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Research Article

Antibacterial and Antibiofilm Efficacy of *Lactobacillus plantarum* Cell-Free Extract Against Some Multidrug Resistant Bacterial Pathogens Associated with Chronic Wounds

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ABSTRACT
The existence of multidrug resistant bacterial pathogens and biofilm formation are critical factors that compromise the management of wound infections and pose significant barrier to successful therapeutic outcomes. The study aimed to evaluate the antibacterial and antibiofilm effects of *L. plantarum* cell-free extract (CFE) against resistant strains of *S. aureus* and *P. aeruginosa* biofilms associated with chronic wounds. Antibacterial susceptibility testing was performed to determine the resistance profile of *S. aureus* and *P. aeruginosa*. The antibacterial activity was evaluated using agar well diffusion method at various concentrations of CFE. Crystal violet microtiter plate assay was used to quantify the biofilm activity of the isolates. High level of resistance to penicillin (3 mm) and ofloxacin (2 mm) was observed among the isolates. Significant antibacterial effects were observed, with inhibition zones of 17.2 mm for *S. aureus* at 100 µL ($p = 0.002$) and 19.7 mm for *P. aeruginosa* at 200 µL ($p = 0.018$). Quantification of biofilm revealed OD_{600} values of 0.48 and 0.72, corresponding to 60% and 46.7% biofilm inhibition for *S. aureus* and *P. aeruginosa* respectively. The findings highlight promising antibacterial and antibiofilm activity of *L. plantarum* CFE against multidrug resistant bacterial pathogens associated with chronic wounds, suggesting its potential as a potential therapeutic agent.

Keywords: Antibacterial activity; Biofilm inhibition; *Lactobacillus plantarum*; *Staphylococcus aureus; Pseudomonas aeruginosa*

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INTRODUCTION

Skin damage can be caused by a variety of different reasons such as cuts, abrasions, chemical burns, fire burns, cold, heat, radiation, surgery, or as a result of underlying diseases such as diabetes. Chronic infected wounds, such as venous or arterial ulcers, diabetic foot ulcers, pressure sores, and nonhealing surgical wounds delay wound healing, have a significant impact on the patients' quality of life, represent a significant cause of morbidity and mortality, and result in enormous healthcare expenditures (Fusieger *et al*., 2020; Moraffah *et al*., 2022). Wound infections are most often caused by biofilm-forming bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae*, *Enterobacter* spp., *Peptostreptococcus* spp., and many others (Sabina *et al*., 2019). These bacteria show slower growth rate, transfer antibiotic resistance genes and inhibit diffusion of antibiotics due to possession of exopolysaccharides (EPS) layer (Costerton and

Stewart, 2001). Systemic administration of antibiotics coupled with surgical interventions to destroy the biofilm matrix is the commonly used wound therapy, but success often not guaranteed.

As biofilms are inherently known to impair wound healing and aggravate chronic wound infections, alternative therapeutic approaches have to be assigned, one could be the bacteriotherapy. The almost new context of utilizing bacteriotherapy which involves the application of non-pathogenic microorganisms (probiotics) to combat the pathogenic microbes have recently attracted the attention of researchers. Lactic acid bacteria (*Lactobacillus rhamnosus, Lactobacillus* acidophilus, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*) are the commonest probiotics known. They show antagonistic and inhibitory effect against pathogenic microbial composition by producing active antimicrobial metabolites (Furtado *et al*., 2019). Application of these probiotics could be a means to prevent infection, regulate inflammation, and potentially improve wound healing. Topical administration of *L. plantarum* accelerated the healing of chronic diabetic foot ulcers and infected burn wounds by altering infection, angiogenesis, macrophage phenotype, and neutrophil response (Argañaraz e*t al*., 2022; Moraffah *et al*., 2022).

Biofilm bacteria aggravate chronic infectious wounds and delay healing resulting to significant morbidity and mortality, enormous healthcare expenditures with remarkable impact on the patients' quality of life (Daeschlein, 2013; Mihai *et al*. 2018). The management of wound infections becomes difficult as bacteria in biofilms have high level of antibiotic resistance. In Nigeria, the control of wound infections has become more challenging due to increasing prevalence of antibiotic resistance as successful strategies that can prevail over this challenge in the wound management are missing. Several researches specifically drug designs based on synthetic analogs have been conducted to tackle antibiotic resistance and virulence traits including biofilm formation, but have not yielded promising results due to toxicity and low bioavailability. However, it is necessary to develop alternative therapies to effectively manage chronic biofilm wound infections. Topical bacteriotherapy using probiotics has shown efficacy in multiple human and animal models at improving numerous aspects of wound healing, but there are many unanswered questions such as type and dosing regimen of the probiotic utilized and a lack of standardized outcome measures. Thus, this research aims to evaluate the effect of *L. plantarum* on elimination of pathogenic bacterial biofilms on chronic infectious wounds to improve wound healing.

