Production of Bacteriocins by *Lactobacillus plantarum* and *Pediococcus acidilactici* Isolated from Cow Milk

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Abstract: A dramatic increase in bacterial resistance towards currently available antibiotics has raised worldwide concerns for public health. Therefore, bacteriocins which are antimicrobial peptides (AMPs) have emerged as a promisingly new group of therapeutic agents for managing infectious diseases and possibly in food preservation. The present study focusses on the isolation of novel bacteriocins from an indigenous sample of cow milk and testing of its antimicrobial activity. Two bacteria isolates (*Pediococcus acidilactici* and *Lactobacillus plantarum)* isolated from raw cow milk gotten from Yenegoa, Bayelsa state, Nigeria produced potent bacteriocins on De-Mann Rogosa and Sharpe agar and these were shown to have inhibitory activity against the pathogenic bacteria *Escherichia coli*. The bacteriocins were heat stable, remaining active at temperatures up to 100°C and functioned well over a wide pH range of 0-10. There was a reduction in activity of the bacteriocins after treatment with proteinase K and peptidase, thus confirming the proteinaceous nature of the compounds. These bacteriocins displayed bacteriostatic and bactericidal activity against *E. coli,* with a minimum inhibitory concentration of 12.5 µg/ml and minimum bactericidal concentration of 25µg/ml which is lower than that of the conventional antibiotics chloramphenicol (50µg/ml) used as positive control. The bacteriocins produced by lactic acid bacteria isolated from cow milk in this work is effective in inhibiting the growth of *E. coli* and thus may be of use as a food preservative in the storage of food or as therapeutic agent for the treatment of infections caused by multi-drug resistant *E. coli.*

Keywords: Bacteriocin, Antimicrobial peptide, drug resistance

INTRODUCTION

The greatest threat to quality and safety of food comes from microbial spoilage (Pal, 2013). Microorganisms growing on food can cause problems such as bad taste, unpleasant smell, and poor appearance. More importantly, the growth of microbes on food may lead to dangerous levels of toxins in the food, resulting in food intoxication and food infections. This makes the food unfit to be eaten by the people, and hence it leads to food scarcity (Pal, 2013). Hence, food spoilage is wasteful, costly and can adversely affect the economy, eroding the confidence of the consumers. Numerous preservation methods are employed to prevent food poisoning and spoilage. These techniques include thermal treatment (pasteurization, heating sterilization), pH and water activity reduction (acidification, dehydration) and addition of preservatives (antibiotics, organic compounds such as propionate, sorbate, benzoate, lactate, and acetate).

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Although these methods have been proven to be highly successful, there is an increasing demand for natural, microbiologically safe products providing the consumers with high health benefits (Deegan *et al.,* 2006).

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rods, catalase-negative, and fastidious organisms, with high tolerance for low pH (Van Geel-Schuttená e*t al.,* 1998). LAB are among the most important microbes which are used in food fermentations, as well as in enhancing taste and texture in fermented food products (Hati *et al.,* 2013). They are characterized by the production of lactic acid as the main product from glucose and growth inhibition substances such as hydrogen peroxide, diacyls, etc. which prevent the proliferation of food spoilage bacteria and pathogens (Alakomi., et al. 2000). In addition, some species of LAB produce antimicrobial peptides known as bacteriocins. To date, several LAB isolates from the *Lactobacillus* genus and their bacteriocins have been applied in food preservation and in the control of human pathogens (Parada *et al.,* 2007).

Bacteriocins are ribosomally synthesized polypeptides possessing bacteriocidal activity that are rapidly digested by proteases in the human digestive tract (Joerger *et al.,* 1986).

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous and generally possessing a narrow specificity of action against strains of the same or closely related species. They can be referred to as Anti-Microbial Peptides (AMPs). Bacteriocin production seems to be aimed to compete against other bacteria which are closely related or present in same ecological niche.

