Antagonistic Activity of Partially Purified Bacteriocins Produced by *Lactobacillus* species Isolated from *Nono* (Fermented Milk)

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Abstract: *Lactobacilli, a* genera belonging to Lactic acid bacteria (LAB) are widely applied in fields related to food, feed, pharmaceuticals and biotechnology. The present study was aimed at isolating bacteriocin producing *Lactobacillus* species from 'Nono'. The samples were screened for the presence of *Lactobacillus* spp based on routine cultural characteristics, general morphological, biochemical tests and phenotypically using the API 50CHL technique. The LAB were screened by inoculating into MRS broth for 48 hours for bacteriocin production. The crude bacteriocins were evaluated for *in vitro* antagonistic activity by agar well diffusion method against foodborne pathogens. The extracted crude bacteriocins were partially purified using 80% cold- acetone saturation. Bacteriocins' activity (arbitrary unit per ml/ AU/mL) as well as stability towards varying temperatures, pH and effect of proteolytic enzymes on the partially purified bacteriocins were determined using standard procedures. The results revealed that *Lactobacillus plantarum 1, Lactobacillus fermentum 1* and *Lactobacillus pentosus* were isolated from *nono.* The extracted bacteriocins exhibited a broad spectrum of activity against *S.aureus (*18.3± 0.6mm) and *E.coli (*20± 0.8mm). The partially purified bacteriocins were heat stable at temperature range of (40℃- 100℃) (6400 -200 AU/ml). They were also stable at pH range of 2-6 (12800 -200 AU/mL). The bacteriocins were sensitive to proteolytic enzymes such as chymotrypsin and proteinase K, but not sensitive to catalase and α-amylase which served as the control enzymes. This further confirms the proteinaceous nature of bacteriocins. Bacteriocin of *Lactobacillus pentosus* exhibited the highest activity against the tested isolates. The result of this research indicates that bacteriocins could be used in controlling contamination causing microorganisms as well as an alternative to the use of chemical preservatives as food additives.

Keywords: Antagonistic Activity, Bacteriocins, Cold-Acetone, *Lactobacillus* species, *Nono*.

INTRODUCTION

Nono is prepared from unpasteurized cow's milk produced from spontaneous fermentations. This product is made by rural communities (Hausa/Fulani cattle herdsmen) in Nigeria and some areas in West Africa (Akabanda *et al.,* 2013). It is an opaque white to milky coloured liquid food drink rich in protein, essential amino acids, phosphorous and vitamins (Nebedum and Obiakor, 2007). A standard way to consume these fermented product is serving alone or with fura (a pearl millet based product). Recent study revealed that *Lactobacillus fermentum* was the most frequently isolated LAB species throughout the fermentation period. The other detected LAB species included *Lactobacillus plantarum, Leuconostoc mesenteriodes, Lactobacillus helveticus, Enterococcus faecium, Enterococcus italicus, Weissella confusa* and *Lactococcus* spp. (Akabanda *et al.,* 2013).

Lactobacillus is the largest genus of the family Lactobacillaceae, belonging to Lactic acid bacteria (LAB). Huang *et al.,* (2018) compiled 196 validly published species and these species are commonly associated with fermented food, such as milk, fruits, meat, sourdough, and vegetables (Tamang *et al.,* 2015). The genus are generally anaerobic, Gram – positive non spore – forming rods that are usually non motile and occasionally nitrate reducers (Hammes and Hertel, 2006). They are catalase negative when growing in complex nutritional environments, such as carbohydrates, amino acids, peptides, fatty acid esters, salt, nucleic acid derivatives and vitamins. For growth, the temperature range is between 2 and 53^{o}C, and the pH range is between 3 and 8. Optimal growth temperature and pH are usually 30 – 40^{o}C and 5.5 – 6.2 respectively (Salvetti *et al.,* 2012). *Lactobacillus* species generally utilize glucose by fermentatively via the Embden – Meyerhof - ParnasPathway (EMP) or glycolysis, and may be either homofermentative, producing more than 85% lactic acid from glucose, or heterofermentative, producing lactic acid, CO_2, ethanol and/or acetic acid in equimolar amounts (Zhang and Cai, 2014).

