevalent in traditional fermented foods where the lactic acid bacteria were isolated.
494 The isolates may have adapted to the mesophilic temperature range found in fermented foods.
495 Significant reduction in the bacteriocin production as the temperature increased was reported by
496 Meera and Devi (2012) and this is in agreement with the findings in this study. Growth
497 temperature seems to play an important role in bacteriocin activity.
498
499 Both isolates showed wide range of pH tolerance and activity as they inhibited the test
500 pathogens between the pH range of 3-10: an indication of acidophilic and slightly alkalinophilic
501 activity. Both isolates showed optimum inhibitions at or near neutral pH and will probably be
502 well suited in traditional fermentation of several foods as starter cultures. It has been reported
503 that some bacteriocins retained their activity at pH 2.0 to 6.0 (Djadouni and Kihal, 2013) and this
504 agrees with this study. Djadouni and Kihal (2013) stated that microbial cells are significantly
505 affected by the pH of their immediate environment because they apparently have no mechanism
506 for adjusting their internal pH.
507
508 It has been suggested that inorganic salts have a synergistic effect on bacteriocin efficacy
509 when added with specific concentrations in foods (Djadouni and Kihal, 2013). Also, Bacterial
510 metabolism is sensitive to salt, because salt exhibits specific ionic and water binding properties
511 (Korkeala *et al.,* 1992). Mahrous *et al.* (2013) reported that in MRS broth, 2 % NaCl increased
512 the activity of bacteriocins isolated from *Lactobacillus pentosus* CH2 against *E. coli* ATCC
513 25922 (14 mm zone of inhibition) while *L. fermentum* M1 had 15 mm zone of inhibition against
514 *Bacillus subtilis* NCIB3610.
515 The higher activity shown against *E. coli* 0157:H7 (a gram negative bacterium) by the
516 two Lactic acid bacteria isolates could be attributed to the nature of Gram negative cell wall
517 which seems to be more susceptible to salt than gram positive cell wall possibly due to the

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thicker peptidoglycan nature of the latter. However, detrimental effects of high concentrations of NaCl have been reported by Verluyten *et al.* (2004).

Storage time affected bacteriocin's inhibitory activity with highest activity shown on the first and second day of storage followed by a gradual decrease in the 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. For *L. tucceti* CECT 5920, the highest bacteriocin activity was recorded within the first 1-3days against the test bacteria. The activity *L. tucceti* CECT 5920 on the 14th day of storage was about was one-sixth when compared with the first day of storage, while for *L. mindensis* TMW, it was almost one-eight. 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 and a 36.61 percent reduction of *S. aureus* NCTC 8325 from the partially purified bacteriocin of *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 diarrhoea which is leading ~~to~~ cause 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 (Sasidharan and Nirmala Menon, 2010). Azu and Onyeagba (2007) 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*. This report agrees with the findings in this work.

Antimicrobial activities indicated that the bacteriocins produced by the isolates have broad spectrum activity against gram positive and gram negative bacteria (*S. aureus* and *E. coli* respectively). Similar results were recorded by Adesokan *et al.* (2009) against *S. aureus, E. coli* and *Pseudomonas aeruginosa.* The inhibitory effect recorded is assumed to be due to

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547 bacteriocin and not H_2O_2 since there was no oxidizing effect on the lactic acid bacterial cells
548 which will destroy the basic molecular structure of the lactic acid bacteria cell proteins because
549 the CFS tested was treated with NaOH and catalase to neutralize the possible oxidizing effects of
550 organic acids and H_2O_2 respectively. Lactic acid bacteria have potentials to inhibit the growth of
551 pathogenic and spoilage bacteria and the possibilities exist for using them to improve the shelf
552 life of different foods. Their antagonistic property is attributed to the low pH, the un-dissociated
553 acid and production of other primary and secondary antimicrobial metabolites (Ten Brink *et al*.,
554 1994).

555 The removal of the test pathogens from the samples was directly proportional to the
556 concentration of the bacteriocin tested (5-50 %). At 50% concentration, *L. tucceti* CECT 5920
557 and *L. mindensis* TMW showed the highest preservative effect from both meat and fish against
558 both test bacteria. The control did not show any inhibitory activity against the test isolates. This
559 result showed that the lactic acid bacteria isolates have the potential of being used as starter
560 culture as their metabolite will confer preservative status on traditional fermented foods. Joshi *et*
561 *al.* (2006) reported that partially purified bacteriocin was found to be preservative against *B.*
562 *cereus* and that the preservative effect in juice, wine and pulp increased with the increase in the
563 concentration of bacteriocin. The findings here indicate that bacteriocin possesses desirable
564 characteristics of a bio-preservative.

565

566 **CONCLUSION AND RECOMMENDATION**

567 The studies on the bio-preservative potential of the two lactic acid bacteria: *L. tucceti*
568 CECT 5920 and *L. mindensis* TMW revealed that both isolates had rapid acidification ability as
569 well as production of bacteriocin with varied levels of antimicrobial activities against the two test
570 pathogens: *S. aureus* NCTC 8325 and *E. coli* 0157:H7 used in this work. Much of the interest in
571 the analysis of LAB produced bacteriocins is driven by their potential applications. The
572 properties of the bacteriocins studied, like the inhibition of pathogenic strains, their stability over
573 a wide pH range, heat resistance and high salt tolerance makes them promising agents in food
574 preservation. In overall performance, both LAB isolates would make good research organisms of
575 study in future works as regards the use of lactic acid bacterial metabolites as better substitutes in
576 food preservation that the conventional chemical preservatives which have several health issues.

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The author recommends that the government and private bodies should invest more in researches involving the preservative potentials of LAB that abound in our traditional fermented foods so as to encourage the application of the bio-active substances of the organisms that emerged from this work in our local industries. Bacteriocins can be used to preserve our locally made fruit juices and possibly vegetable pastes thereby prolonging the availability of such foods round the year and also improving our health status as the use of chemical preservatives which have been associated with several health challenges will be by-passed since bacteriocins are metabolically digested by the human body without any side effect.

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