

Antimicrobial Activity of Bacteriocins Produced by Lactic Acid Bacteria Against Bacteria Isolated From Abattoir Wastewater

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Abstract. This study investigated the bacterial composition and the effect of bacteriocin on bacteria isolated from abattoir wastewater. We isolated bacteria from abattoir wastewater using standard microbiological methods. We isolated lactic acid bacteria from fermented food waste using standard microbiological methods and confirmed their identity by 16S rRNA sequencing. We produced bacteriocins from lactic acid bacteria by submerged fermentation and purified them by ammonium sulphate precipitation and column chromatography. We assayed bacteriocin extracts for antibacterial activity using the agar well diffusion method. Bacteria isolated from abattoir wastewater were *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Salmonella sp*, *Staphylococcus aureus* and *Bacillus cereus*. We identified the lactic acid bacteria that showed significant inhibition as *Lactobacillus plantarum* and *Lactobacillus fermentum*. *Lactobacillus plantarum* and *Lactobacillus fermentum* bacteriocins both showed the highest inhibition against *Staphylococcus aureus*, with zones of inhibition of 16 mm and 15 mm, respectively. Both bacteriocins showed the highest antagonistic activity against isolated bacteria at pH 4 and 40 °C. The activity of both bacteriocins decreased as storage time increased. The results indicate that bacteriocins exhibit significant inhibitory activity against bacterial strains in abattoir wastewater and offer an alternative approach for treating abattoir wastewater.

Keywords: Antimicrobial; Abattoir; Wastewater; Lactic Acid Bacteria; Bacteriocin.

INTRODUCTION

Improper wastewater management has increased the discharge of untreated wastewater into the environment and has amplified natural water resource pollution [1]. The food industry typically uses large amounts of water. Dairy, beverage production, vegetable, fruit, oilseed, seafood, poultry, and other meat-processing processes consume large volumes of freshwater. From 2018 to 2019, the global meat market increased by 6% due to rising per capita meat consumption [2]. Meat production in abattoirs is a major source of animal protein globally, and it generates large amounts of waste. Government agencies should ensure that abattoir staff follow standard operating practices, including maintaining personal hygiene and using personal protective equipment (PPE) while carrying out their

activities, to prevent outbreaks of foodborne diseases. In Nigeria, regulation enforcement has been dismal, resulting in a very pitiable state of affairs in abattoirs across the country. Abattoirs across the country generally suffer from poor drainage systems, inadequate education and training for butchers and workers, poor waste management, inadequate storage facilities, insufficient inspection and monitoring of animals and meat products, and unreliable power supply, among other deficiencies [3]. These inadequacies mean consumers may be exposed to meat contaminated with foodborne pathogens.

High demand for meat correspondingly increases freshwater consumption by meat processing plants. Abattoirs, as a small industry, use large volumes of water to wash and remove faecal waste, stomach contents, and blood. These pro-

cesses require an evacuation system separating solid waste and fats, as well as treatment. Wastes generated from abattoirs usually consist of blood, oil, mineral and organic solids, salts, and chemicals added during the handling operations. The lack of facilities to handle this solid waste and wastewater is a major problem in most abattoirs in Nigeria. Wastewater generated from abattoirs can significantly increase nitrogen, phosphorus, and total solids in receiving water bodies, causing water pollution [4]. Slaughterhouses are known globally to contaminate the environment directly or indirectly through several procedures and may contribute to river deoxygenation, groundwater contamination, and climate change. These pose environmental safety concerns and reflect a public health concern. Wastewater from abattoirs is a major source of environmental pollution. Researchers have previously isolated and identified pathogenic bacteria such as *Clostridium perfringens*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Proteus sp.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella sp.*, *Staphylococcus aureus*, and fungi such as *Penicillium sp.*, *Saccharomyces sp.*, and *Aspergillus sp.* from abattoir effluents in Nigeria [3]. These pathogens cause many food- and water-borne infections. Likewise, animals that come into contact with these pathogens can serve as vectors, transferring them to humans and leading to zoonotic infections. Waste management practices in many Nigerian abattoirs are poor, posing public health and environmental hazards. Open waste disposal and direct discharge of effluents into water bodies are common effluent-disposal methods in many Nigerian abattoirs, exposing nearby populations to serious health risks. Abattoir wastewater can also contribute to greenhouse gas emissions, especially when piled up in ponds. Microbial activity produces methane, which intensifies the greenhouse effect. These emissions increase global warming, leading to climate change and extreme weather events.