Lactic Acid Bacteria (LAB) are known to produce several compounds that enhances taste, smell, colour and texture of foods. They also exhibit the ability to produce some antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide and bactericidal protein or bacteriocins (Syukur *et al*., 2014). These substances have capacity to inhibit pathogenic and food spoilage bacteria. More so, there have been focus on Lactic Acid Bacteria (LAB) bacteriocins due to their apparent importance in food and feed fermentation, and also by being considered as Generally recognized as safe (GRAS) organisms by Food and Drug Administration (FDA) and not least because of good funding in the 1990s and into the twenty – first century by the European Union (Nes, 2011). Bacteriocins have a number of positive attributes that made them especially attractive for various applications. Many LAB bacteriocins have potential applications in the food industry, mainly by inhibiting the growth of foodborne bacterial pathogens such as *L. monocytogens, B. cereus, B. thurigiensis, Salmonella* and *Staphylococcus aureus* (Jamaluddin *et al.*, 2018).

Bacteriocins particularly Nisin, have been shown to have potential in the bio preservation of meat, dairy products, canned food, fish, alcoholic beverages, salads, egg products, high – moisture bakery products, and fermented vegetables either alone, in combination with other methods of preservation or through their incorporation into packaging film/food surfaces (Cotter *et al.,* 2005).

Hence, the present study was designed to evaluate the antagonistic effect of bacteriocins produced by *Lactobacillus* species isolated from *Nono* against selected foodborne spoilage and pathogenic bacteria.

MATERIALS AND METHODS

Collection of samples

Thirty *nono* samples were collected in sterile bottles from commercial producers in *Kofar Wambai* market, Kano. All the samples were transported to Microbiology department of Bayero University kano in ice packs and analyzed immediately. Food products consisting of vegetables and dairy products purchased from *Rimi* market, Kano, were used for isolating the bioassay isolates.

Isolation of *Lactobacillus* species.

Ten (10) ml of each of the collected homogenized samples were added to 90ml of sterilized distilled water for the purpose of serial dilutions process. Aliquots of 10^{-7} (0.1ml) dilutions were aseptically dispensed on sterile plates of MRS (De Man Rogosa Sharpe) agar which was adjusted to pH 5.5 and allowed to set. The plates were incubated at 37°C for 48 hours under anaerobic conditions using anaeroGen, then placed and closed immediately in the anaerobic jar. Discrete colonies were streaked onto fresh agar to obtain pure cultures of each isolate (Adebayo *et al*., 2014). Before experimental use the cultures were propagated twice in MRS broth at 37°C for 48hrs.

Identification of *Lactobacillus* species Using phenotypic characterization

The pure colonies were characterized using colonial, morphological characteristics and biochemical tests which included gram staining, catalase test, oxidase, citrate utilization test, sugar fermentation and motility test. Non spore forming bacilli, non-capsule, catalase negative and gram positive isolates were maintained on MRS agar slants and stored at 4°C for further tests (Savadogo *et al.,* 2004).

Identification of *Lactobacillus* species Using the API 50CHL System

The LAB species were further confirmed for production of acids from carbohydrates and related compounds by use of the API 50CHL system (BIOMÉRIEUX SA, France). All Lactobacillus identification procedures were conducted in accordance with manufacturer's instructions. Portions of pure culture of each isolate were aseptically transferred from a freshly inoculated stock culture using a swab to an ampule of API 50 CHL (10.0ml) basal medium and then emulsified to give a final turbidity equivalent to McFarland standard No.2.

Each tube of the API 50 CHL strip was inoculated with the bacterial suspension using a sterile pipette. The strip was placed in the incubation tray with honeycombed wells each filled with distilled water and covered with mineral oil according to the manufacturer's instructions. After incubation of 48hr, each well was observed for colour changes. Positive result was confirmed by the change of colour of bromocresol purple indicator from purple to yellow, except for well No.26 (for esculin hydrolysis test) by the change of colour from purple to black. While negative result was indicated by a no colour change. The first well on the strip served as the control. The results were analyzed using api-web^{TM} identification software database (BIOMÉRIEUX, France V5.1) to identify *Lactobacillus* species (Pyar and Kok, 2019).

Extraction of Crude Bacteriocins

The *Lactobacillus* spp were grown in 500ml MRS broth at 37°C for 48 hours anaerobically in triplicates. The cultures were centrifuged at 20,000 rpm for 20 minutes at 4°C. To eliminate growth inhibition caused by organic acids, the resulting cell free supernatant fluids was adjusted to pH 7.0 with 1N NaOH. Inhibitory activity of hydrogen peroxide was eliminated by adding 5mg/mL catalase and sterilized by filtration through 0.2µm Millipore filter (Rajaram *et al*., 2010).