Bacteriocins can be used as a preservative in food due to its heat stability, wider pH tolerance and its proteolytic activity. Due to thermo stability and pH tolerance, it can withstand heat and acidity/alkanity of food during storage condition. Torodov and Dicks, (2005) reported that bacteriocins ST28MS and ST26MS produced by *Lactobacillus plantarum* isolated from molasses remained stable after incubation for 2hours at pH values between 2.0 and 12.0 with no decrease in antibacterial activity after incubation for 90 min at 100ºC or 20 min at 121º C. However, because of their proteinaceous nature, there was complete inactivation with reduction in antimicrobial activity after treatment with proteinase K, pepsin and trypsin. Although many types of bacteriocin such as subtilin, cerein, thuricin, plantaricin etc., have been isolated and characterized, and are still in a process of getting commercial status to be used as food preservatives, so far only one bacteriocin; Nisin has been given the status of preservative to be added in food items commercially (Ogunbanwo *et al.,* 2003). Notably, bacteriocins are generally regarded as safe (GRAS) and possess a spectrum of activity narrower than conventional antibiotics (Morency *et al.,* 2001) and have the tendency to penetrate the outer membrane of Gram-negative bacteria. Chemical preservatives used in food products pose several health challenges to consumers such as cardiovascular diseases, chronic diseases and certain types of cancer thus consumption of these chemically preserved foods raises concern amongst consumers who yearn for naturally produced antimicrobial agents. Furthermore, because of increase in prevalence of antibiotics resistant organisms and the side effects of most antibiotics in use, there is need for safe, effective and natural antimicrobial agents which can be utilized both therapeutically and as food preservatives to replace chemical preservatives in foods. This study is geared towards the discovery of such alternatives as it is a developing field of enquiry and will specifically aim at the isolation of lactic acid bacteria from raw cow milk, production and purification of bacteriocins from LAB and evaluation of the antimicrobial potentials of the bacteriocins and their possible use as food preservatives.

MATERIALS AND METHODS

Sample Collection

Samples of raw cow milk were randomly collected from lactating cows in Yenegoa, Bayelsa State Nigeria. The raw milk samples were pasteurized at 63°C for 30 minutes to kill off saprophytic microorganism's present. The milk was left to ferment in a sterile flask under room temperature (25^{O}C) for two days.

Isolation of Lactic Acid Bacteria from Fermented Milk

After the fermentation process, Lactic acid bacteria (LAB) was isolated by streaking an inoculum of the fermented cow milk on deMann, Rogosa and Sharpe (MRS) agar plates (Merck, Germany). The inoculated plates were incubated for 24 hours at 37°C. Single colonies were picked from the plates and subcultured onto MRS agar plates and incubated for 24 hours at 37°C to obtain pure cultures. These were subjected to biochemical tests after the scheme of Cheesbrough (2006) and interpreted using the identification schemes of the 8th Edition of Bergey's manual of Determinative Bacteriology (Harrigan, 1998).

Production of Bacteriocin

The various isolates were grown in 200mls of MRS broth for 24 hours at 37°C. After growth, the media is transferred into several sterile test tubes and centrifuged at 10,000 x g for 20 minutes. The resultant residue with bacteria pellets was discarded and the cell-free supernatant was filtered using a 0.2µm membrane filter and collected for assay.

Preparation of Test Isolates

The test isolate utilised for this study was *Escherichia coli* obtained from stock cultures of the Microbiology Laboratory, Federal University Otuoke. The viability of the test organisms was confirmed by growing on nutrient agar and its identity was confirmed by biochemical tests following the scheme of Cheesbrough (2006). The concentration of the test bacteria was adjusted to the MacFarlands standard by measuring to an optical density (OD) value of 0.1 at 600nm wavelength using a UV/Vis spectrophotometer.

Antimicrobial Assay of Bacteriocin

The antimicrobial activity of the bacteriocins was determined by the agar-well-diffusion assay using Muller-Hinton agar. After incubation at 37^{O}C for 24 hours, the average diameter of inhibition zones obtained was measured before subjecting the bacteriocins to the tube dilution assay to determine the Minimum Inhibitory Concentration (MIC) by spectroscopic methods using different concentrations of bacteriocins ranging from 3.125 to 50 µg/ml and Minimum Bactericidal Concentration (MBC) by subculturing tubes that showed growth inhibition onto fresh nutrient agar as described by Fasoyiro and Adegoke (2007) and Nwachukwu *et al.* (2009). The MIC was reported as the broth containing the lowest concentration of the bacteriocin extract, which showed no growth (was able to inhibit microbial growth) while the MBC is the lowest concentration of the bacteriocin extract that caused cell death.

