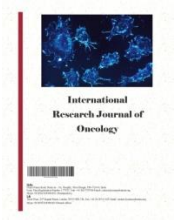

International Research Journal of Oncology

5(3): 10-19, 2021; Article no.IRJO.74057

Determining the Efficiency of Bacteriocins using Enzymes, at Varied pH, Temperature and the Ability of its Immunity Gene to Resist Antibioics Susceptibility

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Lomas Kumar Tomar, National University of Ireland, Ireland.
Reviewers:
(1) Damtew Bekele, Ambo University, Ethiopia.
(2) Seema Garcha, Punjab Agricultural University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/74057

Original Research Article

Received 12 July 2021
Accepted 22 September 2021
Published 28 September 2021

ABSTRACT

The uses of Lactic acid bacteria as probiotics have received considerable attention as a suitable alternative to antibiotics. Bacteriocins produced by LAB are used as biopreservative in foods, with a resultant reduction in the use of chemical preservatives. A typical bacteriocin contains a toxin gene, an immunity gene (which confers immunity to the producing organism), and a lysis gene, which encodes a protein that aids in toxin release from the producing cell. The aim of this research is to determine the efficiency of bacteriocin despite subjecting to different treatment and to find out the ability of bacteriocin producing cell to resist treatment with antibiotics inorder to be considered as a potential effective antitumour agent. Bacteriocins produced by *Weissella cibaria* CBA3612, *Lactobacillus plantarum* LZ95, *Lactobacillus fermentum* 3872, *Leuconostoc pseudomesenteroides* SRA3 and *Lactobacillus plantarum* WCFS1 were subjected to several treatment with proteinase k and catalase, adjusted to pH 5,7,9 at temperature 50^0C, 75^0C and 90^0C then susceptibility of the lactic acid bacteria were tested on antibiotic disc and incubated. After incubation, the bacteriocins were deactivated by the enzymes. Bacteriocins from *Lactobacillus plantarum* showed high

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efficiency on *E. coli* and *Staphylococcus aureus* at pH 5 and showed thermostability but more effective at 50^0C. Most of the LAB were resistant to the antibiotics, this could be attributed to the presence of immunity gene protecting its bacteriocin-producing cell.

Keywords: Bacteriocin; lactic acid bacteria; probiotics; antibiotics; anti tumour bio-preservatives; immunity; genes; cell; enzymes; temperature.

1. INTRODUCTION

Bacteriocins are antimicrobial peptides produced by many lactic acid bacteria (LAB), which are directed mainly to inhibit the growth of related species or species with the same nutritive requirements. Bacteriocins can be used in three different ways in fermented foods: *in situ* production by the addition of a bacteriocinogenic lactic culture, as a co-culture and by the direct addition of the bacteriocin [1]. Bacteriocins are considered an attractive compound to use in the food and pharmaceutical industries for preventing spoilage of food and the growth of pathogenic bacteria [2].

Generally, bacteriocins are peptides or proteins and different bacteriocins have dissimilar antimicrobial spectra [3]. In animal farming, bacteriocins play an important role in controlling the overgrowth of potentially pathogenic bacteria. The lack of maternal bacterial flora or the induction of a proper immune system in newly hatched broiler chickens makes them susceptible to infection [4]. The use of *E. faecium* bacteriocins after hatching increases the survival rate of chickens infected with the poultry pathogen *S. pPullorum* and *E. coli* microcins contribute to the destruction of *S. typhimurium* in adult chickens. There are reports that the introduction of colicin-producing bacteria into the rumen of cows reduces the number of intestinal pathogens in animals [5]. As a rule, probiotic mixtures based on sorbic acid and bacteriocins or bacteriocin-producing crops are used in animal farming. These mixtures are used as additives in feed and drinking water.

Probiotics are non-pathogenic and non-toxic strains, beneficial to the host animal, that are able to survive and maintain metabolic activity in the intestinal environment and remain stable and viable for long storage periods [6]. Probiotics demonstrate the potential for antimicrobial production, competitive pathogen destruction, competition for nutrients, and immune system modulation. Many antibacterial substances, such as bacteriocins, short-chain fatty acids, and hydrogen peroxide are produced by probiotics to inhibit gastrointestinal pathogens.