Antibacterial Assay

The antibacterial activity of the crude bacteriocin was determined using the agar well diffusion method (Ochei and Kolhatkar, 2008). The bioassay bacteria were standardized and compared with standard. About $1.5x10^8$ CFU/mL of the bacteria to be tested for sensitivity were inoculated (1%) into 20 ml of nutrient agar and then poured into Petri dishes. Then 100 µl of crude bacteriocin was filled in 7-mm diameter sealed wells cut in the nutrient agar. Once solidified, the dishes were stored for two hours in a refrigerator. The inoculated plates were then incubated for 24 hours at 37°C, and the diameter of the inhibition zone were measured using meter rule in millimeter. The antibacterial activity were assayed in triplicates (Ochei and Kolhatkar, 2008).

Partial Purification of Bacteriocins

Cold-Acetone Precipitation

The method described by Pal *et al*., (2009) with slight modifications was adopted. Crude bacteriocins of *Lactobacillus* species from 1 L of culture broth was maintained at ~0 ˚C; ice-cold acetone was added to the extent of 80% saturation with constant stirring and incubated for 15 min at 4℃. The mixture (Protein precipitate) were centrifuged at 10,000rpm for 20 minutes and the precipitate was re-suspended in 25ml of 20mM sodium acetate buffer. The reconstituted bacteriocins concentrate were checked for antibacterial activity using Agar well diffusion method.

Determination of Bacteriocin Titre (MIC)

The titres of bacteriocins produced were prepared by two – fold serial dilutions in saline solution. Ten (10) sterile test tubes containing 1ml each of normal saline were arranged serially and labeled. One milliliter (1ml) each of of the partially purified bacteriocins was transferred to the second tube and mixed well. From the first tube, 1ml was transferred to the third tube. The process was performed up to the last test tube. Then aliquots of 100µl from each dilution were placed in wells in plates seeded with standardized bioassay strains. These plates were incubated at 37^oC for 24hrs and examined for the presence of 2mm or larger clear zones of inhibition around the wells. The antibacterial activity of bacteriocin was defined as the reciprocal of the highest dilution showing inhibition against indicator strain x 100 µl and was expressed as activity units per ml (AU/mL) $1/10^n$ x 100 (Gopakumaran *et al.,* 2017).

Effect of Temperature, pH and Proteolytic Enzymes on Partially Purified Bacteriocins

The partially purified bacteriocin samples were characterized by studying the effects of pH, heat stability and proteolytic enzymes (Sure *et al*., 2016).

Heat stability of Bacteriocins

The effects of temperature on the partially purified bacteriocin was tested by heating the bacteriocin from 40ºC to 100ºC with 20ºC increment using a water bath. A control was maintained by incubating the bacteriocin sample at 37 ºC. Five (5) ml aliquots of each treatment was taken after 15 minutes (Sure *et al*., 2016). The residual activities was then assayed, using heat treated partially purified bacteriocin against test organisms while untreated bacteriocin samples was used as control.

Effects of varying pH on Bacteriocins

To test the stability at different pH, partially purified bacteriocin was mixed with equal volume of buffers with pH values ranging from 2- 10 with an increment of 2 and incubated for 30min at 37ºC. The residual activities were then assayed, using pH adjusted partially purified bacteriocin *against* test organisms while pH unadjusted bacteriocin samples was used as control (Sure *et al*., 2016).

Effect of proteolytic enzymes on Bacteriocins

The effect of the proteolytic enzymes on the inhibiting activity of partially purified bacteriocin was carried out on solid medium. To ensure the proteineous nature of the inhibiting substances, the proteolytic enzymes proteinase k, chymotrypsin, trypsin, α-amylase and catalase were used. Each enzyme was dissolved in plug phosphates buffer (10 mM, pH 7.0) with a concentration of 10mg/ ml and sterilized by filtration (0.45 µm). During the treatment by Proteinase K, trypsin, pepsin, chymotrypsin, catalase and α-amylase. The filtrate containing these enzymes were incubated for 1hour at 37°C. The sensitivity of an antibacterial substance to a given enzyme is appreciated by determining the residual activity by measurement of the diameter of zone of inhibition (Saidi *et al*., 2011).

