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**Article**

**Keywords:** MRSA, human microbiota, antibiotic resistance, novel antimicrobial peptides, cytotoxicity analysis, bacteriocin stability

**Posted Date:** October 14th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-11349586/v1

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**Additional Declarations:** No competing interests reported.

# Exploring the therapeutic potential of bacteriocin-producing skin and oral microbiota for combating methicillin-resistant *Staphylococcus aureus*

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## Abstract

**Background:** Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with multidrug-resistant (MDR) infections and poses a serious health hazard, necessitating the development of novel antimicrobial compounds. Bacteriocins are antimicrobial peptides produced by human microbiota to combat pathogens. This study aimed to explore the potential activity of bacteriocins against clinical isolates of MRSA.

**Methods:** In this cross-sectional study, a total of 203 bacterial isolates were obtained from the skin and oral cavity microbiota of healthy individuals, and each isolate was systematically evaluated for bacteriocin production using a suite of microbiological assays. *S. aureus* isolates from the human microbiota were molecularly characterized for the 16S rRNA, *nuc*, and *mecA* genes. Bacteriocin extract stability was determined under different conditions of pH, temperature, enzymatic activity, and cytotoxicity was observed against the ATCC cell lines PC3, A549, and HCT116. Antibacterial activity of the extracts was monitored against 30 MRSA clinical isolates.

**Results:** Six *S. aureus* isolates from microbiota produced bacteriocins, which were stable at a pH of 6.5–7 and temperature of 25–30 °C, and under certain enzymatic treatments. The agar well diffusion assay showed that the six *S. aureus* bacteriocin extracts inhibited two MRSA clinical isolates. Bacteriocin extracts had a minimum inhibitory concentration of ≥162.5 µg/mL, 425µg/ml, 162.5µg/ml, 225µg/ml, 121.875µg and 168.75 µg/ml (all $p < 0.05$). The bacteriocins showed no cytotoxicity against PC3, A549, and HCT116 cell lines at a concentration of 2 µL, although a varied pattern was found at 8 µL. Bacteriocins produced through optimized scale-up fermentation were purified into active fractions (F5, F6, F7, F8, F11, F12), which demonstrated robust stability and potent antimicrobial effects, yielding statistically significant minimum inhibitory concentration (MIC) values (121.8–425 µg/mL) against MRSA strains.

**Conclusions:** Bacteriocins from the human microbiota are novel antimicrobial peptides that show remarkable action against some clinical isolates of MRSA. They have substantial potential for an alternative therapy against MDR bacteria.

**Abbreviations:** MRSA = methicillin-resistant *Staphylococcus aureus*, MDR = multidrug-resistant, MIC = minimum inhibitory concentration, ATCC = American type culture collection.

**Keywords** MRSA, human microbiota, antibiotic resistance, novel antimicrobial peptides, cytotoxicity analysis, bacteriocin stability

## 1. Introduction

*Staphylococcus aureus* is one of the frequently found bacteria in human nostrils and skins and is associated with a variety of human infections. It is well-known for its high antibiotic resistance due to genetic mutations.[1] Methicillin-resistant *S. aureus* (MRSA) infections are associated with high morbidity and mortality due to their multidrug-resistant (MDR) nature and pose a significant global public health menace.[2,3] MRSA, first discovered in 1960, has disseminated throughout the world and become a highly antibiotic-resistant pathogen.[4] The dissemination of staphylococcal infections can be reduced by stringent infection control policies and by finding the right treatment options.[5]

The emergence of MDR bacteria demands the exploration of alternative empirical therapies by the scientific community. Bacteriocins, antimicrobial peptides produced by some bacteria, are bacteriostatic or bactericidal to similar or closely related pathogens. They have gained special interest due to their potential for an antibacterial effect against MRSA.[6-8] Bacteriocins present several advantages over commonly available antibiotics due to their smaller size, cationic nature, and narrow spectrum of activity, which helps them to target specific bacteria while preserving the human microbiota.[9] They can cause bacterial cell death by rupturing the integrity of the cell membrane.[10] Because of these distinct qualities, bacteriocins are prospective therapeutic agents against drug-resistant infections.[11]

The microbiota of the human skin and oral cavity consists of diverse microbial species. The oral microbiota has evolved to coexist in a highly competitive environment, and bacteriocin production by some oral microbes provides them an adequate opportunity to survive in this environment.[12] The oral microbiota is potentially a rich source of bacteriocin-producing strains with action against MRSA, considering the frequency of MRSA colonization and infections in the mouth.[13] Many recent investigations have focused on the isolation and identification of bacteriocin-producing bacteria in the human flora. Bacteriocin-producing *Lactobacillus* species have shown activity against common oral bacterial strains such as *Streptococcus mutans*. Some studies have suggested that the bacteriocin-producing bacteria in the oral cavity may serve as a

source of new antibiotics.[14,15] The bacteriocins synthesized by bacteria found on the skin and in the mouth have demonstrated significant effectiveness in suppressing MRSA and other pathogens with wide-ranging impact, rendering them highly desirable for potential therapeutic uses.[16]

The identification of bacteriocins may facilitate the development of specific antimicrobial therapies that are highly effective against several pathogens and show less toxicity compared to commercially available antibiotics. This study aimed to extract and screen bacteriocin-producing isolates from the microbiota of human skin and oral cavity to examine the activity of these antimicrobial peptides against MRSA clinical isolates. By examining the skin and oral microbiota, we would be able to access an extensive range of microorganisms and discover different bacteriocins that possess strong antibacterial properties against MRSA.