Currently, many probiotics are used in everyday life, including lactic acid bacteria, non-pathogenic strains of E. coli, Bacilli, and yeast [7]. Although the main application of bacteriocins is in the food industry to combat spoilage and foodborne bacteria, in recent years the use of bacteriocin has been shifted to the diagnosis and treatment of cancer, as well as resistance to plant diseases and growth stimulation [8]. Class I also includes a group of compounds—thiopeptides that have multiple biological activity (antibacterial, antiviral, antiparasitic, and immunosuppressive). Antibacterial thiopeptides interfere with protein synthesis by binding to the 50S ribosome subunit or elongation factors [9]. Thiopeptides are usually active in the nanomolar range, but their poor water solubility and low bioavailability make it difficult to use them in clinical settings, despite their high activity. Thiopeptide derivative GE2270A is currently the only bacteriocin of this type undergoing clinical trials in the treatment of gastrointestinal infections caused by *Clostridium difficile* [10]. The mannose phosphotransferase system (Man-PTS) is the main mannose permease in bacteria, but it is also a known receptor for class IIa bacteriocins (pediocin-like group), as well as class IId lactococcin A (LcnA) and lactococcin B (LcnB). Class IIa bacteriocins are potent against Clostridium difficile, but not against Lactococcus spp. In contrast, LcnA-like bacteriocins act only against Lactococcus lactis strains. Garvicin Q (GarQ) is a class IId bacteriocin with little similarity to LcnA-like bacteriocins and a relatively broad antimicrobial spectrum, including Clostridium difficile and Lactococcus spp among others [10].

A group of bacteriocins similar in structure to thiopeptides are modified thiazole/oxazole-microcins-boromycins. Their distinctive features include the presence of macrocyclic amidine, decarboxylated C-terminal thiazole, and several rare β-methylated amino acid residues. The botromycins discovered to date are produced by bacteria of the genus Streptomyces spp. and are potent agents against multidrug-resistant microorganisms, such as MRSA and vancomycin-resistant enterococci (VRE). Botromycins also inhibit protein synthesis by

interacting with the bacterial 50S ribosomal subunit [11,12].

The most widely studied natural source of bacteriocins is soil. Many bacteriocins extracted from rhizosphere and soil bacteria are used for plant protection. Thus, Pseudomonas putida BW11M1, isolated from basal microbial communities of banana, produces putidacin, which destroys the plant pathogen P. putida GR12-2R3. Other examples include bacteriocin Bac 14B (B. subtilis 14B), which is effective against the causative agent of a disease caused by Agrobacter tumefaciens, and Bac GM17 (B. clausii GM17), which has broad-spectrum antibacterial and antifungal activity. In addition, some plant pathogens produce antibacterial substances. The phytopathogenic strain Erwinia carotovora NA4 isolated from affected vegetables and fruits produces euriniocin NA4, a pathogen of tomato Clavibacter michiganensis ssp. Michiganensis—bacteriocin michiganin A, which inhibits the growth of C. michiganensis subsp. Sepedonicus, which causes ring rot of potatoes. Most of the soil bacteriocins are synthesized by representatives of the genus Bacillus and are actively used as bioinsecticides and biopesticides, as well as growth stimulants [13].

Many physicochemical factors seemed to affect bacteriocin production as well as its activity, despite the fact that antimicrobial peptides have an inhibition spectrum narrower than that of antibiotics [1]. The study is relevant, since it identifies information related to the detection of Bacteriocin efficiency in terms of biopreservatives and possibly treatment of diseases.

2. MATERIALS AND METHODS

2.1 Bacteriocin Production

Bacteriocin produced and screened from our previous research was preserved at 4ºC in the department of Microbiology, Kaduna State University and used for further studies [14].