RESULTS

Isolation of *Lactobacillus* Species

Lactobacillus spp selectively isolated on MRS agar appeared as creamy, smooth and round colonies. Their microscopic appearance revealed them as gram positive rods (plates I).

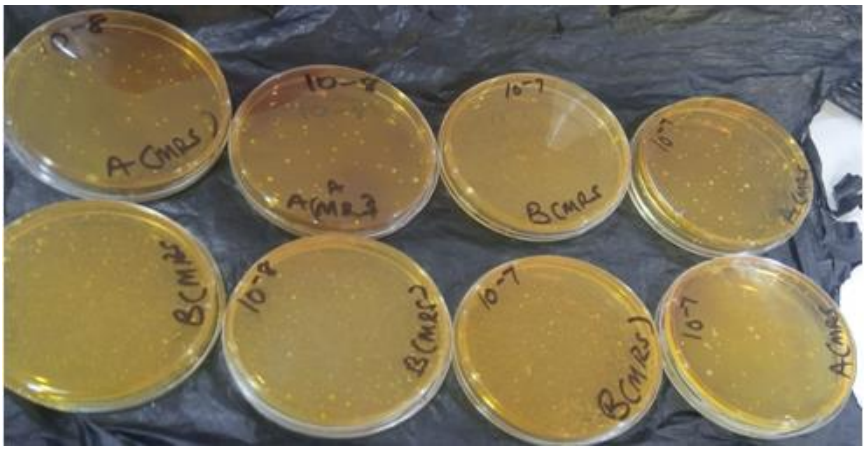

Plate 1: Morphology of *Latobacillus* species on MRS Agar

Identification of *Lactobacillus* spp using (API 50 CHL) kit.

The results of carbohydrates fermentation profile of API 50 CHL identification kit are shown in Table 1.The results obtained are in agreement with characteristics of *Lactobacillus* species. API 50 CHL database indicates that NK2, NL1, and N64, were *Lactobacillus plantarum 1, Lactobacillus fermentum 1,* and *Lactobacillus pentosus,* with percentage 1.Ds of 99.9%, 99.7%, and 98.6%, respectively.

Antibacterial Activity of Crude Bacteriocin

The result of the antibacterial activity of bacteriocins extracted from *Lactobacillus* spp against indicator foodborne pathogens is presented in Table 2. The inhibitory effects were higher on *E. coli* (16±0– 20±0.8) than *S. aureus* (16±0.8–18.3±0.6).

Table 1: Sugar Fermentation by Isolates Using API50CHL

S/NO	Carbohydrates	NK2	NL1	N64
1.	Control	-	-	-
2.	Glycerol	-	-	-
3.	Erythirol	-	-	-
4.	D – arabinose	-	-	-
5.	L – arabinose	+	-	+
6.	Ribose	+	+	+
7.	D – xylose	-	-	+
8.	L – xylose	-	-	-
9.	Adonitol	-	-	-
10.	β-metil – D – xyloside	-	-	-
11.	Galactose	+	+	+
12.	D – glucose	+	+	+
13.	D – fructose	+	+	+
14.	D – mannose	+	-	+
15.	L – sorbose	-	-	-
16.	Rhamnose	+	-	-
17.	Dulcitol	-	-	-
18.	Inositol	-	-	-
19.	Manitol	+	-	+
20.	Sorbitol	+	-	+
21.	α-methyl-D-mannosidse	+	-	-
22.	α-methyl-D-glucoside	-	-	+
23.	N-acetyl-glucosamine	+	-	+
24.	Amigdalin	+	-	+
25.	Arbutin	+	-	+
26.	Esculin	+	-	+
27.	Salicin	+	-	+
28.	Cellobiose	+	-	+
29.	Maltose	+	+	+
30.	Lactose	+	+	+
31.	Melibiose	+	+	+
32.	Saccharose	+	+	+
33.	Trehalose	-	-	+
34.	Inulin	-	-	-
35.	Melezitose	+	-	+
36.	D-raffinose	+	+	+
37.	Amidon	-	-	-
38.	Glycogen	-	-	-
39.	Xylitol	-	-	-
40.	β-gentiobiose	+	-	+
41.	D –turanose	+	-	+
42.	D – lyxose	+	-	-
43.	D – tagarose	-	-	-
44.	D – fuccose	-	-	-
45.	L – fuccose	-	-	-
46.	D – arabitol	-	-	-
47.	L – arabitol	-	-	-
48.	Gluconate	+	-	-
49.	2 – keto – gluconate	-	-	-