## 2. Materials and methods

### *2.1. Study design and ethics*

This is a cross-sectional study conducted after obtaining ethical approval from the research and biosafety committee of the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore. The research followed the ethics guidelines and principles of the Declaration of Helsinki.[17] Informed consent was obtained from each volunteer before skin and oral swabs were collected to isolate the microbiota.

### *2.2. Sample collection and processing*

We collected 45 skin and 45 oral cavity swabs from healthy individuals using sterile swabs. The skin swabs were taken from the moist skin of the toe web, axillary vault, and the popliteal fossa. In the case of oral cavity swabs, the participants were instructed to refrain from eating, drinking, or performing oral hygiene measures for at least one hour prior to specimen collection. The specimens were taken from various sites, including the tongue, buccal mucosa, and gingival crevices, to ensure a representative collection of oral microbiota. The swabs were immediately transferred to a sterile buffer–salt solution transport medium to maintain the viability of the microorganisms during transportation to the laboratory.

### *2.3. Isolation and cultivation of skin and oral microbiota*

For the isolation and cultivation we used all the culture media manufactured by Oxoid (Hampshire, UK), as well as Mitis Salivarius Agar (MSA) from Neogen (Lansing, MI, USA). Upon arrival at the laboratory, the swabs were streaked onto nutrient agar and blood agar to isolate aerobic bacteria. MSA was used to selectively isolate *Staphylococcus* and *Streptococcus* species (viridans streptococci such as *Streptococcus mitis, Streptococcus salivarius,* and *Enterococcus faecalis*). MSA plates were incubated aerobically and in the presence of 5% $CO_2$ (for microaerophilic bacteria) at 37 °C for 24 hours to allow the growth of different bacterial colonies. After incubation, the colonies exhibiting distinct morphological characteristics were selected for further purification and identification. The bacterial isolates were identified according to the guidelines of Bergey's Manual of Determinative Bacteriology.[18] *S. aureus* isolates were further confirmed using specific identification tests such as the coagulase test and DNase test.[19]

*2.4. Molecular characterization of Staphylococcus aureus*

Molecular characterization was performed by identifying the 16S rRNA gene, confirmatory *nuc* gene, and *mecA* gene in *S. aureus* using PCR. DNA was extracted from the *S. aureus* isolates using a commercial kit (GeneAll, Hanam-si, South Korea), and a PCR Master Mix 2× (Thermo Fisher Scientific, Waltham, USA) was used to amplify the genes with specific primers (Table 1). The amplified genes were detected using 1.5% agarose gel electrophoresis, and the DNA ladder was used to evaluate the size of the amplified products.

**Table 1**

The list of primers used for identifying 16S rRNA, *nuc*, and *mecA* genes of *Staphylococcus aureus*.[20]

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| 16S rRNA | 5'AGTTTGATCCTGGCTCAG3' | 5'GTGTGTACAAGGCCCGGGAAC3' |
| *Nuc* | 5'GCGATTGATGGTGATACGGTI3' | 5'AGCCAAGCCTTGACGAACTAAAGC3' |
| *mecA* | 5'TCCAATTACAACTTCACCAGG3' | 5'CCACTTCATATCTTGTAACG3' |

*2.5. Antibiotic profile of Staphylococcus aureus*

*Staphylococcus aureus* isolates were further tested for methicillin resistance with oxacillin 1 µg, vancomycin 30 µg and cefoxitin 30 µg using the Kirby–Bauer disk diffusion method. The isolates were cultured in nutrient broth, and the inoculum was standardized by comparing its turbidity to a 0.5 McFarland standard for antibiotic susceptibility testing (AST). The bacterial suspension was evenly spread on Mueller–Hinton agar using sterile cotton swabs, and antibiotic discs were placed on the plate. The plates were then incubated at 37°C for 24 hours and the AST results were interpreted.[21]

*2.6. Selection of medium for the production of bacteriocins*

To evaluate the maximum production of bacteriocins and appropriate medium from human microflora, four previously reported media were used includes LB (Oxide Thermo Fisher Scientific Massachusetts, US), Nutrient broth (Lab M Limited, UK), Brain Heart Infusion broth (Oxide Thermo Fisher Scientific Massachusetts, US) and MRS broth (NEOGEN Lansing MI, USA). Four culture media were prepared, sterilized, and inoculated with fresh microflora, and cell-free supernatants were collected at 24, 48, and 72 hours. These supernatants were screened against clinical MRSA, and antimicrobial activity was assessed by measuring zones of inhibition in millimeters.

*2.7. Production of bacteriocins from the isolates*

The bacterial isolates identified from the skin and oral cavity were selected for further screening for bacteriocin production. To induce maximum bacteriocin production, each strain was inoculated into 10 mL of De Man–Rogosa–Sharpe broth and incubated at the optimal growth temperature of 37 °C for 24 hours in a shaking incubator (Becton Dickenson, Franklin Lakes, NJ, USA) at 70 rpm under aerobic conditions.[22]

*2.8. Bacteriocin extraction and concentration*

After incubation, the bacterial cultures were centrifuged at 4000× *g* for 10 minutes and then the supernatants were collected. This process effectively separated the bacterial cells from the extracellular components, which also included the bacteriocins. The supernatants were carefully filtered by using a sterile filter paper of 0.22 µm pore size to collect the supernatant-contained bacteriocins and remove any residual bacterial cells or debris.[23] Then, the bacteriocin-containing supernatants were concentrated under rotary evaporator. The concentrated supernatants were used for screening against clinical isolates of MRSA.

