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ISOLATION AND CHARACTERIZING BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM SOIL SAMPLE AND ASSESSING ITS EFFICACY AGAINST WOUND PATHOGENS

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ABSTRACT: Bacteriocins are ribosomally synthesized antimicrobial peptides, which are secreted to act against closely related bacterial species without affecting the producing strain. Bacteriocins of lactic acid bacteria (LAB) have been given much attention because some of them exhibit high activity against pathogenic organisms. LAB was isolated from soil and assessed for bacteriocin production and antimicrobial activity against wound pathogens like Klebsiella spp, Salmonella spp, Staphylococcus aureus and Escherichia

coli. using the agar-well diffusion method. Four different LAB species were isolated and identified using standard techniques. The isolates were cultured into Brain Heart Infusion medium for bacteriocin production. The result showed that all the test isolates were susceptible to 50% of the produced bacteriocin while resistant to 50% as well. The produced bacteriocin presents a broad spectrum of activity against possible pathogenic organisms. The identification of this substance active against important pathogens addresses an important aspect of antimicrobial resistance in treatment of wound infections.

Keywords: *Bacteriocins, Lactic Acid Bacteria, Pathogens, Soil.*

Introduction

In highly competitive natural settings, such as soil or environments impacted by waste, the synthesis of antimicrobial agents is a vital survival strategy for coexisting microbial populations (Agu et al., 2015; Awari et al., 2023). Bacteriocins stand out among these agents as exceptionally targeted and potent antagonists that play a central role in bacterial interactions (Sahl, 1994). These ribosomally synthesized peptides and proteins are produced by various bacteria to inhibit the growth of taxonomically related species, while the host cell maintains immunity through specialized self-protection systems (Sahl, 1994; Motta & Brandelli, 2008). Because they are biochemically diverse, bacteriocins vary significantly in their molecular mass, mechanisms of action, and inhibitory ranges (Klaenhammer, 1998).

Beyond their ecological significance in managing microbial competition in harsh or polluted habitats, these peptides are increasingly valued as medical alternatives for infection control and as biopreservatives in the food industry (Agu et al., 2014; Anthony et al., 2009; Settanni & Corsetti, 2008). Their utility in food science stems from their robust nature; many are heat-stable, function across wide pH scales, and are easily broken down by digestive enzymes, ensuring they remain active during storage but are safe for human consumption (Gautam & Sharma, 2005; Cleveland et al., 2001). Historically, this field began with Gratia's 1925 discovery of bacterial toxins, followed by the identification of nisin in 1928 and its analogue, subtilin, in 1948 (Gratia, 1925; Hurst, 1967; Hansen, 1993).

Genetically, the ability to produce bacteriocins is often linked to plasmids, which provide a competitive edge by allowing a population to eliminate neighboring strains that lack these protective genetic elements (Avonts & De Vuyst, 2001). While both Gram-positive and Gram-negative bacteria produce these toxins, those derived from lactic acid bacteria (LAB) have garnered the most interest due to their potential as natural preservatives (Riley & Wertz, 2002; Ennahar et al., 1999). The genus *Lactobacillus* is a prominent group of LAB found in diverse niches, ranging from fermented products to industrial waste sites (Agu et al., 2023). These Gram-positive, non-spore-forming rods primarily generate lactic acid, which lowers environmental pH to inhibit pathogens and extend the shelf life of food products (Aasen et al., 2000; Ivanova et al., 2000).

Modern classification systems categorize these substances into four distinct groups, ranging from the Class I lantibiotics like nisin to the larger, heat-labile proteins of Class III and the complex molecules of Class IV (Klaenhammer, 1993). Currently, nisin is the only bacteriocin widely approved for global food use, though pediocin-based products are also commercially available (Twomey et al., 2002; Rodríguez et al., 2002). Unlike traditional antibiotics, bacteriocins offer a more surgical approach to pathogen control with a lower likelihood of fostering widespread resistance. Consequently, this research focuses on isolating bacteriocin-producing LAB from soil to evaluate their potential in treating wound-associated infections, expanding on previous environmental microbiological surveys (Agu et al., 2015; Awari et al., 2023).