MATERIALS AND METHODS

Study Area

The study was carried out in Yola, Adamawa state in the North Eastern part of Nigeria.

Study Design

The study was conducted for a period of five months between September, 2024 to January, 2025.Wound swab samples were aseptically collected using sterile swab sticks from patients with chronic wound and burns at Modibbo Adama University Teaching Hospital, Yola. One hundred millilitre (100 ml) of fresh cow milk was obtained aseptically in a sterile container from breastfeeding cows at Sebore farms, Mayo Belwa in Adamawa State. All the samples were transported to the Microbiology Laboratory at Department of Microbiology, Modibbo Adama University, Yola for isolation and identification of *S. aureus*, *P. aeruginosa*, and *L. plantarum*

Identification of the Isolates

Isolation and identification were performed following standard bacteriological techniques described by (Cheesbrough, 2006). Wound samples were aseptically inoculated on Mannitol Salt agar and Cetrimide agar for the isolation of *S. aureus* and *P. aeruginosa* respectively. *Lactobacillus plantarum* was isolated from fresh cow milk using de Man Rogosa and Sharpe (MRS) agar. Ciprofloxacin (4 ppm) was added unto the MRS culture medium to prevent contamination and inhibit the growth of *Streptococci* and *Staphylococcus*. The plates were incubated at 37°C for 24 hours for morphological observation of growth and the isolates were identified to species level using biochemical techniques (Martinez *et al*., 2013).

Molecular Characterization of the Isolates

Genomic DNA was extracted using the Zymo Research Quick-DNA Miniprep Kit following the manufacturer's instructions. Then PCR amplification was done using forward: 5'-AGAGTTTGATCCTGGCTCAG-3'and reverse: 5'-

GGTTACCTTGTTACGACTT-3' universal primers targeting the 16S rRNA gene (Barghouthi, 2011) with Taq DNA polymerase, dNTPs, $MgCl_2$, and buffer used as the reaction mixture. The condition for amplification included cycling condition of initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The PCR products were visualized using 1.5% agarose gel electrophoresis. Following amplification, the PCR products were sequenced and then subjected to comparative analysis using publicly available NCBI BLAST database.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed using Kirby Bauer disc diffusion method according to the Clinical Laboratory Standard Institute (CLSI) (CLSI, 2023). Briefly, fresh overnight isolated colonies of *S. aureus* and *P. aeruginosa* were suspended in separate tubes of saline solution. The suspension was gently swirled to match the 0.5 McFarland turbidity standard (approximately 1.5 x 10^8 CFU/mL). A sterile cotton swab was dipped into the standardized inoculum and then evenly swabbed over the entire Mŭller-Hinton agar plate surface. The plate was allowed to seed for 5 minutes at room temperature prior to application of antibiotic disks. The antibiotic impregnated disks (levofloxacin (5 µg), gentamicin (10 µg), meropenem (10 µg), ofloxacin (5 µg), ceftazidime (30 µg), penicillin (6 µg), cefotaxime (30 µg), doxycycline (30 µg), linezolid (30 µg) and piperacillin-tazobactam (100/10 µg) were then placed onto the agar surface using pair of forceps and the plates were incubated at 37^0C for 24 hours. After incubation, the diameter of the zone of inhibition (in mm) around each disk was measured using a ruler and the measurement were compared to CLSI interpretative chart to classify the organism as susceptible or resistant to each antibiotic.

Antibacterial Testing of *L. plantarum* Cell Free Extract (CFE) on the Isolates

The antimicrobial activity of *L. plantarum* strain was examined by agar well diffusion method as described by the CLSI (CLSI, 2023). Briefly, *L. plantarum* was cultured in MRS broth and incubated at 37^0C for 24 hours. The culture was centrifuged at 5000 rpm for 10 minutes, and the resulting supernatant was sterile-filtered using a 0.22 µm membrane to obtain the CFE.