*2.9. Methanol extraction of bacteriocins*

We added 15 mL of Milli Q water to the isolated extract of each promising strain and dissolved it. Then, 2 g of DuPont AmberLite XAD polymeric adsorbent resin was added and kept at room temperature for 24 hours. The extracts were centrifuged at 4500 rpm for 10 minutes at a low temperature to remove excessive water and to separate the XRD pellet. Then, 20 mL methanol was added to each tube with an XAD pellet and kept overnight. The solution was centrifuged, and the supernatant was used for further antibacterial analysis and cytotoxicity assay by dissolving 500 µL of it in MeOH and 200 µL in dimethyl sulfoxide (DMSO), respectively, and placing them in a Genevac EZ-2 solvent evaporator (ATS Life Sciences, Warminster, UK).

*2.10. Antimicrobial resistance profile of clinical isolates of MRSA*

Thirty clinical isolates of MRSA from a cancer hospital were collecetd and labeled them MRSA-1 to MRSA-30. Eleven of the 30 (37%) isolates were from cancer patients and 19 (63%) were from non-cancer patients. The MRSA isolates were tested against a panel of 12 commonly used antibiotics (Oxoid) to detect the antimicrobial resistance (AMR) of the isolates. Antibacterial discs of linezolid (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), vancomycin (30 µg), fusidic acid (10 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 U), oxacillin (1 µg), co-trimoxazole (1.25 µg), ciprofloxacin (5 µg), and tetracycline (30 µg) were used in this study. The ATCC 25923 *S. aureus* strain was the control.[21]

*2.11. Screening of bacteriocin against MRSA isolates*

The bacteriocins produced by skin and oral microbiota were screened against the 30 MRSA clinical isolates using MRS agar followed by agar well diffusion assay. Briefly, the MRSA isolates were streaked onto agar plates, and wells were made using a sterile cork borer. After sealing the wells, cell-free supernatants obtained from overnight cultures of the bacteriocin-producing isolates were added to the wells, the plates were incubated at 37 °C for 24 hours in the incubator, and oral microaerophilic isolates were incubated in a jar with 5% $CO_2$. After incubation for 24 hours, zones of inhibition were observed. The inhibition zones around the wells indicated the presence of bacteriocin activity.

*2.12. Bacteriocin characterization*

The bacteriocin-producing isolates were selected for further characterization. The stability of the bacteriocins was assessed by subjecting them to various pH levels, temperatures, and protease enzyme conditions. The bacteriocin extracts, mixed with an equivalent volume of dimethyl sulfoxide (DMSO), were used as a negative control and vancomycin as a positive control in the assays.[24,25] To determine the pH stability of the bacteriocins, aliquots of the concentrated bacteriocin samples were adjusted to pH values of 5, 6, 7, 8, and 9 using the appropriate buffering system. The bacteriocin extracts were incubated for 30 minutes at 37 °C. Bacteriocin activity was assessed by performing tests for antibacterial activity against MRSA isolates. The pH value at which the bacteriocin showed maximum activity was considered the optimum pH for stability.

To evaluate heat stability, aliquots of the concentrated bacteriocin samples were subjected to different temperatures: 4 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 80 °C. The samples were incubated for three hours, and the residual bacteriocin activity was assessed using antimicrobial assays. The temperature at which the bacteriocin retained maximum activity was considered the optimum temperature for stability.[26] Methanol extracts of all bacteriocin-producing isolates were incubated with proteinase K, trypsin and pepsin, lipase, alpha-amylase, and RNase and were tested for antimicrobial activity against clinical isolates of MRSA.

*2.13. Minimum inhibitory concentration and cytotoxicity analysis*

The minimum inhibitory concentration (MIC) of bacteriocins against MRSA was evaluated using the broth microdilution technique. The culture supernatant containing bacteriocins was filter-sterilized using a membrane filter of 0.2 µm pore size to remove any residual cells or debris. The resulting sterile filtrate was considered the stock solution of the bacteriocin. MRSA broth was prepared, and 96-well TPX plates (BKMAM, Changde, China) were used to determine the MIC. The bacteriocin solution was serially diluted two-fold in MRS broth. An overnight culture of the target bacterial strain was prepared in LB broth. The culture was adjusted to a specific cell density of 0.5 McFarland, and 50 µL of bacterial culture was inoculated into each well of a plate containing the bacteriocin solutions diluted two-fold. We used well 11 containing only the MRSA isolate as a positive control and well 12 with MRS broth as a negative control [27]. The assay was run in triplicate. Cytotoxicity analysis of the bacteriocins characterized was performed against ATCC cell lines—prostate adenocarcinoma cell line PC3, lung carcinoma

epithelial cell line A549, and colorectal carcinoma cell line HCT116—as per standard protocol.[28,29]

*2.14. Scale up fermentation, XAD and methanol extraction*

Selected isolates (1a, 1b, 4b, 4c, 5a, 6b) were scaled up from seed cultures to 5 L MRS broth, sampled at multiple time points for LC-MS, and incubated for eight days before extraction. Antimicrobial metabolites were isolated from both supernatant (XAD-16 resin) and pellet (methanolic extraction), concentrated, and analyzed by LC-MS to determine extracellular and intracellular production.

*2.15. Reverse-phase silica gel column fractionation*

XAD-derived extracts from isolates (1a–6b) were combined after showing similar LC-MS metabolic profiles and subjected to reverse-phase silica gel chromatography. Sixteen fractions (F1–F16), each eluted with 500 ml of increasing acetonitrile concentrations (0–100%), were collected. All fractions were dried by lyophilization to obtain concentrated samples. These concentrated fractions were then screened for antibacterial activity against clinically isolated MRSA strains R5 and R13.