Vulnerability of Bacteriocin to Enzyme Action

The vulnerability of the bacteriocin to breakdown/denaturation by different enzymes namely: proteinase K, peptidase, catalase and lysozyme was tested to verify its proteinaceous nature. This was achieved by introducing 100 µl of an enzyme into a test tubes. To this, 1ml of the bacteriocin extract is added to bring about a final concentration of 1mg/ml. This is repeated for all enzymes tested and all preparations were shaked and incubated at 37°C for one (1) hour before determining the antimicrobial activity by agar well diffusion assay method as discussed above. One control without enzyme treatment was tested.

Thermal Stability Test of Produced Bacteriocin

To assess the thermal stability of the produced bacteriocin, 2mls each of the crude bacteriocin extract was measured into four (4) test tubes. These were exposed to temperatures of 40, 60, 80 and 100°C for 20 minutes in a water bath. The heated bacteriocin was cooled to room temperature (25°C) and tested for its antimicrobial activity against *E. coli* using the agar well diffusion assay method as discussed above. One control without temperature treatment was tested.

pH Stability Test of Produced Bacteriocin

To assess the pH stability of the produced bacteriocin, 2 mls each of the crude bacteriocin extract was measured into four (4) test tubes. Using a micropipette, the pH of each tube was adjusted using concentrated HCl and NaOH to 2,4,8 and 10 respectively. This was incubated for 2 hours at 25°C before determining the antimicrobial activity by agar well diffusion assay method as discussed above. One control without pH adjustment was tested.

RESULTS AND DISCUSSION

Isolation of Lactic Acid Bacteria from Fermented Milk and Screening for Bacteriocin Production

A total of 8 different isolates were obtained after streaking of fermented milk on deMann, Rogosa and Sharpe (MRS) agar plates. These were inoculated into MRS broth for bacteriocin production. After incubation and centrifugation, the filtrate was analysed for antimicrobial activity by the well-in-agar assay using *E.coli*. The result is presented in table 1. below.

Of the eight (8) LAB isolates obtained from fermented milk on MRS agar, only two (Isolates C and F) showed significant antimicrobial activity against the test organism with zones of inhibition of 26mm and 18mm respectively.

Table 1. Zones of inhibition (mm) produced by bacteriocins against *E.coli.*

Isolates	Zone of Inhibition
A	-
B	-
C	26
D	-
E	-
F	18
G	-
H	-
Control (Chloramphenicol)	30

Identification of Bacteriocin Producing Isolates

The isolates (C and F) producing potent antimicrobial agents (bacteriocins) were subjected to Gram staining and other biochemical tests to aid in their identification. The result is presented in Table 2. below.

Table 2. Gram staining and biochemical characteristics of bacteriocin producing isolates

Isolates	**Tests**							**Suspected Organism**
	Gram reaction	Shape	Citrate test	Bile Esculin test	Catalase test	Oxidase test	Indole test	
C	+ve	Rod	-ve	+ve	-ve	-ve	-ve	*Lactobacillus plantarum*
F	+ve	Cocci in tetrads	-ve	+ve	-ve	-ve	-ve	*Pediococcus acidilactici*

Upon conduction of biochemical tests on isolates C and F, the results indicate that the bacteriocin producing organisms are *Lactobacillus plantarum* and *Pediococcus acidilactici.* These two organisms are known to be potent producers of bacteriocin. Moreno *et al.,* (2000) reported the production of pediocin by *Pedicoccus* spp. while Messi *et al.,* (2001) reported on the production of plantaricin 35d by *Lactobacillus plantarum.*

Minimum Inhibitory Concentration (MIC) Test

The minimum inhibitory concentration of the produced bacteriocins was determined by the tube dilution assay and the result is presented in Table 3. below.