2.2 Isolation of Lactic Acid Bacteria (Lab)

Lab isolated from wara, daddawa, ogi and nono (*Lactobacillus plantarum* WCFS1, *Lactobacillus plantarum* LZ95, *Weissella cibaria* CBA3612, *Lactobacillus fermentum* 3872 and *Leuconostoc pseudomesenteroides* SRA3) from our previous research (14) were used for this study.

2.3 Effect of Enzymes on Antimicrobial Activity of the Bacteriocins

The effect of enzymes on the antimicrobial activity of the Bacteriocin produced was carried out using the method of Chen and Yanagida [15]. 10 mls of the crude bacteriocin was treated with 100 μλ of proteinase K (pH 7) and catalase (pH 7). While sterile distilled water was used as control. After treatment with the various enzymes, antimicrobial activity was assayed by agar well diffusion [16].

2.4 Assessment of the Effect of pH on Antimicrobial Activity of the Bacteriocins

The effects of pH (native and ranging from 4 to 10) on antibacterial properties of the bacteriocin were studied. The cell free supernatant of Bacteriocins was divided into two parts: one with native pH (pH= 2 to 4 resulting from bacterial growth on broth medium) and another with altered pH (pH= 5 to 9 by addition of NaOH). After these treatments, the residual antibacterial activity was determined by the agar well diffusion method [16].

2.5 Heat Stability of Produced Bacteriocin

A volume of 5ml of the bacteriocins were poured in different test tubes and heated in a water bath at 50, 75 and 90^{0}C for 15 minutes. The heat treated bacteriocin samples were then assayed for antimicrobial activity by the agar well diffusion method [17].

2.6 Determination of Antibiotic Susceptibility Pattern on the LAB

The standardized concentrations (Mcfarland standard 0.5) of inocula of LAB culture were inoculated in MRS broth at 37 °C for 24 hours. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the MRS agar plate and allowed to dry before placing the diffusion discs containing antibiotics. Susceptibility of the LAB was performed by disc diffusion [18]. method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines using the following antimicrobial drugs: erythromycin (10 µg), pefloxacin (10 µg), streptomycin (30 µg), amoxacillin (30 µg), ciprofloxacin (10 µg), septrin (30 µg), gentamycin (30 µg), rocephin (25 µg), zinnacef (20 µg), and ampiclox (30 µg) were placed on the surface of the agar plates. Precaution was taken to ensure

that there was uniform contact between the antibiotic disc and agar plate. The plates were then incubated at 37 °C for 24 hours [17].

3. RESULTS

3.1 Characteristics of the Produced Bacteriocin by Lactic Acid Bacteria

Temperature and pH played an important role in the antimicrobial properties of the bacteriocins. The temperature of the bacteriocin adjusted to (50, 75 and 90^{0}C) showed thermostability of the bacteriocins produced. The maximum inhibition zone was measured at 50^{0}C while there was very low activity recorded at 100^{0}C. Bacteriocin produced by W3 and D3 showed high activity to *E. coli* and *S. aureus* despite heat applied. Increase in temperature (90^{o}C for 20 minutes), decreased the effectiveness of bacteriocin on methicillin resistant *S. aureus* as shown in Table 1,2, and 3. pH alteration between 5 to 7 had no effect on antibacterial activities which arose due to the presence of bacteriocin components, but in the alkaline range (8 to 10), these activities were reduced. The maximum inhibition zone was measured at pH5 by all bacteriocin produced (D1, D3, O3, W3, N2). Many of the bacteriocins and bacteriocin-like substances seem considerably more tolerant of acid than alkaline pH extremes as seen in Figs 1,2,3 and 4. When the bacteriocins were subjected to enzymatic reaction, they were denatured by the enzymes.