*2.16. Antibacterial activity of fractions against MRSA, characterization MIC and cytotoxicity analysis*

Fractions F1–F16 dissolved in DMSO were tested for antibacterial activity against MRSA using broth dilution assays, standardized to 0.5 McFarland ($1\times10^8$ CFU/ml) and measured at 600 nm.
MIC was determined using a broth dilution assay in 96-well plates by exposing standardized MRSA inoculum ($1\times10^8$ CFU/ml, 0.5 McFarland) to serial concentrations of each promising fraction. After 24 hours of incubation at 37°C, optical density at 600 nm was measured, and the MIC was defined as the lowest concentration showing no visible bacterial growth. Cytotoxicity of all fractions was evaluated on PC3, A549, and HCT116 cell lines, using DMSO and $H_2O_2$ as negative and positive controls. Active fractions were further characterized for stability across varying pH levels, temperatures, and enzymatic treatments. Proteinaceous nature and robustness of bacteriocins were confirmed through enzyme digestion assays followed by antimicrobial testing.

*2.17. Data analysis*

Statistical analysis was performed with IBM SPSS v. 21.0 (SPSS Inc., Chicago, IL, USA). A one-sample *t*-test was used to determine the significance of the results obtained from the antimicrobial assay and analysis of variance (ANOVA) was performed to determine the characterization of bacteriocins. The post-hoc Tukey's honest significant difference test was also performed. GraphPad Prism v. 9.3.1 (GraphPad Software Inc., San Diego, CA, USA) was used for descriptive statistics and graphical representations to illustrate the results. The significance level (α) of 0.05 was used as the threshold for statistical significance in all tests.

## 3. Results

### *3.1. Isolation of human microbiota*

A total of 203 bacteria were isolated from the skin and oral cavity specimens. The skin microbiota consisted of 119 bacterial isolates, comprising 62 (52.1%) *S. aureus* and 57 (47.9%) *Streptococcus* sp. isolates. The oral microbiota consisted of 84 bacterial isolates, comprising 18 (21.4%) *S. aureus*, 49 (58.3%) *Streptococcus* sp., and 17 (20.3%) *Micrococcus* sp. isolates. Overall, the skin and oral cavity microbiota bacterial isolates included 80 (39.4%) *S. aureus*, 106 (52.2%) *Streptococcus* sp., and 17 (8.4%) *Micrococcus* sp. isolates.

### *3.2. Molecular identification of isolates*

The isolates were identified molecularly by amplification of the 16S rRNA gene. *S. aureus* was confirmed and MRSA was identified with PCR targeting of the species-specific genes *nuc* and *mecA*. The confirmatory *nuc* gene was detected in all 80 isolates of *S. aureus*. The *mecA* gene was detected in 57 (71.3%) *S. aureus* isolates, comprising 11/18 (61.1%) oral and 46/62 (74.2%) skin microbiota isolates. The size of the amplified fragments was 1500 base pairs (bp) for 16S rRNA, 162 bp for *mecA*, and 270 bp for *nuc* (Figure 1).