Then 0.5 ml of standardized inoculum of *S. aureus* and *P. aeruginosa* were inoculated on separate Mŭeller-Hinton agar plates using sterile cotton swabs. Six uniform wells were made on each of the agar plate using 5mm sterile corkborer. Serial dilutions of various concentrations of the cell free extract of *L. plantarum* (200, 100, 50, 25, 12,5, 6.25µL) were then added into the wells in each of the plates. After incubation at 37^0C for 24hours. Ciprofloxacin and MRS broth were used as positive and negative control respectively. The zone of Inhibition was measured using a meter ruler. This process was repeated in triplicates for both isolates.

Antibiofilm Inhibitory Assay

The antibiofilm activity of *L. plantarum* cell-free extract against *S. aureus* and *P. aeruginosa* was determined using the crystal violet microtiter plate assay as described by Sharma *et al*. (2019). Briefly, overnight cultures of *S. aureus* and *P. aeruginosa* were adjusted to 0.5 McFarland standard and diluted in 1:100 in Trypticase Soy broth supplemented with 1% glucose. For the assay, 100 µL of the bacterial suspension was dispensed into each well of a sterile 96- well flat-bottom microtiter plate followed by 100 µL of *L. plantarum* cell-free extract at varying concentrations (200, 100, 50, 25, 12,5, 6.25µL). Positive control well includes bacterial suspension without CFE and broth without bacteria was used as the negative control. Plates were then incubated at 37^0C for 24 hours under static conditions. After incubation, the wells were gently washed with phosphate buffered saline to remove planktonic cells, stained with 0.1% crystal violet for 15 minutes, and then rinsed thoroughly. The bound dye was solubilized with 95% ethanol, and biofilm mass was quantified by measuring absorbance at 600 nm using microplate reader. Percentage inhibition of biofilm formation was calculated by comparing optical density (OD) values of treated wells against the positive controls.

Below are the standard CV assay guidelines (Sharma *et al*., 2019)

OD < 0.3 – Non – biofilm formers

0.3 ≤ OD < 0.5 – Moderate biofilm formers

0.5 ≤ OD < 1.0 – Strong biofilm formers

RESULTS

Biochemical and Molecular Characteristics of the Isolates

All the three isolates show the presence of bacterial DNA. All the isolates showed a band at approximately 1000 base pairs which confirms the presence of the DNA (Figure1) and the result was compared to the GenBank database using BLAST.

Antibiotic Resistant Profile of the isolates

Antibiotic susceptibility profile of the antibiotics tested against the isolates showed susceptibility as well as resistance. Generally, the isolates showed highest susceptibility to linezolid (100%) followed by gentamicin (Figure 2A) and meropenem (90%) and ceftazidime (73%) (Figure 2B). *S. aureus* was specifically observed to be most resistant to penicillin, cefotaxime and clindamycin with zone diameter less than 6 mm while *P. aeruginosa* appeared to be highly resistant to ofloxacin, ciprofloxacin and gentamicin having zone diameter of 8 mm (Table 2).

Antibacterial activity of *L. plantarum* CFE against the isolates

The result indicates a strong correlation between the concentration of *L. plantarum* cell-free extract and inhibition of bacterial growth (Table 3). *Staphylococcus aureus* exhibited growth inhibition at 100 µL with zone of inhibition of 17.2 mm (P= 0.002), while *P. aeruginosa* was observed to be inhibited at a higher threshold (200 µL) with zone of inhibition of 19.7 mm in diameter, confirming it greater resistance to *L. plantarum* compared to *S. aureus* (P=0.018) (Table 3).

Antibiofilm Efficacy of *L. plantarum* CFE

The result for antibiofilm efficacy of *L. plantarum* CFE revealed that *S. aureus* formed strong biofilm with an OD_{600} of 1.20 in the control group. The biofilm inhibitory effect was found to be significant at 100 µL of CFE (OD_{600} =0.48 with 60% reduction). However, *P. aeruginosa* had a control OD_{600} of 1.35, showing strong biofilm production. A marked reduction of biofilm formation was observed at 200 µL of CFE (OD_{600} = 0.72 with 46.7% reduction). Lower rates of biofilm inhibition were found at lower concentrations (6.25- 50 µL) of the CFE.