Conventionally, abattoir wastewater is treated by physical, chemical and biological methods before discharge or reuse. Several studies have examined and treated this type of wastewater using treatment plants, either through aerobic methods or anaerobic techniques. Other treatment methods for purifying slaughterhouse wastewater include sand filtration and electrocoagulation. However, these methods reduce indicator organisms by only about one logarithmic span.

A new treatment approach could use extracellular extracts, such as bacteriocins, to treat waste. Bacteriocins are antimicrobial peptides produced by certain bacteria. Bacteriocins produced by lactic acid bacteria are of particular interest because they can control contaminants in food, feed, or waste [5]. Bacteriocins are ribosomally synthesised bactericidal or bacteriostatic peptides produced by certain bacteria [6]. Although Gram-negative bacteria can produce bacteriocins, the vast majority of bacteriocins characterised so far are produced by Gram-positive bacteria. Lactic Acid Bacteria (LAB) are Gram-positive, non-sporulating, facultative anaerobic, cocci or rods which produce lactic acid during fermentation. They are mostly used as starter cultures in producing acidophilus milk, yoghurt, buttermilk, cottage cheese, hard cheeses, and soft cheeses [7]. They also process meats, alcoholic beverages, and vegetables to produce products such as sausages, cured hams, wine, beer, fortified spirits, pickles, and sauerkraut. LAB are generally recognised as safe, which has attracted industrialists to use them in developing new products. LAB also produce other metabolites, such as organic acids, hydrogen peroxide, and vitamins, during fermentation.

This research investigated the efficiency of bacteriocins produced by lactic acid bacteria in inhibiting bacteria isolated from abattoir wastewater.

METHODS

Collection of Samples. The researchers aseptically collected abattoir wastewater samples from the Ubakala abattoir in Umuahia, Abia State, using sterile containers. We aseptically collected local fermented food products (fufu and ogi) and effluent samples from cassava and maize processing plants in Umuahia, Abia State. We took them to the Microbiology Laboratory at Michael Okpara University, Umudike, for analysis.

Isolation and Purification of Bacteria from Wastewater Samples. We prepared ten-fold serial dilutions of the abattoir wastewater samples and inoculated them onto Nutrient agar, MacConkey agar, Salmonella–Shigella agar, and Eosin Methylene Blue agar. We incubated the culture plates at 37°C, subcultured distinct colonies to confirm their purity, and identified the isolates.

Identification of Bacterial Isolates. We identified bacterial isolates based on morphological characteristics and biochemical tests. We observed

morphological characteristics for each bacterial colony after 24 hours of growth. We performed Gram staining, Endospore staining, motility tests, and biochemical tests to identify the bacterial isolates. The biochemical tests included the catalase test, Coagulase test, Methyl red test, Indole test, Citrate test, Voges-Proskauer test, Oxidase test, and Sugar fermentation tests [8].

Isolation of Lactic Acid Bacteria (LAB). To isolate LAB, 0.1 mL aliquots of fermented Fufu and Ogi effluent samples were plated on MRS agar plates after ten-fold serial dilution in 0.1% peptone water. Samples were incubated in an anaerobic jar at 37 °C for 72 hours. Distinct colonies were sub-cultured onto MRS agar to obtain pure cultures [9].

Characterisation of Lactic Acid Bacteria (LAB). Lactic acid bacterial strains were characterised based on their morphological, biochemical and physiological properties. We examined each isolate microscopically after Gram staining and Spore staining for identification. After morphological identification, we carried out biochemical tests such as the catalase test, oxidase test, and sugar fermentation tests. Probable identities were confirmed using Bergey's Manual of Systematic Bacteriology [8].

Antibacterial screening of lactic acid bacteria against isolated bacteria. We evaluated the antibacterial activity of the LAB isolates against the isolated pathogens using the agar well diffusion method. We seeded 5 mL of an overnight culture of each test organism onto Mueller–Hinton agar plates. We cut wells into the agar using a sterile 5 mm diameter cork borer and dispensed 0.03 mL of each LAB isolate into the respective wells. We then incubated the plates at 37 °C for 24 hours and measured the zones of inhibition after incubation [9].