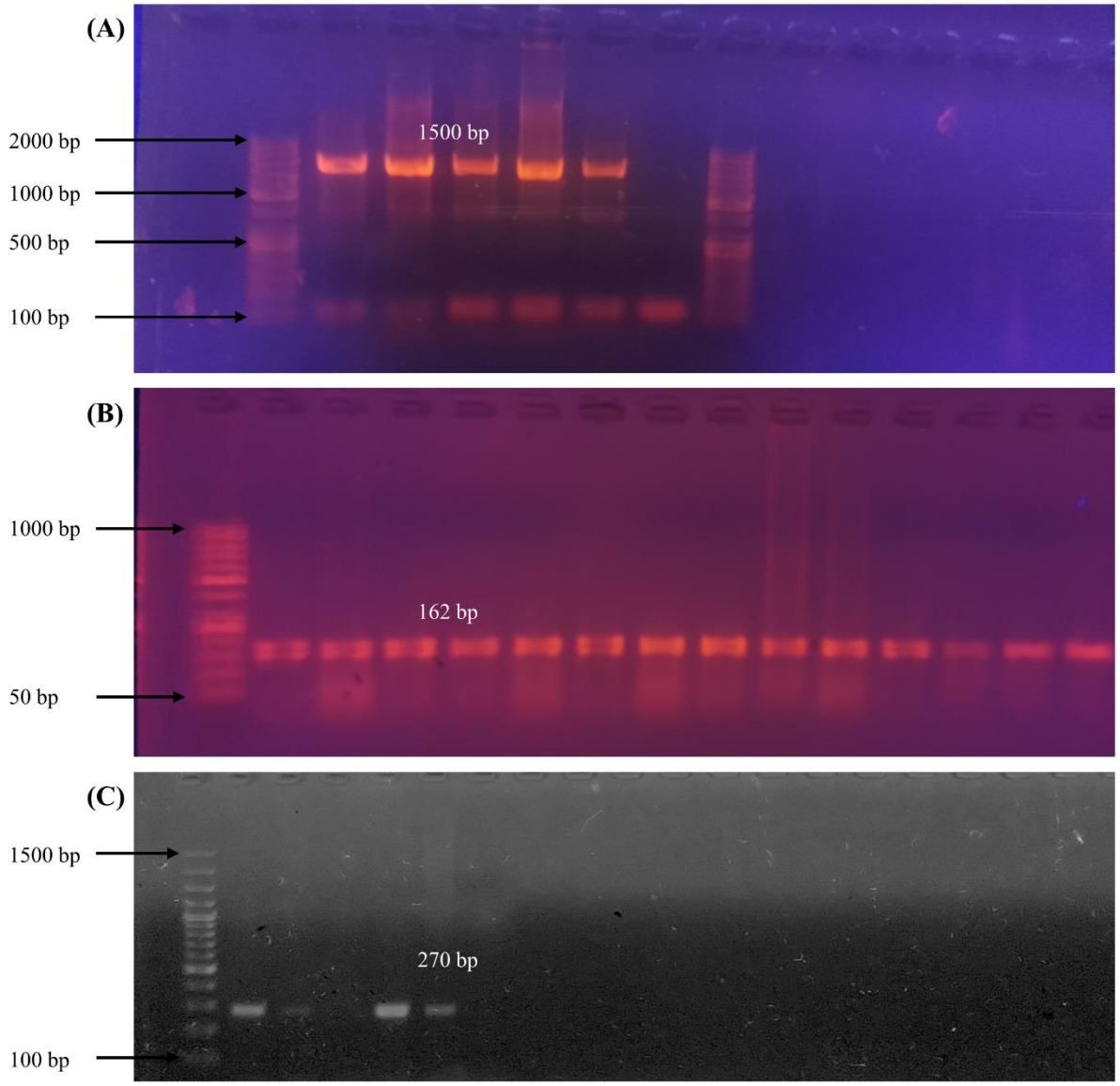



**Figure 1.** Molecular detection of *S. aureus* genes of human microbiota using agarose gel electrophoresis. (a) Amplification of the 16S rRNA gene using a 2000 bp DNA ruler. (b) The amplified *mecA* gene products included a DNA ruler of 1000 bp. (c) Amplification of the confirmatory *nuc* gene using a DNA ruler of 1500 bp.

*3.3. Antimicrobial resistance of clinical isolates of MRSA*

The AMR profile of clinically isolated MRSA from cancer patients demonstrated a high rate of resistance to penicillin (11/11, 100%), erythromycin (9/11, 79%), ciprofloxacin (8/11, 73%), and fusidic acid (7/11, 61%). The MRSA isolated from non-cancer patients also showed high resistance to penicillin (19/19, 100%), followed by erythromycin (16/19, 83%), ciprofloxacin

(14/19, 75%), and fusidic acid (12/19, 64%). However, there were no statistically significant AMR correlations ($p$ = 0.9831) between MRSA infections in cancer and non-cancer patients. Remarkably, none of the clinical isolates of MRSA were resistant to vancomycin, linezolid, and chloramphenicol (Figure 2).

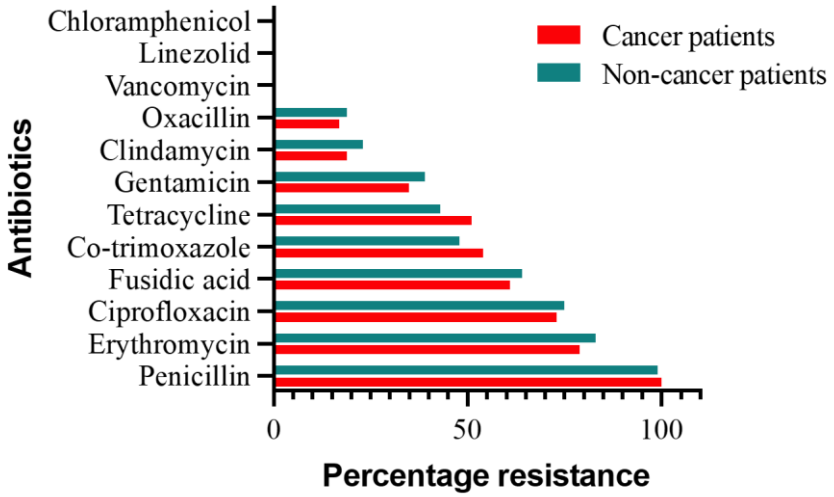



**Figure 2.** Antimicrobial resistance profile of clinical isolates of methicillin-resistant *Staphylococcus aureus* obtained from cancer patients and non-cancer patients.

*3.4. Selection of medium for the production of bacteriocins*

Four media LB, nutrient broth, BHI, and MRS were tested for bacteriocin production from human microflora (skin, oral cavity) by collecting cell-free supernatants at 24, 48, and 72 hours and screened them against clinical MRSA. The largest zones of inhibition were produced by supernatants grown in MRS medium, indicating superior bacteriocin yield. This aligns with previous studies showing that the enriched composition of MRS supports optimal bacteriocin production.

*3.5. Production of bacteriocins and screening for their activity against MRSA*

All the microbiota isolates (n = 203) were inoculated into MRS broth for bacteriocin production. The agar well diffusion assay was used to screen the cell-free supernatant for production of bacteriocins with activity against the 30 clinical isolates of MRSA. The screening results revealed that only six *S. aureus* isolates out of the 203 microbiota isolates exhibited inhibitory activity against MRSA-13 (isolates 1a, 1b, 4b, and 4c) and MRSA-5 (isolates 5a and 6b). These

isolates demonstrated the ability to produce bacteriocins that effectively inhibited the growth of MRSA clinical isolates (Figure 3).