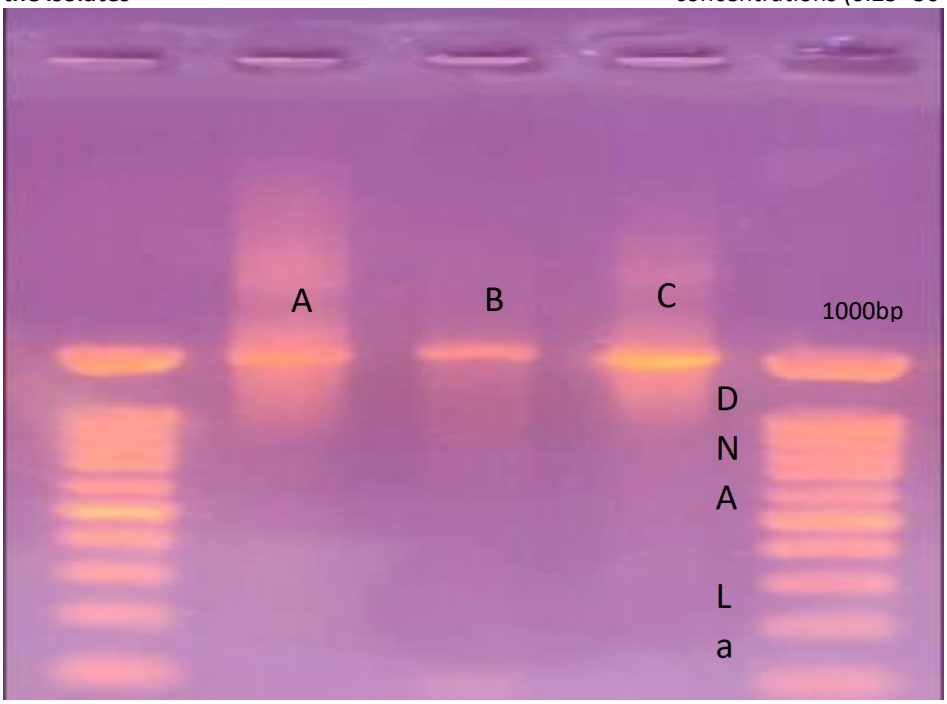

Plate 1: *PCR for 16S rRNA Gene Amplification of S. aureus, P. aeruginosa and L. plantarum respectively binding at 1000bp*