Table 1. Inhibitory activity of bacteriocin at 50^{0}C at different time interval (mm)

E.coli	**D1**	**D3**	**O3**	**W3**	**N2**
10 mins	14.03±0.35c	14.46±0.05 ^{c}	8.00±0.00 ^{a}	10.50±0.45^{b}	12.50±0.54 ^{bc}
20 mins	13.34±0.45 ^{b}	13.94±0.35 ^{b}	0	7.05±0.46 ^{a}	12.05±0.77 ^{b}
30 mins	12.36±0.19^{ab}	12.50±0.07 ^{b}	0	0	10.00±0.00 ^{a}
S. aureus					
10 mins	10.00±0.00^{b}	14.00±0.10 ^{c}	7.05±0.45 ^{a}	7.00±0.00 ^{a}	11.50±0.34^{b}
20 mins	0	14.00±0.55 ^{c}	7.00±0.30 ^{a}	0	10.50±0.74^{b}
30 mins	0	14.00±0.00 ^{b}	7.55±0.43 ^{a}	0	8.00±0.54 ^{a}
Methicillin_resistant_*S. aureus*					
10 mins	0	7.00±0.56 ^{a}	7.05±0.54 ^{a}	7.00±0.05 ^{a}	7.35±0.36 ^{a}
20 mins	0	7.50±0.46 ^{a}	7.50±0.35 ^{a}	7.00±0.54 ^{a}	7.56±0.86 ^{a}
30 mins	0	0	0	0	0
Klebsiella_pneumonia					
10 mins	0	12.50±0.74 ^{b}	14.30±0.35^{c}	10.05±0.46^{a}	15.05±0.65^{c}
20 mins	0	10.00±0.00 ^{b}	13.06±0.75^{c}	8.00±0.05 ^{a}	13.55±0.35^{c}
30 mins	0	10.00±0.00 ^{b}	11.55±0.26^{c}	7.05±0.0.95^{a}	12.05±0.45^{c}

Data are mean ± SEM of triplicate determination. Data followed by different superscript alphabet along the same row are significantly different (p<0.05); D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides, No inhibition (0)

Table 2. Inhibitory activity of bacteriocin at 70^{o}c at different time interval (mm)

E.coli	**D1**	**D3**	**O3**	**W3**	**N2**
10 mins	14.05±0.34 ^{c}	14.05±0.45 ^{c}	0	7.57±0.05 ^{a}	10.54±0.64^{b}
20 mins	11.44±0.57 ^{b}	12.96±0.43 ^{b}	0	7.06±0.54 ^{a}	8.06±0.45 ^{a}
30 mins	11.06±0.34 ^{c}	10.35±0.57 ^{b}	0	0	7.55±0.54 ^{a}
S. aureus					
10 mins	0	14.30±0.34 ^{c}	7.05±0.54 ^{a}	0	10.05±0.35 ^{b}
20 mins	0	12.55±0.67 ^{b}	7.55±0.55 ^{a}	0	8.00±0.00 ^{a}
30 mins	0	10.50±0.50 ^{b}	0	0	7.50±0.55 ^{a}
Methicillinresistant*S. aureus*					
10 mins	0	0	7.05±0.45	0	0
20 mins	0	0	7.55±0.55	0	0
30 mins	0	0	0	0	0
K. pneumonia					
10 mins	0	12.05±0.23 ^{b}	14.35±0.45 ^{c}	10.00±0.50^{a}	15.55±0.46^{c}
20 mins	0	10.55±0.54 ^{b}	12.06±0.06 ^{c}	7.05±0.56 ^{a}	12.50±0.34^{c}
30 mins	0	8.05±0.56 ^{a}	10.07±0.43 ^{b}	0	12.54±0.36^{c}

Data are mean ± SEM of triplicate determination. Data followed by different superscript alphabet along the same row are significantly different (p<0.05); D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides, No inhibition (0)

Table 3. Inhibitory activity of bacteriocin at 90^{0}C at different time interval (mm)