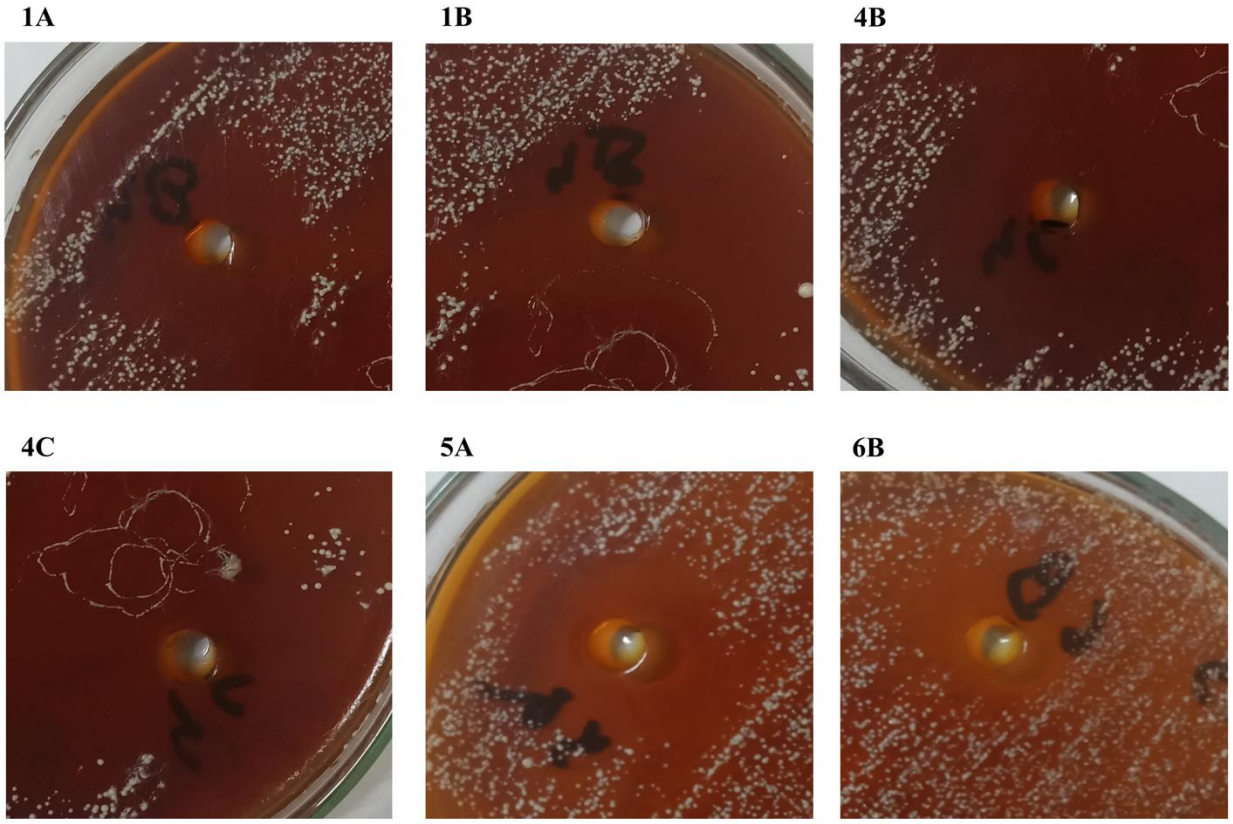



**Figure 3.** Zones of inhibition of bacteriocin-producing microbiota isolates against MRSA clinical isolates. The cell-free supernatant of bacteriocin-producing human microbiota *S. aureus* isolates 1A, 1B, 4B, and 4C inhibited the growth of the clinical isolate MRSA-13 and the bacteriocin-producing *S. aureus* isolates 5A and 6B inhibited the growth of the clinical isolate MRSA-5.

*3.6. Characterization of bacteriocins*

Bacteriocins produced by 1a, 1b, 4b, 4c, 5a, and 6b isolates were further characterized by subjected to different pH levels, temperature variations, and enzymatic treatments. The results indicated that the bacteriocins remained active and retained their antimicrobial activity across the range of conditions tested, suggesting their potential for practical applications. The stable pH range for the bacteriocins isolated was 6.5 to 7 and the temperature range was 25–30 °C. Enzymatic treatment showed significant results compared to the controls (positive and negative), with treatment with protease enzymes resulting in lower bacteriocin activity. The results of one-

sample *t*-tests indicated that the compound stability maximum was at the temperature of 30 °C ($p$ = 0.2133) and pH of 7 ($p$ = 0.1778), and that varying these two factors would change the compound's activity. In the case of enzymatic treatment, the $p$-values for proteinase K, trypsin, and pepsin were 0.0002, 0.0002, and 0.0003, which is less than ($\alpha = 0.005$), indicating that after treatment with protease enzymes, resulted in no significance (Figure 4).

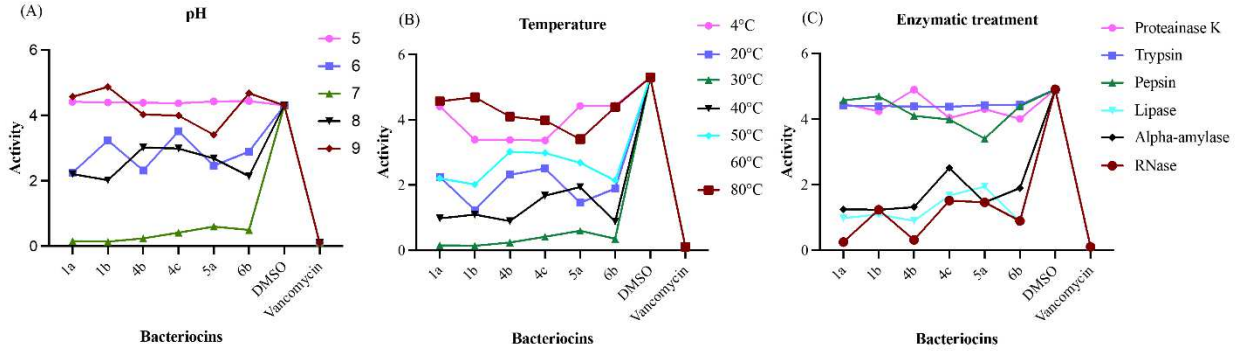



**Figure 4.** Stability assessment of bacteriocins to validate their resistance and characterization using pH, temperature, and enzymatic treatments. The bacteriocin extracts were mixed with DMSO (negative control) and vancomycin (positive control) in each assay. (A) The pH stability of bacteriocins at different pH levels ranging from 5 to 9. (B) The temperature steadiness of bacteriocins at different temperature intervals from 4 °C to 80 °C. (C) The effect of treatment with various enzymes on bacteriocins.