Molecular Identification of Lactic Acid Bacteria. Lactic acid bacteria exhibiting significant inhibitory activity were identified using prokaryotic 16S rRNA universal primers 27F (50 - AGAGTTTGATCCTGGCTCAG-30) and 1492R (50 - GGTTACCTTGTTACGACTT-30) performed with the following PCR procedure: 3 min at 95 °C for pre-deformation; 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 90 s; and a final step of 72 °C for 5 min. Inqaba Biotech Company sequenced the PCR products. We evaluated homology between the obtained sequences and those in GenBank using BLAST analysis on the NCBI website. [10].

Bacteriocin Extraction and Purification. We produced bacteriocin according to the following protocol: We inoculated 0.1% of each lactic acid bacteria isolate into 60 mL of MRS broth and incubated the cultures at 37 °C for 24 hours. We then centrifuged the cultures at 10,000 × g for 30 minutes. We treated the cell-free supernatant with Chelaton-EDTA III and heated it at 80 °C for 10 minutes to remove other organic substances. We then filtered the supernatant through a 0.22 µm filter. The filtrate was precipitated with ammonium sulphate (70% saturation) overnight at 4 °C. After precipitation, it was centrifuged at 10,000× g for 30 minutes and dissolved in double-distilled water. The dissolved bacteriocin was filtered through a 0.22-µm filter and dialysed using a 1000 Da cut-off membrane. The precipitate was resuspended in a minimal volume of phosphate buffer (5 mL, pH 5.0) [11]. Furthermore, it was applied on a diethylaminoethyl-cellulose column (1.5 × 40.0 cm) equilibrated with 0.1 mol/L Tris-HCl buffer (pH 9) and eluted with a linear salt gradient of NaCl (0-1 mol/L). The active fractions were pooled, concentrated by ammonium sulphate, loaded onto a Sephadex G-75 column (1.594 0 cm) equilibrated with 0.1 mol/L Tris-HCl buffer (pH 9), and eluted with the same buffer at a flow rate of 0.5 ml/min; the eluted fractions were assayed for bacteriocin activity [12]. Final purification was done by reverse-phase high-performance liquid chromatography (RP-HPLC) (Agilent) using a C-18 reverse-phase column. The elution program was as follows: an initial gradient from 0 to 60% of solvent B (0.1% acetic acid in acetonitrile) from 0 to 20 min, followed by a gradient from 60 to 100% of solvent B from 20 to 25 min, and finally using 100% solvent B for 5 min. The flow rate was 1 ml/min and a UV detector monitored elution at 220 nm [13]. The fractions were collected and analysed for antimicrobial activity.

Antibacterial assay of bacteriocins. The antibacterial activity of the purified bacteriocin extracts was assayed against three isolated bacteria by the agar well diffusion method. We added 0.1ml of bacteriocin to 10 mm wells on Mueller-Hinton agar previously inoculated with 0.1ml of isolated bacterial suspensions. We incubated the plates at 37 °C for 48 hours and examined the zones of inhibition [14].

Effect of pH on bacteriocin activity. We adjusted bacteriocins produced by each lactic acid bacteria isolate to a pH range of 4 to 9 using diluted acid and base solutions and allowed them to

stand at room temperature for 2 hours. We determined bacteriocin activity against the test organisms using the agar well diffusion method.

Effect of Temperature on bacteriocin activity. We subjected the bacteriocin to different temperatures (40 °C, 60 °C, 80 °C, and 100 °C). We determined the effect of these temperatures on bacteriocin activity against the indicator organisms using the agar well diffusion method.

Effect of Storage on Bacteriocin Activity. We stored the bacteriocin produced by each LAB isolate at 37 °C for 96 hours and determined its activity every 24 hours against the isolates using the agar well diffusion method.

Statistical Analysis. Data were analysed using analysis of variance (ANOVA) and Tukey HSD pairwise comparison in the R statistical programming package.

RESULTS AND DISCUSSION

Identification of Bacteria. Identified bacteria were *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Salmonella sp*, *Bacillus cereus* and *Staphylococcus aureus*. Different studies have reported isolating pathogenic bacteria from abattoir effluent. Previous research reported *Escherichia coli* and *Staphylococcus* species in different abattoirs investigated in Rivers State, Nigeria [15]. The presence of pathogenic bacteria in untreated abattoir wastewater can be attributed to the large volume of organic waste generated in abattoirs.

Antimicrobial Assay of Lactic Acid Bacteria. Screening of lactic acid bacteria isolates using the agar well diffusion method against three bacteria isolated from abattoir wastewater showed that two strains, L1 and L4, significantly inhibited the organisms (Figure 1). We tested the antimicrobial activity of the lactic acid bacteria isolates against *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacter aerogenes*.