E.coli	D1	D3	O3	W3	N2
10	10.05±0.46 [b]	7.05±0.56 [a]	0	7.50±0.46 [a]	7.54±0.46 [a]
20	8.00±0.46 [a]	0	0	0	0
30	7.05±0.35 [a]	0	0	0	7.57±0.45 [a]
S. aureus					
10	0	8.50±0.35 [a]	0	0	8.45±0.35 [a]
20	0	7.25±0.42 [a]	0	0	7.64±0.43 [a]
30	0	7.67±0.76 [a]	0	0	7.78±0.54 [a]
Methicillin resistant *S. aureus*					
10	0	0	0	0	0
20	0	0	0	0	0
30	0	0	0	0	0
Klebsiella Pneumoniae					
10	0	7.05±0.32 [a]	7.50±0.34 [a]	0	9.05±0.46 [b]
20	0	0	0	0	7.00±0.35
30	0	7.45±0.65 [a]	7.56±0.55 [a]	0	7.55±0.46 [a]

Data are mean ± SEM of triplicate determination. Data followed by different superscript alphabet along the same row are significantly different (p<0.05); D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides, No inhibition (0)

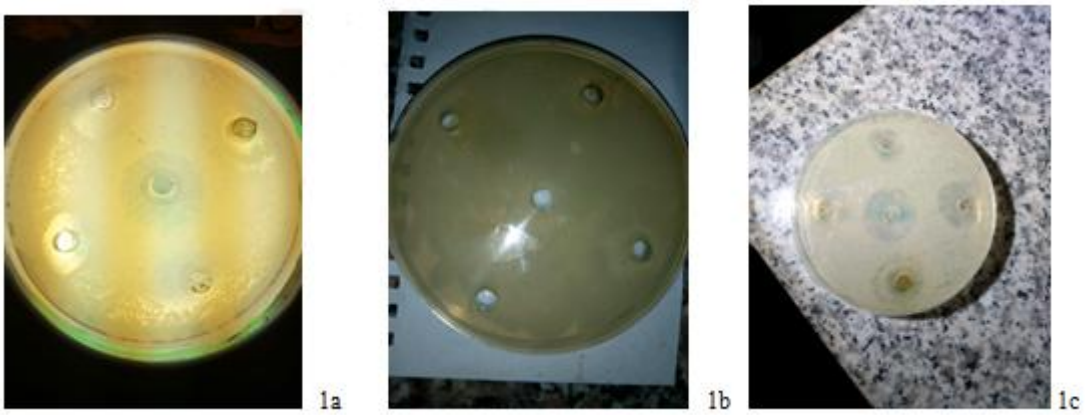

Plate 1. Bacteriocin activity on test bacteria (1a: E.*coli*, 1b: *Klebsiella*, 1c: S.*aureus*) at pH 5