Key: NK2 = *Lactobacillus plantarum1*, *NL1 = Lactobacillus fermentum1*, *N64 = Lactobacillus pentosus*,

Table 2: Antibacterial Activity of Crude Bacteriocins

Isolates No:	Zone of inhibition (mm)	
	S. aureus	***E.coli***
NL1	17±0	17±0
NK2	16±0.8	16±0
N64	18.3±0.6	20±0.8

Key: Values in mean ± SD
NL1 – N64 = Crude bacteriocins from different isolates

Partial Purification of Bacteriocins

Determination of Bacteriocins Titre

The inhibitory activity in (AU/mL) of bacteriocins measured after partial purification (80% ice – cold acetone precipitation) shows bacteriocin titres of 12800 AU/mL (N64), 6400 AU/mL (NL1) and 3200 AU/mL (NK2) against the tested isolates Figure 1.

Effects of Temperature, pH and Proteolytic Enzymes on Bacteriocin Activity

All the bacteriocins were found to be heat stable at a temperature range of 40°C – 100°C for 15min. The highest activities were recorded at 40°C for N64; *E. coli* (6400 AU/mL) and *S. aureus* (3200 AU/mL). While for NL1 and NK2 bacteriocins, (3200 Au/ml) was recorded for both *S. aureus* and *E. coli*. The least activity was at 100°C (200-400 AU/mL) figure 2.

The activity of bacteriocins were not inhibited after treatment in pH range of 2 – 10 (figure 3). The activity was highest at pH 2 – 6 (12800 AU/mL – 3200 AU/mL). While the activity reduced from pH 8 – 10 (400 AU/mL– 200 AU/mL).

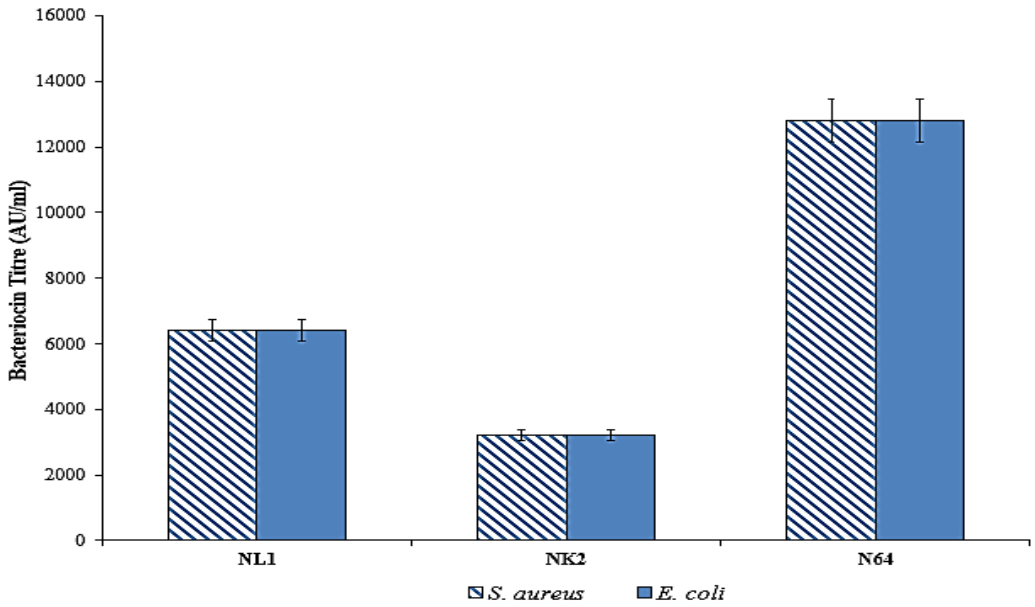

Figure 1: Bacteriocin Titres (AU/mL)