Table 3. Minimum inhibitory concentration (MIC) test

DILUTION(µg/ml)	**ANTIMICROBIAL AGENTS**		
	Bacteriocin from *Lactobacillus plantarum*	Bacteriocin from *Pediococcus acidilactici*	Control (Chloramphenicol)
50	No growth (Clear)	No growth (Clear)	No growth (Clear)
25	No growth (Clear)	No growth (Clear)	No growth (Clear)
12.5	No growth (Clear)	No Growth (Turbid)	No growth (Clear)
6.25	Growth (Turbid)	Growth (Turbid)	Growth (Turbid)
3.125	Growth (Turbid)	Growth (Turbid)	Growth (Turbid)
MIC	**12.5 µg/ml**	**12.5 µg/ml**	**12.5 µg/ml**

The MIC test result shows a value of 25µg/ml for bacteriocin produced by *Pediococcus acidilactici* and a value of 12.5 µg/ml for the bacteriocin produced by *Lactobacillus plantarum.* These results are comparable to those reported by Goh and Phillip (2015) who obtained an MIC of 18.5 µg/ml against *E.coli* using bacteriocins produced by *Weissella confusa* A3. Furthermore, the MIC results are quite similar to the value of 12.5 µg/ml obtained by the chloramphenicol control.

Minimum Bactericidal Concentration (MBC) Test

The minimum bactericidal concentration (MBC) of the produced bacteriocins was determined by transferring an inoculum from the MIC test tubes showing growth inhibition (clear) onto nutrient agar plates using a wire loop and incubated for 24hrs at 37°C. After incubation, the plates are checked for colony formation and result is shown in Table 4. below.

Table 4. Minimum Bactericidal Concentration (MBC) test

Dilution(µg/ml)	**Antimicrobial agent**		
	Bacteriocin from *Lactobacillus plantarum*	Bacteriocin from *Pediococcus acidilactici*	Chloramphenicol
50	No growth	No growth	No growth
20	No growth	No growth	Growth
12.5	Growth	-	Growth
MBC	25 µg/ml	25 µg/ml	50 µg/ml

A minimum bactericidal concentration (MBC) of 25µg/ml was obtained for bacteriocin produced by *Pediococcus acidilactici* and a value of 25µg/ml for the bacteriocin produced by *Lactobacillus plantarum.* These results are much lower than those reported by Goh and Phillip (2015) who obtained an MBC value of 74 µg/ml against *E.coli* using bacteriocins produced by *Weissella confusa* A3. This indicates that the bacteriocins produced in this study are more potent and active than those reported by Goh and Phillip (2015). Also, the MBC results of the produced bacteriocins are lower than those of the chloramphenicol control which gave a value of 12.5 µg/ml.

Vulnerability of Produced Bacteriocin to Enzyme Action

The produced bacteriocins were subjected to treatment by the enzymes; proteinase K, peptidase, catalase and lysozyme to test ability of these enzymes to inactivate the bacteriocins. After treatment with the enzymes, the bacteriocins were incubated at 37°C for one (1) hour before determining the antimicrobial activity by agar well diffusion assay method with results shown in Table 5. below .

Table 5. Vulnerability of Produced Bacteriocin To Enzyme Action

Enzyme	**Antimicrobial agent**			
	Bacteriocin from *Lactobacillus plantarum*	Bacteriocin from *Lactobacillus plantarum* (UNTREATED CONTROL)	Bacteriocin from *Pediococcus acidilactici*	Bacteriocin from *Pediococcus acidilactici* (UNTREATED CONTROL)
Proteinase K	8mm	25mm	7mm	17mm
Peptidase	9mm	24mm	8mm	16mm
Catalase	23mm	24mm	17mm	18mm
Lysozyme	25mm	26mm	16mm	17mm

The antimicrobial activity of the bacteriocins was reduced by treatment with proteinase K and peptidase enzyme but was not affected by catalase and lysozyme. This confirms the peptide nature of the produced bacteriocins (anti-microbial peptides) as proteinase k and peptidase enzymes are known to denature proteins. The catalase and lysozyme did not affect the activity of the bacteriocins as these enzymes do not lyse proteins. This result is similar to those obtained by Abdelahhad *et al.,* (2013) who produced the bacteriocin salivaricin from *Streptococcus salivarius* NU10 and tested the action of the enzymes proteinase k, lyticase, catalase and peptidase on it. The bacteriocin produced by Abdelahhad *et al.,* (2013) was denatured by proteinase k and peptidase but not by catalase and lyticase.