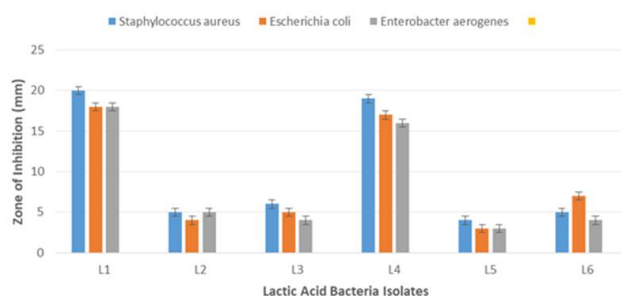


Figure 1 – Antimicrobial Assay of Lactic Acid Bacteria Isolates

Identification of Lactic Acid Bacteria. We investigated the morphological, physiological, and molecular characteristics of lactic acid bacterial isolates L1 and L4, which exhibited significant inhibitory activity against the isolated bacteria. We identified them as *Lactobacillus plantarum* B19 and *Lactobacillus fermentum* HB3-3.

Antimicrobial Activity of Bacteriocins. Purified bacteriocin extracts of *Lactobacillus plantarum* and *Lactobacillus fermentum* were assayed for antibacterial activity against three test organisms. *Lactobacillus plantarum* bacteriocin exhibited the highest inhibitory activity against *Staphylococcus aureus* with an inhibition zone of 16mm and the least inhibitory activity against *Escherichia coli* (11 mm). *Lactobacillus fermentum* bacteriocin also showed the highest inhibitory activity against *Staphylococcus aureus* (15 mm) and the least antibacterial activity against *Enterobacter aerogenes* (12 mm). Figure 2 displays this.

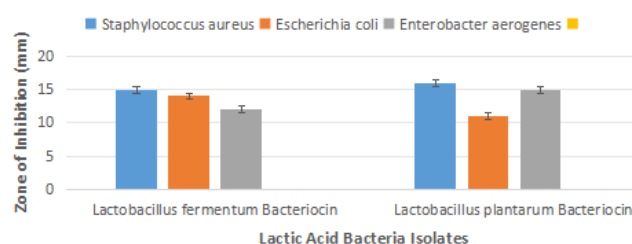


Figure 2 – Antibacterial Assay of Purified Bacteriocins

Effect of pH on the Antimicrobial Activity of Purified Bacteriocin Extracts. We determined the antibacterial activity of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacteriocins at different pH levels. The antibacterial activity of both bacteriocins decreased as pH increased. Both bacteriocins showed the highest activity against the test organisms at pH 4, similar to previous research [16]. *Lactobacillus plantarum* bacteriocin exhibited the highest activity against *Staphylococcus aureus* at pH 4, with a zone of inhibition of 16 mm, and the least inhibition against *Enterobacter aerogenes* at pH 9 (2 mm). Inhibition against isolated bacteria differed significantly ($P=0.05$). *Lactobacillus fermentum* bacteriocin also showed the highest inhibitory activity against *Staphylococcus aureus* (15 mm) and the least inhibitory activity against *Enterobacter aerogenes* at pH 9 (2 mm). *Lactobacillus fermentum* bacteriocin showed significant differences in inhibition of the different

spoilage organisms at different pH levels ($P=0.05$). Figures 3 and 4 show these results.

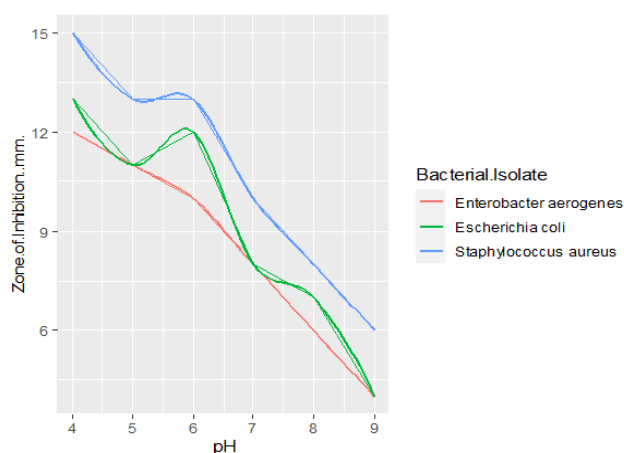


Figure 3 – Effect of pH on *Lactobacillus fermentum* Bacteriocin Antibacterial Activity