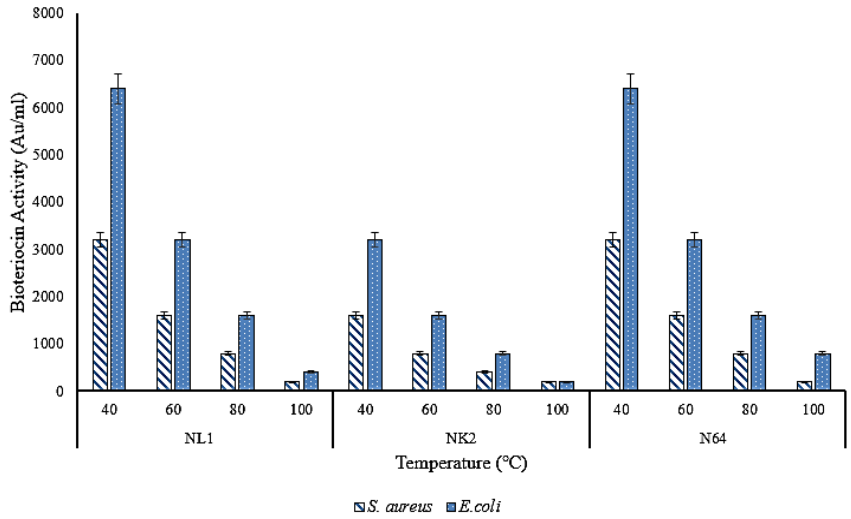

Figure 2: Effect of temperature treatment on activity partially purified bacteriocins

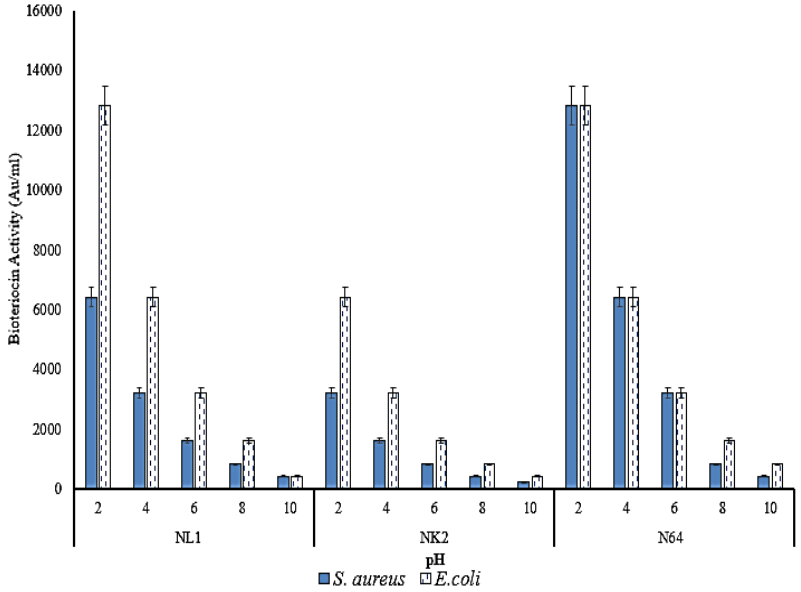

Figure 3: Effect of pH Treatment on Activity of Partially Purified Bacteriocins

Treatment of bacteriocins with proteolytic enzymes completely inhibited the activity of the bacteriocins, while catalase and amylase had no effect on bacteriocin activity indicating the protein nature of bacteriocins (Table 3).

Table 3: Effect of Proteolytic Enzymes on Activity of Partially Purified bacteriocin

Enzyme	**Bacteriocin Activity**		
	NL1	**NK2**	**N64**
Proteinase K	-	-	-
Chymotrpsin	-	-	-
Trypsin	-	-	-
Pepsin	-	-	-
α-amylase	+	+	+
Catalase	+	+	+

Key:

- = No Inhibition + = Inhibition

DISCUSSION

The research revealed that most of the strains isolated from *Nono* were different species of the genus *Lactobacillus* identified phenotypically based on their carbohydrates fermentation profiles (API50 CHL). They were identified as *L. plantarum 1*, *L. fermentum 1*, and *L. pentosus*. This result is in agreement with the findings of Jones and Versalovic (2009). API 50 CHL identification kit has been reported as an important tool for Lactobacilli identification. It can be used for taxonomic identification which is based on phenotypic characteristics to identify different species of Lactobacillus (Pyar and Kok, 2019). Many studies had reported the diversity of LAB in milk and milk products. Syukur *et al.,* (2014) isolated different *Lactobacillus* spp from 'Dadih' (fermented buffalo milk) in Indonesia.