This study aimed to isolate and characterize bacteriocin-producing lactic acid bacteria from soil samples and to evaluate the antimicrobial efficacy of the produced bacteriocins against selected wound-associated pathogenic microorganisms.

MATERIALS AND METHOD

Study Area

This study was carried out in Awka metropolis, Anambra State. Anambra State is in the Southeast geo-political Zone of Nigeria. It lies between latitude 6°06'N and 6°15'N and longitudes 7°05'E and 7°15'E. The physical size and structure of Awka

capital city makes it suitable to play several roles and serve several functions in the socio-economic and socio-political development of Anambra State especially its administrative functions as the capital city of the State (Nwanna and Ezenagu, 1995).

Sample Collection

Three distinct soil specimens were obtained from separate locations within the Nnamdi Azikiwe University (UNIZIK), Awka metropolis. Each sample was clearly labelled, aseptically transferred into sterile plastic containers, and conveyed to the Applied Microbiology and Brewing Laboratory of Nnamdi Azikiwe University, Awka, for microbiological analysis.

Isolation of Microorganisms

Soil samples were handled under aseptic conditions using sterile spatulas. Exactly 1 g of each sample was suspended in 9 mL of sterile peptone water and thoroughly mixed to obtain a uniform suspension. Serial tenfold dilutions ranging from 10^{-1} to 10^{-6} were prepared by sequential transfer of 1 mL aliquots into fresh diluent using sterile pipettes. From selected dilutions (10^{-3}, 10^{-4}, and 10^{-5}), 0.1 mL was inoculated onto de Man, Rogosa and Sharpe (MRS) agar supplemented with natamycin (50 µg/L) using the spread plate technique. Plates were incubated at 30°C for 24 hours, after which colony morphology was examined. Distinct colonies were subcultured to obtain pure isolates, which were maintained on nutrient agar slants and preserved at 4°C for subsequent analyses (Cheesbrough, 2010).

Identification and Characterization of Isolates

Bacterial isolates were identified through a combination of macroscopic and microscopic examinations, Gram reaction, and standard biochemical assays. The biochemical tests employed included catalase, citrate utilization, methyl red, Voges–Proskauer, oxidase, coagulase, triple sugar iron (TSI), motility, indole, and ornithine decarboxylation tests, following established protocols (Cheesbrough, 2010).

Gram Staining

Gram staining was performed to differentiate bacterial isolates into Gram-positive and Gram-negative groups using standard differential staining procedures

(Cheesbrough, 2006). Fresh overnight cultures grown at 37°C were used to prepare smears on clean glass slides. The smears were heat-fixed and sequentially treated with crystal violet, Gram's iodine, ethanol for decolourization, and safranin as counterstain. After air-drying, slides were examined microscopically under oil immersion (×100 objective), and organisms were classified based on staining characteristics.

Methyl Red Test

The methyl red assay was conducted to detect stable acid production from glucose fermentation by enteric bacteria. Test organisms were inoculated into glucose phosphate peptone broth and incubated at 37°C for 48 hours. Following incubation, a few drops of methyl red indicator were added. The immediate appearance of a red coloration indicated a positive reaction (Cheesbrough, 2006).

Catalase Test

Catalase activity was assessed to distinguish catalase-producing organisms from non-producers. A sterile test tube containing hydrogen peroxide was inoculated with bacterial colonies using a sterile glass rod. The rapid release of oxygen bubbles signified a positive catalase reaction, while absence of effervescence indicated a negative result (Cheesbrough, 2006).

Citrate Utilization Test

Citrate utilization was evaluated using Simmons' citrate agar to determine the ability of isolates to utilize citrate as a sole carbon source. Test organisms were streaked onto citrate agar slants and incubated at 37°C for 48 hours. A change in the medium from green to blue was interpreted as a positive result (Cheesbrough, 2006).

Urease Test

Urease production was assessed by inoculating isolates into urea-containing medium supplemented with phenol red indicator. After overnight incubation at 37°C, a shift in color from yellow to pink or red indicated urease activity due to ammonia production following urea hydrolysis (Cheesbrough, 2006).