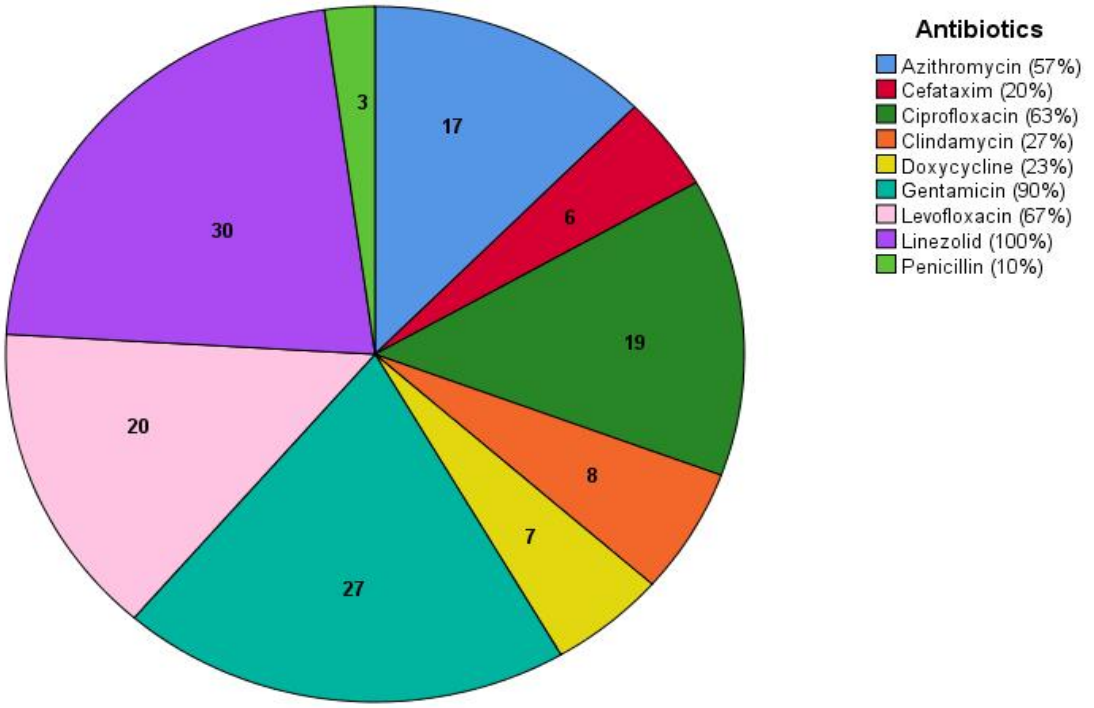

Figure 2A: Percentage Resistance Profile of *S. aureus* Isolate

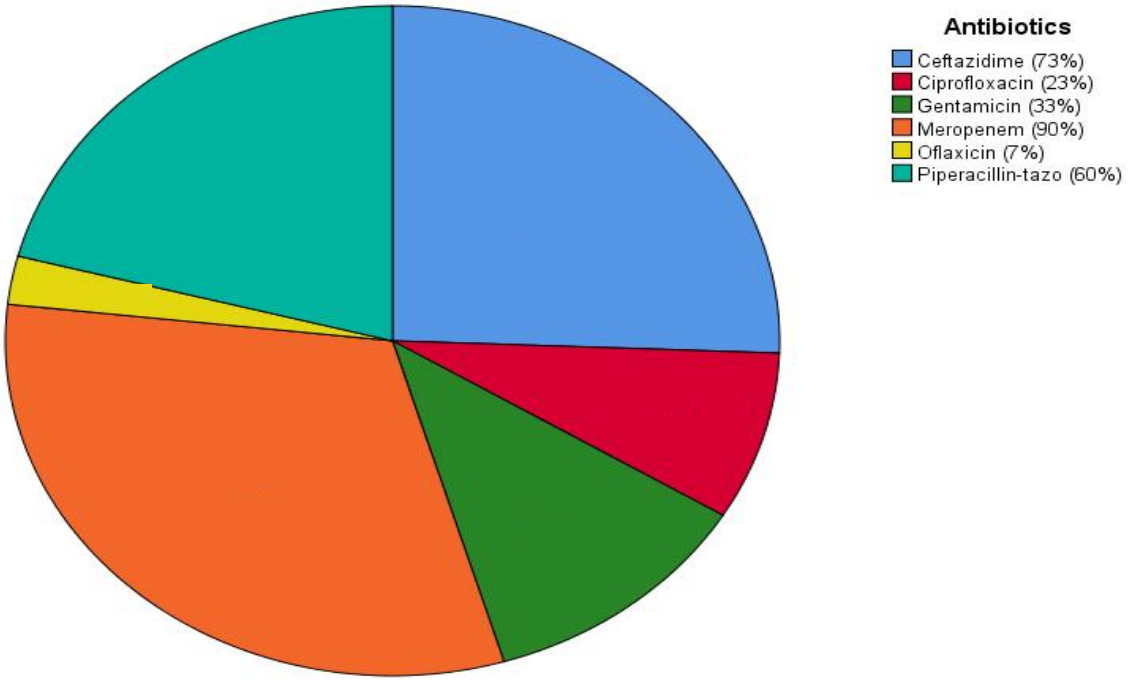

Figure 2B: Percentage Antibiotic Resistance Profile of *P. aeruginosa* Isolate

Table 1. Antibacterial Activity of *L. plantarum* Cell Free Extract

CFE Volume (µL)	Mean Zone of Inhibition (mm) *S. aureus*	S.D *S. aureus*	Mean Zone of Inhibition (mm) *P. aeruginosa*	S.D *P. aeruginosa*	P-value (ANOVA)
6.25	3.5	±0.5	2.1	±0.5	0.045
12.5	6.9	±0.6	4.7	±0.6	0.038
25	10.4	±0.4	8.2	±0.4	0.032
50	13.8	±0.3	11.6	±0.4	0.028
100	**17.2**	±0.2	15.1	±0.2	0.002
200	20.5	±0.4	**19.7**	±0.3	0.018

Key: S.D- Standard deviation, ANOVA – Analysis of Variance

Table 2. Antibiofilm Activity of *L. plantarum* CFE against *S. aureus* and *P. aeruginosa*

CFE Volume (µL)	OD_{600} (*S. aureus*)	Inhibition (%)	OD_{600} (*P. aeruginosa*)	Inhibition (%)
6.25	1.12	6.7	1.28	5.2
12.5	1.00	16.7	1.20	11.1
25	0.88	26.7	1.12	17.0
50	0.72	40.0	1.00	25.9
100	**0.48**	**60.0**	0.88	34.8
200	0.42	65.0	0.72	**46.7**
Control (0µL)	1.20	-	1.35	-