Crude Bacteriocins i.e. (cell free supernatant) from 3 *Lactobacillus* spp showed various degree of activity against the Gram positive and Gram negative foodborne isolates used. Higher inhibitory activity was observed against *E. coli* (20 $\pm$ 0.8) than *S. aureus* (18.3$\pm$0.6). The higher activity of bacteriocin against *E. coli* may be due to the variation in the structure of these bacteriocins and/or differences in the structure of the putative target molecules (receptors). Bacteriocins with high structural similarly may even differ remarkably in their antimicrobial spectrum of activity (Drider *et al.,* 2006; Pal *et al.,* 2009). Stevens *et al.,* (1991) reported that bacteriocins are not frequently active against Gram negative bacteria because the outer membrane of this class of bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents and dyes from reaching the cytoplasmic membrane. Also Cleveland *et al.,* (2001) earlier reported that bacteriocins are supposed to act only on closely related species which limits their application as a natural preservation, but in contrast, our study revealed that bacteriocins produced by *Lactobacillus* spp were active against both Gram positive and gram negative isolates suggesting their broad spectrum of activity. Similar results were obtained by Sharma *et al.,* (2011) in their research, purification and characterization of bacteriocin produced by *Lactobacillus* sp. A 75 isolated from fermented chunks of 'phaseolus radiata'. Thus, this proves that bacteriocins from different lactic acid bacteria have specific inhibition spectra.

This present study further reveals that partially purified bacteriocin (cold – acetone precipitation) had a higher bacteriocin activity (12800 AU/mL) against indicator strains compared to the crude bacteriocins. The increase in inhibitory potential and bacteriocin activity after partial purification suggests that the targeted proteins have been concentrated while other interfering proteins have been removed from the samples which otherwise may shield the effect of the bacteriocins and thus it is more active against the indicator strains resulting in added inhibition thus enhancing its effect in favorable direction i.e for safer bio preservation of food (Gautam and Sharma, 2015). Various physicochemical factors seemed to affect bacteriocins activity; in temperature stability studies, it was found that partially purified bacteriocins was stable at temperature range of 40^{o}C – 100^{o}C but not 121^{o}C. Similar results were obtained in the case of bacteriocins produced by *Bacillus subtitlis* R75 as well as *L. plantarum* LE5 and LE7 *(*Sure *et al.,* 2016 and Gautam and Sharma, 2015). Thermal stability of partially purified bacteriocins opens an avenue for its use in food products which needs to be operated at high temperatures. It can also slow the activity of foodborne pathogens which are heat resistant and hence could be exploited as a potent source of bacteriocin. It is thus noteworthy that thermal stability enhances the use of bacteriocin as food preservatives because many food processing steps requires high temperature.

The activity of bacteriocins exhibited against the indicators strains are pH dependent. The highest antibacterial activity was observed at an acidic pH range (2-6) while it decreased at pH 8 – 10.

This is in agreement with the work of Ogunbawo *et al.* (2013) in their work titled characterization of bacteriocin produced by *L. plantarum* f1 *and L. brevis ogi*. This results also proves that bacteriocins can be used in fermented acidic foods like pickle or yoghurt (Todorov and Dicks, 2004).

Furthermore, the proteinaceous nature of the bacteriocins were confirmed after they lost activity upon treatment with proteolytic enzymes. Treatment with amylase and catalase did not change the antibacterial activity of all the bacteriocins. Thus suggesting that the bacteriocins were not glycosylated. Similar findings have been reported earlier (Pal *et al.*, 2009; Gautam and Sharma, 2015). This results further adds to the fact that bacteriocin upon consumption in food is broken down by digestive juices secreted in the digestive tract of humans thus rendering it completely safe for human consumption (Cleveland *et al.*, 2001).The sensitivity of bacteriocins to proteolytic enzymes further confirms its GRAS status by the US food and drug administration.

CONCLUSION

This research revealed the presence of *Lactobacillus* spp in Nigerian fermented milk 'Nono'. The *Lactobacillus* spp based on API 50CHL profiles were identified as *L. plantarum 1, L. fermentum 1,* and *L. pentosus.* Bacteriocins extracted from the isolated *Lactobacillus* spp had antibacterial activity against the tested foodborne pathogens. Therefore this partially purified bacteriocins possessed antagonistic activity and hence could be exploited further for their use in food safety and quality.

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