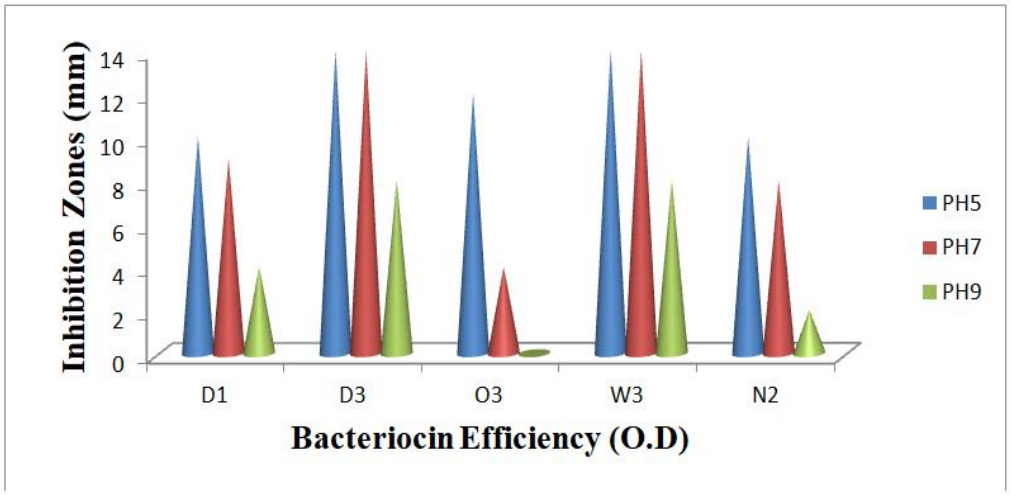

Fig. 1. Effects of Bacteriocin on *E.coli* at different pH

D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides

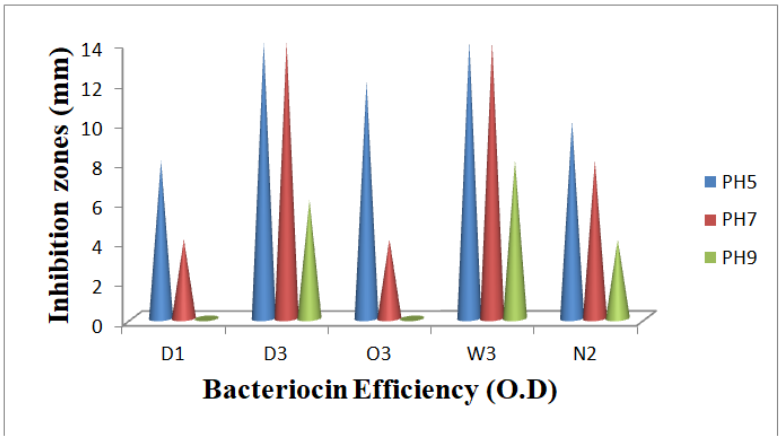

Fig. 2. Effects of Bacteriocin on *Staphylococcus aureus* at different pH

D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides,

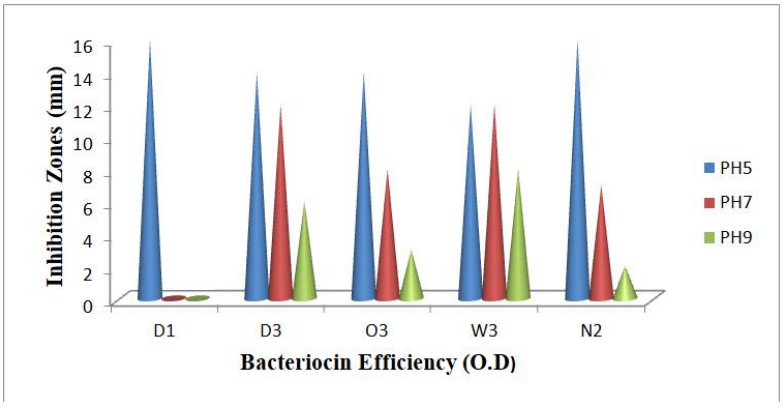

Fig. 3. Effects of Bacteriocin on Methicilene resistant *Staphylococcus aureus* (MRSA) at different pH

D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides

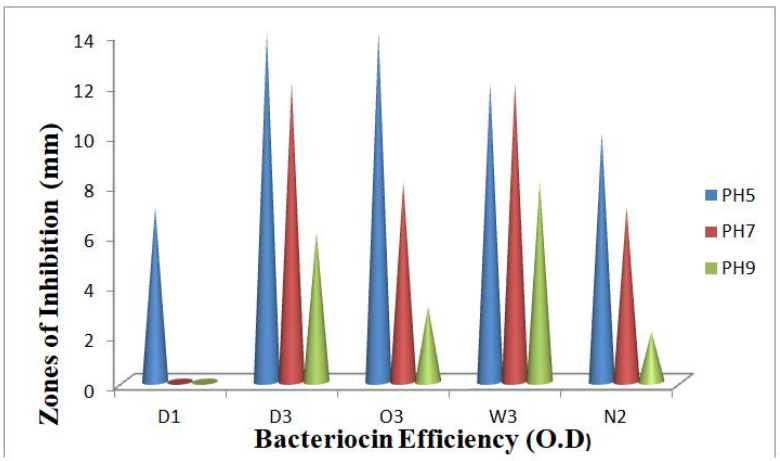

Fig. 4. Effects of Bacteriocin on *K.pneumoniae* at different pH

D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides

Table 4. Antibiotic susceptibility pattern of the lactic acid bacteria in (%)