DISCUSSION

This study evaluated the efficacy of *L. plantarum* as a potential alternative antimicrobial agent against *S. aureus* and *P. aeruginosa* in the context of biofilm-associated chronic wounds. In this study, the isolates obtained were *S. aureus* and *P. aeruginosa,* which have been well recognized in chronic wound infections, known for their biofilm-forming capacity (Bhattarai and Christopher, 2025). The antibiotic susceptibility testing revealed a concerning level of resistance among the isolates. *Staphylococcus aureus* and *P. aeruginosa* exhibited high resistance rates to penicillin and ofloxacin respectively. However, these are the commonly used antibiotics for the treatment of chronic wounds. This finding is consistent with recent studies by Sharma *et al*. (2019) and Said *et al*. (2020) that highlighted the growing threat of multidrug resistant strains in chronic wounds. The rise of antibiotic-resistant bacteria poses a significant threat to public health, and alternative therapeutic approaches are urgently needed.

The cell-free extract of *L. plantarum* in this study showed significant antimicrobial activity against both pathogens. Interestingly, the antibacterial activity was found to be more evident against *S. aureus* which required lower concentration (100µL) for inhibition compared with *P. aeruginosa* (200µL). This difference in susceptibility is often linked to resistance phenotypes. It could be attributed due to structural differences in the bacterial cell walls, with Gram negative bacteria including *P. aeruginosa* reported to be more resistant to antimicrobial compounds due to the presence of cell membrane and other complex features (Singh *et al.,* 2022). However, Nezhadi and Ahmadi (2024) as well as Short *et al*. (2023) reported similar findings that demonstrated the effectiveness of *L. plantarum* cell-free extract in inhibiting multidrug resistant strains associated chronic wounds including *S. aureus* and *P. aeruginosa*

For antibiofilm activity of *L. plantarum* CFE*,* the present study demonstrated a dose dependent in biofilm formation by the isolates. Both isolates demonstrated strong biofilm production under laboratory conditions. This is in line with studies by Abu Elez *et al*. (2023) and Carventes-Alagon *et al*. (2025) who reported biofilm formation significantly contributed to antimicrobial resistance, increased severity of the infection, and delayed healing. Significant inhibition was observed at 100µL for *S. aureus* and 200µL for *P. aeruginosa*, expressed by

OD_{600} reductions of 60% and 46.7% respectively. The findings of this study indicate that its ability to inhibit biofilm formation varies with pathogen type and the amount of CFE present. Recent studies have shown significant inhibitory effect of *L. plantarum* against biofilm forming pathogens especially the multidrug resistant ones (Bai *et al*., 2022; Bhattarai and Christopher, 2025). These pathogens are highly sensitive to disruption by *L. plantarum* as it interferes with quorum sensing and also downregulate resistant genes in *P. aeruginosa* as documented by Keim *et al*., 2024.This effectiveness is attributed to its ability to produce postbiotic metabolites such as organic acids, bacteriocins, and quorum sensing inhibitors (Short *et al*., 2023; Keim *et al*., 2024).

The relationship of antibacterial and antibiofilm activity of *L. plantarum* CFE highlights its potential as a supplementary treatment in chronic wound management. Its natural origin coupled with less risk of developing resistance make it a strong candidate for probiotic-based formulations in managing chronic wound. The findings from this research strengthen the increasing focus on microbiome-based approaches for management of infectious diseases.

Although the findings are encouraging, the study has its limitations. The *in vitro* assay used may not accurately reflect the complex wound environment. Furthermore, the specific bioactive compounds responsible for the antimicrobial and antibiofilm effects were not isolated or characterized. To bridge these gaps and advanced toward clinical translation, future research should investigate on *in vivo* validations, molecular characterization of bioactive compounds, and comprehensive safety profiling.

CONCLUSION

Key findings revealed a high level of resistance among *S. aureus* and *P. aeruginosa* where they exhibited high rate of resistance to ceftazidime and ciprofloxacin respectively. Both isolates demonstrated strong biofilm formation. Additionally, the research highlights the antibacterial and antibiofilm activity of *L. plantarum* CFE in inhibiting biofilm producing multidrug resistant *S. aureus* and *P. aeruginosa*. The implications of these findings are profound.

Conflict of Interest

No conflict of interest

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Author's Contribution

All authors contributed significantly to the work. A.S. Sa'id led the data collection and coordinated the laboratory activities. J. U. Ewansiha performed the data analysis and interpreted the results. Laurat Tahir and Mohammad Bashir contributed to the literature review.

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