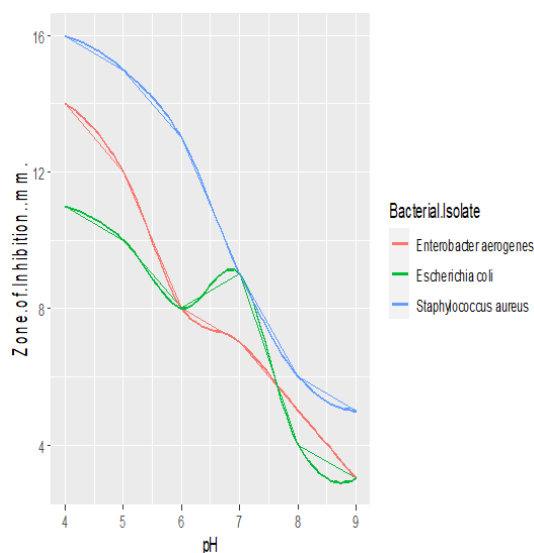


Figure 4 – Effect of pH on *Lactobacillus plantarum* Bacteriocin Antibacterial Activity

Effect of Temperature on the Antimicrobial Activity of Purified Bacteriocin Extracts. We determined the antibacterial activity of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacteriocins at different temperatures. Both bacteriocins showed the highest activity against the test organisms at 40 °C. *Lactobacillus fermentum* bacteriocin showed the highest inhibitory activity against *Staphylococcus aureus* at 40 °C, with an inhibition zone of 15 mm. *Lactobacillus plantarum* bacteriocin showed the highest activity against *Enterobacter aerogenes* at 40 °C, with an inhibition zone of 16 mm. Both bacteriocins significantly inhibited the test organisms ($P=0.05$). The purified bacteriocin extracts showed the

least activity between 80 °C and 100 °C against *Staphylococcus aureus*, *Escherichia coli*, and *Enterobacter aerogenes*. Figures 5 and 6 show these results.

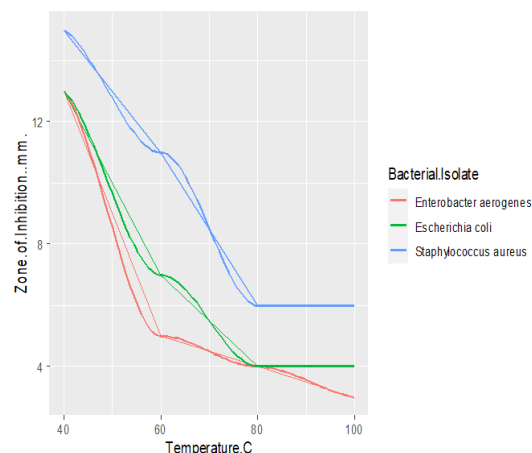


Figure 5 – Effect of Temperature on *Lactobacillus fermentum* Bacteriocin Antibacterial Activity

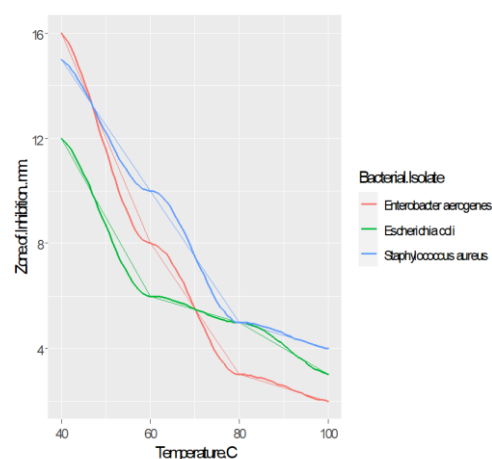


Figure 6 – Effect of Temperature on *Lactobacillus plantarum* Bacteriocin Antibacterial Activity

Effect of Storage on the Antibacterial Activity of Purified Bacteriocin Extracts. We determined the antibacterial activity of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacteriocins after different storage periods at 37 °C. *Lactobacillus plantarum* bacteriocin showed the highest inhibition against the test organisms at 0 hours, with the highest inhibition against *Staphylococcus aureus* (17 mm). Bacterial growth inhibition decreased as storage time increased. Inhibition of the test organisms differed significantly over 96 hours ($P=0.05$).