Antibiotics (μg)	D1	D3	O3	W3	N2
Gentamycin (10)	94.3	92.5	93.8	94.2	90.1
Rocephin (25)	85.7	89.5	92.0	89.4	59.8
Ciprofloxacin (10)	95.5	98.2	96.0	98.3	74.3
Streptomycin (30)	82.6	94.0	90.3	94.0	58.0
Septrin (30)	83.2	84.5	93.0	84.7	53.4
Erythromycin (10)	90.4	89.5	93.6	89.0	58.2
Pefloxacin (10)	13.0	10.4	13.4	14.2	22.3
Zinnacef (20)	20.0	15.0	18.3	23.7	20.0
Ampiclox (30)	15.2	18.0	13.0	23.0	13.4
Amoxacillin (30)	12.0	12.8	10.5	13.2	10.4

D1= Weissella cibaria, D3= Lactobacillus plantarum, O3= Lactobacillus fermentum, W3= Lactobacillus plantarum, N2= Leuconostoc mesenteroides, Percentage ranging from (≥ 90 %) is considered sensitive while intermediate, I (50-60 %) and resistant, R (≤ 25 %) respectively.

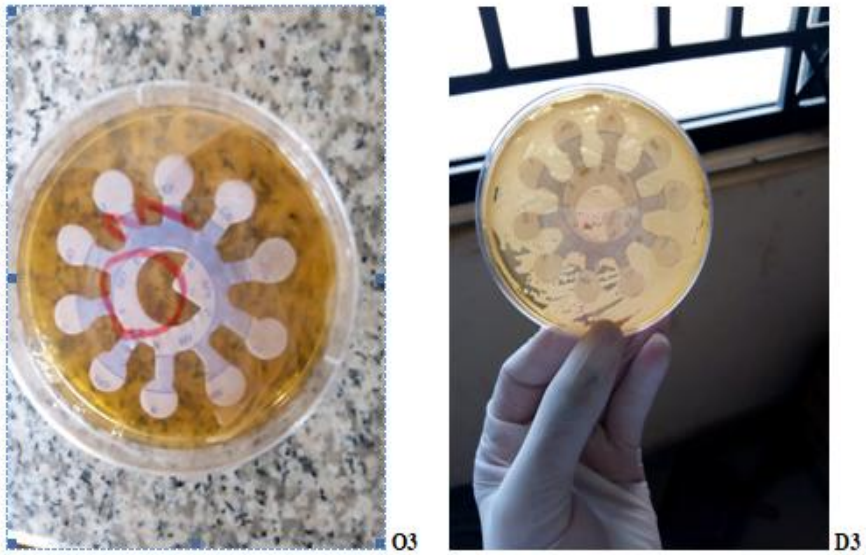

Plate 2. Antibiotic Sensitivity on *Lactobacillus fermentum* (O3) and *Lactobacillus plantarum* (D3)

3.2 Antibiotic Susceptibility Pattern of the Lactic Acid Bacteria

The antibiogram of the identified LAB is shown in Table 4. Among the tested antibiotics the highest susceptibility of the lactic acid bacteria was shown by Ciprofloxacin, Gentamycin, Streptomycin followed by Erythromycin and Rocephin. *Lactobacillus plantarum* which was the predominant isolate gave high susceptibility to Ciprofloxacin 98.3%, Gentamycin 94.3 and Streptomycin 94.0. All the zones were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S (≥ 90 %); intermediate, I (50-60 %) and resistant, R (≤ 25 %), respectively. Growth of isolates was not inhibited by pefloxacin, zinnacef, amoxicillin and ampiclox.

4. DISCUSSION

Heating the bacteriocin to 50^0C at different time interval, with pH 4 (Table 1) did not affect the efficiency of the bacteriocin. After heat treatment of bacteriocin from *L. plantarum* (Table 2) at 70^0C for 20 min and 90^0C for 20 min (Table 3) there was no change in their activity. The thermo-tolerance feature might be related to the molecular structure of the bacteriocin, usually composed by small peptides and the several positions of their genes. Similar observation was reported by Begley *et al*. (2009). The bacteriocins produced were rapidly digested by the enzymes. This gives credence to the theory by Begley [19] who revealed that sensitivity to proteolytic enzymes of bacteriocins evidences their protein nature.