Preparation of Crude Bacteriocin

Bacteriocin production was achieved by cultivating selected bacterial isolates in 200 mL of brain heart infusion (BHI) broth at 30°C on a rotary shaker set at 125 rpm. After incubation, bacterial cells were removed by centrifugation at 10,000 rpm for 15 minutes. The resulting supernatant was passed through 0.1 µm membrane filters to obtain cell-free extracts, which were stored at 4°C in sterile containers until antimicrobial testing.

Test Microorganisms

Clinical wound isolates of *Staphylococcus aureus* and *Escherichia coli* were employed as indicator organisms for bacteriocin activity assessment. These strains were sourced from the culture repository of the Department of Microbiology, Nnamdi Azikiwe University, Awka, and were reactivated in tryptone yeast extract glucose (TYG) broth for 24 hours prior to use.

Bacteriocin Activity Assay

Antibacterial activity was determined using the agar well diffusion technique in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (2000). Indicator organisms were standardized to 0.5 McFarland turbidity and uniformly inoculated onto Mueller–Hinton agar plates. Wells measuring 5 mm in diameter and approximately 4 mm in depth were aseptically bored into the agar. Each well was filled with 100 µL of bacteriocin extract at varying concentrations. Plates were allowed to stand for 3 hours to facilitate diffusion before incubation at 37°C for 24 hours, as previously described by Shahidi (2004) and Aibinu et al. (2007). Zones of inhibition were measured in millimeters. Gentamycin (32 µg/mL) served as the positive control, while sterile distilled water was used as the negative control.

Results

The biochemical characteristics of lactic acid bacteria (LAB) isolated from soil samples are summarized in Table 1, while Table 2 presents the biochemical profiles of the selected wound pathogens used as indicator organisms. The antimicrobial effects of the extracted bacteriocins against the test pathogens are shown in Table 3. Of the four bacteriocin-producing LAB isolates recovered, only two demonstrated inhibitory activity against the tested pathogens.

Table 1: Biochemical test result of bacteria isolates

Isolate	**Gram Stain**	**Catalase test**	**Urease test**	**Methyl red test**	**Citrate Utilization**	**Probable Bacterium**
LAB 1	+ve Rods	-	+	-	-	*Lactobacillus*spp
LAB 2	+ve Rods	-	-	-	-	*Lactobacillus*spp
LAB 3	+ve Rods	-	+	-	-	*Lactobacillus*spp
LAB 4	+ve Rods	-	+	-	-	*Lactobacillus*spp

Table 2: Biochemical Test Result of the Test Organisms

Isolate	**Form**	**Surface**	**Colour**	**Margin**	**Elevation**	**Opacity**	**Gram**	**Cat**	**Mot**	**Ind**	**MR**	**VP**	**Cit**	**Lac**	**Glu**	**Suc**	**Fru**	**Mal**	**Oxi**	**Ure**	**Identity**
1	Irregular	Glistening	Cream	Entire	Raised	Opaque	- Rod	+	-	-	+	-	+	+	+	+	(-)	+	-	+	*Klebsiellapneumoniae*
2	Circular	Smooth	Yellowish	Entire	Raised	Opaque	+ cocci	+	+	-	+	-	-	AG	AG	A	A	AG	-	+	*Staphylococcus aureus*
3	Circular	Smooth	Greyish/ white	Lobate	Low convex	Translucent	- Rod	+	+	-	+	-	+	-	+	-	+	+	-	-	*Salmonella enterica*
4	Circular	Smooth	Whitish	Entire	Convex	Translucent	- Rod	+	+	+	+	-	-	+	+	var	-	-	-	-	*Escherichia coli*

Key:
Gram: Gram reaction
Cat: Catalase test
Mot: Motility test
Ind: Indole test
MR: Methyl-red test
VP: Voges-Proskauer test
Oxi : Oxidase
Cit: Citrate Utilization test
SugarFermentation Tests:
Lac: Lactose Fermentation
Glu: Glucose Fermentation
Suc: Sucrose Fermentation
Fru: Fructose Fermentation
Mal: Maltose Fermentation
Ure: Urease

Table 3: Antimicrobial Activities of the Bacteriocins against Selected Pathogens

Isolate	**LAB 1**	**LAB 2**	**-VE CONTROL**	**+VE CONTOL**
Staphylococcus aureus	18mm	15mm	-ve	28mm
Escherichia coli	35mm	17mm	-ve	40mm
*Klebsiella*spp	31mm	18mm	-ve	36mm
Salmonella spp	16mm	21mm	-ve	25mm