### *3.7. Minimum inhibitory concentration*

The MICs were assessed by visual inspection or by measuring the optical density (OD) of the bacterial cultures using a spectrophotometer at a wavelength of 595 nm. The MIC determination was replicated to ensure the accuracy and reproducibility of the results. Positive and negative controls were included in each assay to validate the assay's performance. One-way ANOVA showed that the MICs of the bacteriocins of 1a (162.5 µg/mL, $p$ = 0.000), 1b (425 µg/mL, $p$ = 0.000), 4b (162.5 µg/mL, $p$ = 0.004), and 4c (225 µg/mL, $p$ = 0.000) isolates against MRSA-13 were statistically significant. The MICs of the bacteriocins of 5a (121.875 µg/mL, $p$ = 0.025) and 6b (168.75 µg/mL, $p$ = 0.014) isolates against MRSA-5 were also statistically significant. A post-hoc test using Tukey's honest significant difference showed statistically significant differences between the MICs of 1a, 1b, and 4c bacteriocins against MRSA-13 and between the MICs of 5a and 6b bacteriocins against MRSA-5. No significant differences were found between

the MICs of 4b, 5a, and 6b bacteriocins against MRSA-13 and 1a, 1b, 4b, and 4c bacteriocins against MRSA-5.

*3.8. Cytotoxicity analysis of bacteriocins characterized*

Cytotoxicity analysis of the bacteriocins characterized was performed against the ATCC cell lines of PC3 prostate adenocarcinoma cells, A549 lung carcinoma epithelial cells, and HCT116 colorectal carcinoma cells [28]. The cells were treated with two concentrations of bacteriocin extract: 2 µL and 8 µL. DMSO was used as a control in the analysis. All six bacteriocin extracts at 2 µL concentration showed minimal or no toxicity to all the cell lines. At the 8 µL concentration of the bacteriocin extracts, a variable pattern of cytotoxicity against the cell lines was observed, with HCT116 showing the highest survival rate (Figure 5).

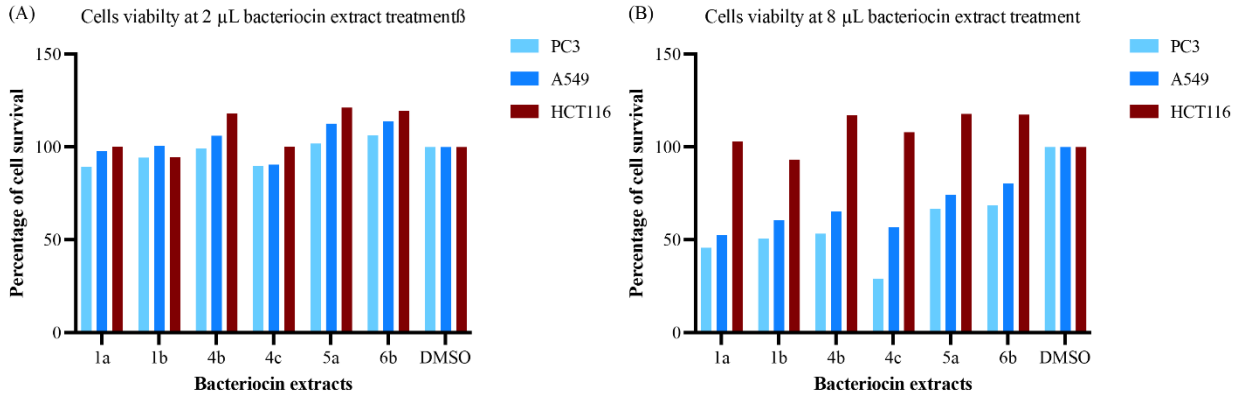



**Figure 5.** Bacteriocin cytotoxicity to PC3 prostate adenocarcinoma cells, A549 lung carcinoma epithelial cells, and HCT116 colorectal carcinoma cells. (A) Viability of PC3, A549, and HCT116 cells at 2 µL concentration of bacteriocin extracts. (B) Viability of PC3, A549, and HCT116 cells at 8 µL concentration of bacteriocin extracts.

*3.9. Scale up fermentation, XAD and methanol extraction, LC-MS analysis*

Isolates 1a, 1b, 4b, 4c, 5a, and 6b were scaled up from seed cultures into 5 L MRS broth, sampled at multiple time points (0, 4, and 8 days), and analyzed by LC-MS. After eight days, XAD-4% extraction was performed, followed by centrifugation, concentration, and LC-MS profiling, which revealed consistent metabolites with retention time 5.4 min and distinct molecular masses for each isolate. Since all XAD extracts showed similar compound profiles, they were combined for collective reverse-phase column fractionation.

*3.10. Antibacterial activity of reverse phase column fractions against clinical MRSA*

Dried XAD extracts from isolates 1a–6b (having dried weight ranges from 16.7–31.2 g) were combined, passed through a reverse-phase silica gel column, and eluted into 16 fractions using 0–100% acetonitrile gradients (500 ml each). All fractions were dried, dissolved in DMSO, and tested for antibacterial activity against MRSA strains R5 and R13 using broth microdilution assays. Fractions F5, F6, F7, and F11 showed activity against R13, while F4, F5, F8, and F12 were active against R5; the remaining fractions showed no antibacterial activity (Figure 6).