The alteration of pH (fig 1) from 5, 7 and 9 had little effect on the efficiency of the bacteriocin to food pathogens. This result is similar to Kim [20] who also discovered that *Lactobacillus* species had their highest antibacterial activity at pH 7, whereas a considerable decrease was observed at both acidic and alkaline pH as also reported by Bibalan [21] stated that in the alkaline range (8 to 10), the antibacterial activities were reduced. The alteration of pH reduced the efficacy of the bacteriocin on methicillin resistance *Staphylococcus aureus* at pH 7.0 and no activity at pH 9.0. The widest diameter (Fig 2) 14mm zone of inhibition was obtained in bacteriocin from *Lactobacillus plantarum* LZ95 and WCFS1 on *Staphylococcus aureus* and 14mm on *E. coli*, while the smallest diameter (4mm) was obtained by bacteriocin from *Lactobacillus fermentum*. The result is in conformity to the report by Savadogo [22], who reported that bacteriocins from *Lactobacillus fermentum* showed little activity on *Staphylococcus aureus* (9mm) and *E. coli* (9mm). Bacteriocin produced by *Leuconostoc mesenteroides* showed relatively high efficiency 10mm on *E.coli* and 10mm on *Staphylococcus aureus* which is similar to the report by Savadogo [22] that measured (10mm) for *Staphylococcus aureus* and (8mm) for *E. coli*.

Test of lactic acid bacteria against antibiotic susceptibility (Table 4) showed high sensistivity to gentamycin, rocephin, ciprofloxacin, streptomycin, septrin and erythromycin but were all resistant to pefloxacin, zinnacef, ampiclox and amoxicillin. According to Chaudhary and Saharan [23], the study of antibiotic resistance pattern is important for selection and evaluation of safe probiotic strain. Udhayashree [16] reported that *Lactobacillus fermentum* showed resistance to streptomycin and ciprofloxacin. This finding is similar to previous reports by Halami [24] which stated that LAB are normally resistant to the principlale types of antibiotics. While previous reports from Gueimonde [25],reported that there was sensistivity to all penicillins and β-lactamase studied, i.e. amoxicillin, ampicillin, augmentin, and penicillin G. However, it showed resistance to a second-generation cephalosporin antibiotic, cefaclor. When inhibitors of the protein synthesis were used, it exhibited susceptibility or moderate susceptibility to chloramphenicol, erythromycin, clindamycin, and tetracycline. However, it displayed resistance to kanamycin and streptomycin.

Recent research by Bindiya [26] showed that Bacteriocin possesses high variety of chemical structures which allows it to affect various vital functions of a living cell (transcription, translation, replication, and cell wall biosynthesis), but most act by forming membrane channels or pores that violate the energy potential of the cell[2]. From this research, it can be deduced that Bacteriocin that was able to show high activity despite pH and temperature alterations and the producing cell showed high resistance to antibiotics should be considered as a potential anti cancer agent when used properly to replace chemical preservatives in food or pharmaceutical industries.

5. CONCLUSION

The Bacteriocins tends to be more active against the food pathogens at pH 5 and 50^0C, it could be concluded that bacteriocin can be used to control these contaminants. The stability of the bacteriocin after the change in temperature and pH indicated that the bacteriocins belong to the class II bacteriocin. Enzymes used in denaturing the bacteriocins deactivated the protein when its activity was assayed.

Bacteria present in fermented foods comprise a potential source of high antibiotic resistance genes which when ingested could influence the establishment and dynamics of antibiotics resistance bacteria in our body. It is thus important to determine the effect of antibiotics on the growth of probiotic strains, especially if the product is considered as a possible probiotic products but this could be an advantage if the product is for treatment.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The authors appreciate Microbiology department of Kaduna State University for provision of materials that were appropriate to achieve this work. The efforts of Mr Ahmed Yahaya to provide funds and encouragement to ensure the manuscript for this paper was prepared and processed is also appreciated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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