DISCUSSION

This study evaluated the antimicrobial potential of bacteriocins synthesized by lactic acid bacteria (LAB) isolated from soil samples. Among the four bacteriocin-producing LAB isolates obtained, inhibitory activity against the selected test organisms was observed in only two isolates. The relatively low recovery of active bacteriocin producers may be attributed to several methodological and biological variables. Factors such as the composition of the growth medium, incubation parameters, choice of indicator organisms, and the sensitivity of the antimicrobial detection technique have all been reported to significantly influence bacteriocin detection and activity (Sezer & Güven, 2009).

Antibacterial activity was assessed using the agar well diffusion assay against representative foodborne and clinical pathogens. The cell-free culture supernatants demonstrated inhibitory effects against both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and

Pseudomonas spp. Importantly, the antimicrobial effect persisted following catalase treatment and pH neutralization, indicating that inhibition was not attributable to hydrogen peroxide production or organic acids such as lactic acid. This observation strongly suggests that the antibacterial activity was primarily mediated by bacteriocins present in the culture filtrates.

Traditionally, bacteriocins are regarded as narrow-spectrum antimicrobial peptides with activity largely confined to phylogenetically related organisms, a property that has been cited as a limitation to their broader application as preservatives or therapeutic agents (Cleveland et al., 2001). However, findings from the present study contradict this generalization, as the bacteriocins produced by the LAB isolates exhibited inhibitory effects against a diverse range of bacterial species. Comparable observations have been reported for bacteriocins derived from *Bacillus subtilis* R75, which demonstrated activity against multiple bacterial genera, underscoring the diversity and specificity of bacteriocin action among different microbial producers (Sharma et al., 2011).

Given the proteinaceous nature of bacteriocins, susceptibility to proteolytic degradation has been widely documented (Sharma et al., 2011). Interestingly, despite the fact that all indicator organisms used in this study are known producers of extracellular proteases (Thangam & Rajkumar, 2002; Bhagwat et al., 2015), bacteriocin activity was not universally abolished. This suggests that only specific proteases are capable of degrading particular bacteriocins, depending on their molecular structure and amino acid composition. Among the tested organisms, *Escherichia coli* exhibited the highest sensitivity to the bacteriocins, indicating its suitability as a model indicator organism for subsequent investigations.

Bacteriocins have long been recognized as promising alternatives to conventional antibiotics due to their affordability, efficacy, and low toxicity to humans and animals (Lewus et al., 1991; Miles et al., 1992; Cleveland et al., 2001). Previous studies have further demonstrated their therapeutic potential in the management of animal diseases (Ogunbanwo et al., 2004; Cole et al., 2006). Collectively, the present findings demonstrate that bacteriocins produced by soil-derived LAB possess a broad inhibitory spectrum against pathogens of veterinary and clinical relevance, while

potentially sparing beneficial microbiota. These attributes support their candidacy as antimicrobial agents and growth-enhancing additives in agricultural and farm environments. Nonetheless, further molecular characterization and in vivo evaluations are required before definitive applications can be recommended.

CONCLUSION

This study successfully identified and characterized bacteriocin-producing *Bacillus* species using standard biochemical profiling techniques. The findings clearly demonstrate that bacteriocins synthesized by bacteria isolated from soil and food-related environments are capable of inhibiting pathogenic microorganisms, thereby confirming their antimicrobial efficacy. These results reinforce the growing body of evidence supporting bacteriocins as safe, natural, and effective antimicrobial compounds.

Given their low cost, high efficiency, and minimal toxicity to humans and animals, bacteriocins continue to attract attention as viable alternatives to conventional antibiotics. The broad-spectrum activity observed in this study further enhances their potential utility as antimicrobial agents and growth promoters in farm and livestock production systems. Overall, lactic acid bacteria isolated from soil exhibited appreciable inhibitory effects against wound-associated pathogens. As interest in bacteriocins and LAB continues to expand in both human and veterinary medicine, the outcomes of this study highlight their promise as valuable antimicrobial resources and justify further research into their development and application.

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