Thermal Stability Test of Produced Bacteriocin

The produced bacteriocins were subjected to temperatures of 40, 60, 80 and 100°C for 20 minutes in a water bath. The heated bacteriocin was cooled to room temperature (25°C) and tested for its antimicrobial activity using the well in agar assay method. The result is shown in Table 6.

Table 6. Thermal Stability Test of Produced Bacteriocin

Temperature	Antimicrobial agent			
	Bacteriocin from *Lactobacillus plantarum*	Bacteriocin from *Lactobacillus plantarum* (UNTREATED CONTROL)	Bacteriocin from *Pediococcus acidilactici*	Bacteriocin from *Pediococcus acidilactici* (UNTREATED CONTROL)
40°C	26mm	26mm	17mm	18mm
60°C	25mm	25mm	18mm	18mm
80°C	26mm	25mm	18mm	17mm
100°C	24mm	25mm	16mm	17mm

The antimicrobial activity of the bacteriocins was not affected by treatment up to 100°C. This confirms the temperature stability of the bacteriocins and their ability to remain active after being subjected to high temperatures. This result is in tandem with that recorded by Abdelahhad *et al.,* (2013) who did not observe any change in the antimicrobial activity of the bacteriocin salivaricin from *Streptococcus salivarius* NU10 when subjected to temperature ranges of 4-80°C in one hour.

pH Stability Test of Produced Bacteriocin

The produced bacteriocins were adjusted to different pH ranges of 2,4,8 and 10 respectively using concentrated HCl and NaOH. 40, 60, 80 and 100°C for 20 minutes in a water bath. This was incubated for 2 hours at 25°C before determining the antimicrobial activity by agar well diffusion assay method. The result is shown in table 7. below.

Table 7. pH stability test of produced bacteriocin

pH	Antimicrobial agent			
	Bacteriocin from *Lactobacillus plantarum*	Bacteriocin from *Lactobacillus plantarum* (UNTREATED CONTROL)	Bacteriocin from *Pediococcus acidilactici*	Bacteriocin from *Pediococcus acidilactici* (UNTREATED CONTROL)
2	24mm	26mm	16mm	18mm
4	24mm	26mm	17mm	18mm
8	26mm	26mm	18mm	18mm
10	25mm	26mm	18mm	18mm

The antimicrobial activity of the bacteriocins was not affected by increase in acidity or alkalinity. This confirms the pH stability of the bacteriocins and their ability to remain active within different pH ranges. This result is in tandem with that recorded by Goh and Phillip (2015) who did not observe a reduction in the antimicrobial activity of the bacteriocins produced by *Weissella confusa* A3 against *E.coli* after adjusting the pH to between 2 and 10.

CONCLUSION

Bacteriocins of *Pediococcus acidilactici* and *Lactobacillus plantarum* isolated from cow milk in Bayelsa state was produced in MRS broth after an enrichment step of pasteurization to remove saprophytic organisms and then fermentation for 48 hours. These bacteriocins displayed bacteriostatic and bactericidal activity against *E. coli,* with a minimum bactericidal concentration of 25µg/ml which is lower than that of the conventional antibiotics chloramphenicol (50µg/ml). The antimicrobial peptide nature of the produced bacteriocin was proved through its inactivation by the proteolytic enzymes proteinase K and peptidase. Furthermore, the produced bacteriocins were shown to be heat stable up to a temperature of 100°C and stable over a wide pH range of 2-10.

This study has revealed additional information on antimicrobial peptides produced by *Pediococcus acidilactici* and *Lactobacillus*

plantarum that can expedite the further development of specific antimicrobial agents in this era of increasing antibiotic resistance.

It is recommended that further studies should be conducted to first purify and characterize the produced bacteriocins and optimize the bacteriocin producing organisms for improved yield. Also, the mode of action of these bacteriocins should be investigated to understand their antimicrobial activity and facilitate its development. This is because these novel bacteriocins isolated from an indigenous milk source has potentials as an antimicrobial in the food industry for food preservation or as a new antibiotic in the health sector.

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