Lactobacillus fermentum bacteriocin showed the highest inhibition against the test organisms at 0

hours, with the highest inhibition against *Staphylococcus aureus* (16 mm). Inhibition of bacterial growth decreased as storage time increased. Inhibition of the test organisms differed significantly over 96 hours ($P=0.05$). Figures 7 and 8 show these results.

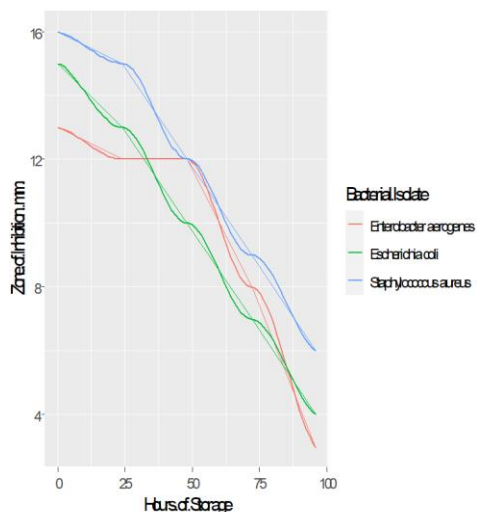


Figure 7 – Effect of Storage on *Lactobacillus fermentum* Bacteriocin Antibacterial Activity

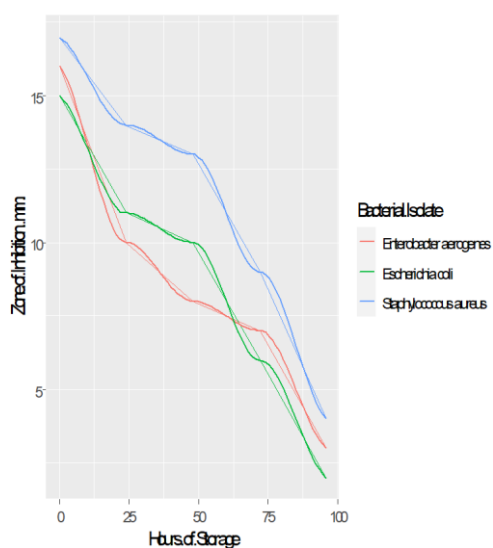


Figure 8 – Effect of Storage on *Lactobacillus plantarum* Bacteriocin Antibacterial Activity

CONCLUSIONS

The research revealed pathogenic bacteria in abattoir wastewater. These microorganisms are a concern because of their environmental impact and potential effects on public health. Increasing antibiotic resistance increases the risk these organisms pose to nearby populations. All this

points to the need for effective wastewater management to limit pathogen discharge into soil, water bodies, and farmlands. The investigated abattoir currently employs no wastewater treatment procedure; this is a common challenge across slaughterhouses in Nigeria, except in a few locations. Conventionally, abattoir wastewater is treated with physical, chemical and biological techniques. However, these methods have disadvantages, such as chemical toxicity and high costs.

The results suggest that bacteriocins produced by lactic acid bacteria can inhibit microbial growth. Lactic acid bacteria were isolated from fermented food effluents, highlighting the potential to convert waste into useful products. *Lactobacillus plantarum* and *Lactobacillus fermentum* bacteriocins significantly inhibited the growth of bacteria isolated from the wastewater. Bacteriocins are a potentially safe alternative to chemicals and antibiotics for mitigating pathogen effects.

As a matter of urgency, abattoirs in Nigeria should apply robust wastewater treatment solutions. The government must invest heavily in this area as it has a weighty bearing on public health. The government should install incinerators, anaerobic digesters, and other essential equipment in all abattoirs to prevent environmental pollution. Abattoir operators should adopt waste-recycling practices to improve waste management. The government should modernise abattoirs by providing clean water, effective drainage systems, and adequate refrigeration facilities. Regulatory agencies should monitor and regulate private abattoirs to ensure compliance with established standards.

Researchers could incorporate bacteriocins into the biological stage of wastewater treatment. They should assess, evaluate, and test the applicability of these antimicrobial peptides to reduce pathogenic strains in abattoir wastewater through pilot-scale studies to determine feasibility. To achieve this, further studies should characterise producer strains, including their mechanisms of action and optimised conditions for maximum activity. Researchers should explore using bacteriocins in synergy with other antimicrobials. Other natural antimicrobials, such as plant-derived essential oils and extracts, could, when used with bacteriocins, produce enhanced antagonistic activity against pathogens.

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