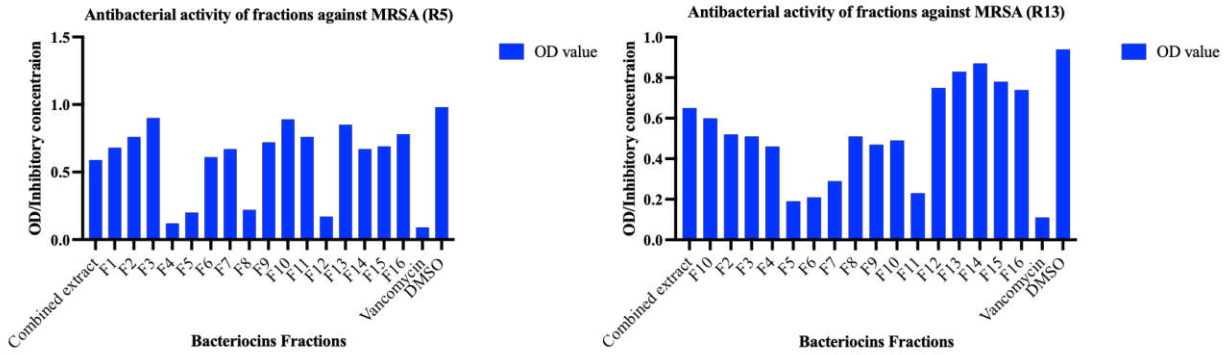



**Figure 6.** Antibacterial activity analysis of fraction F1-F16 against clinically isolated MRSA R13 and R5.

*3.11. Characterization of purified fractions containing bacteriocins*

Bacteriocin fractions (F1–F16) from isolates 1a, 1b, 4b, 4c, 5a, and 6b were further characterized for stability against pH, temperature, and enzymatic treatments. Fractions F5, F6, F7, F8, F11, and F12 retained antimicrobial activity across a temperature range of 25–30°C and pH 6.5–7, indicating optimal stability at 30°C and neutral pH. Enzymatic treatments with proteases (proteinase K, trypsin, pepsin) significantly reduced activity, while lipase, α-amylase, and RNase had minimal effect. One-sample t-test analysis showed *p*-values of 0.2151 and 0.1742 for temperature and pH, confirming non-significant variation and high stability. Protease treatments had *p*-values <0.005, indicating significant sensitivity to enzymatic degradation. These results demonstrate that the bacteriocins are heat- and pH-stable, with selective protease sensitivity, supporting their potential as effective antimicrobial agents (Figure 7).

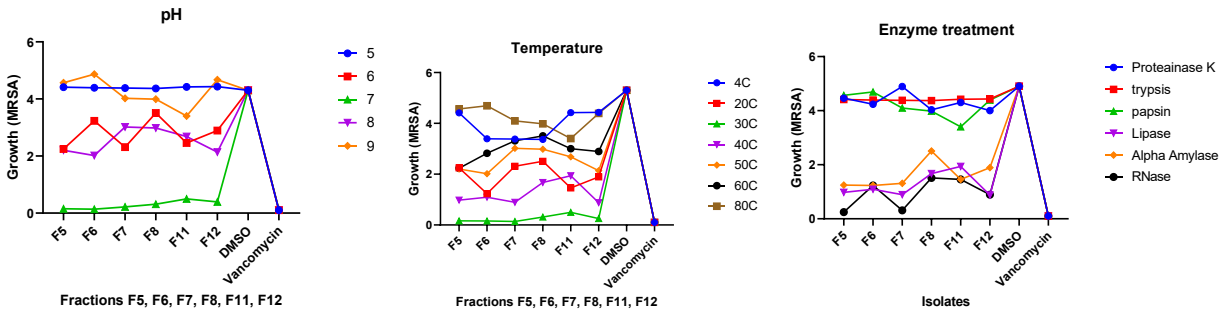



**Figure 7.** Stability assessment of fractionated bacteriocins to validate their resistance and characterization using pH, temperature, and enzymatic treatments. The bacteriocin extracts were mixed with DMSO (negative control) and vancomycin (positive control) in each assay. The pH stability of bacteriocins at different pH levels ranging from 5 to 9. The temperature steadiness of bacteriocins at different temperature intervals from 4 °C to 80 °C. The effect of treatment with various enzymes on bacteriocins.

*3.12. Minimum Inhibitory Concentration and cytotoxicity analysis of characterized bacteriocins fractions*

Cytotoxicity analysis of characterized bacteriocins fractions F1-F16 were performed against the ATCC Cell lines of Prostate adenocarcinoma cells PC3, Lung carcinoma epithelial cells A549 and cells were treated with 2ul volume of extracts. DMSO used as negative control and $H_2O_2$ were used as positive control. By observing the cell survival percentage and cell toxicity among fractions F1 to F16 were non-toxic to the PC3 cell line, however, in case of A549 Fraction 4 was shown 85% toxicity to the cell line and all fractions were non toxic to the HCT116 colorectal carcinoma cells lines. DMSO has shown 100 percent survival of the cells and $H_2O_2$ shown to the A549, PC3 and HCT116. H2O2 exhibited 99% toxicity to the A549, HCT116 and PC3 cell lines (Figure 8). The MICs of bacteriocin fractions against MRSA were determined by measuring the optical density (OD) of the bacterial cultures using a spectrophotometer at a wavelength of 595 nm with replicates ensuring accuracy and controls validating the assay. Against R-13, fractions F5, F6, F7, and F8 showed MICs of 121.5, 365.65, 162.5, and 225 µg/mL, respectively, all statistically significant ($p \leq 0.005$). Against R-5, fractions F11 and F12 showed MICs of 121.75 and 168.75 µg/mL, respectively ($p < 0.05$). Post-hoc Tukey analysis revealed significant differences between F5, F6, F8 against R-13 and F11, F12 against R-5, while other comparisons

were not significant. These results highlight the variability in potency among the bacteriocin fractions.

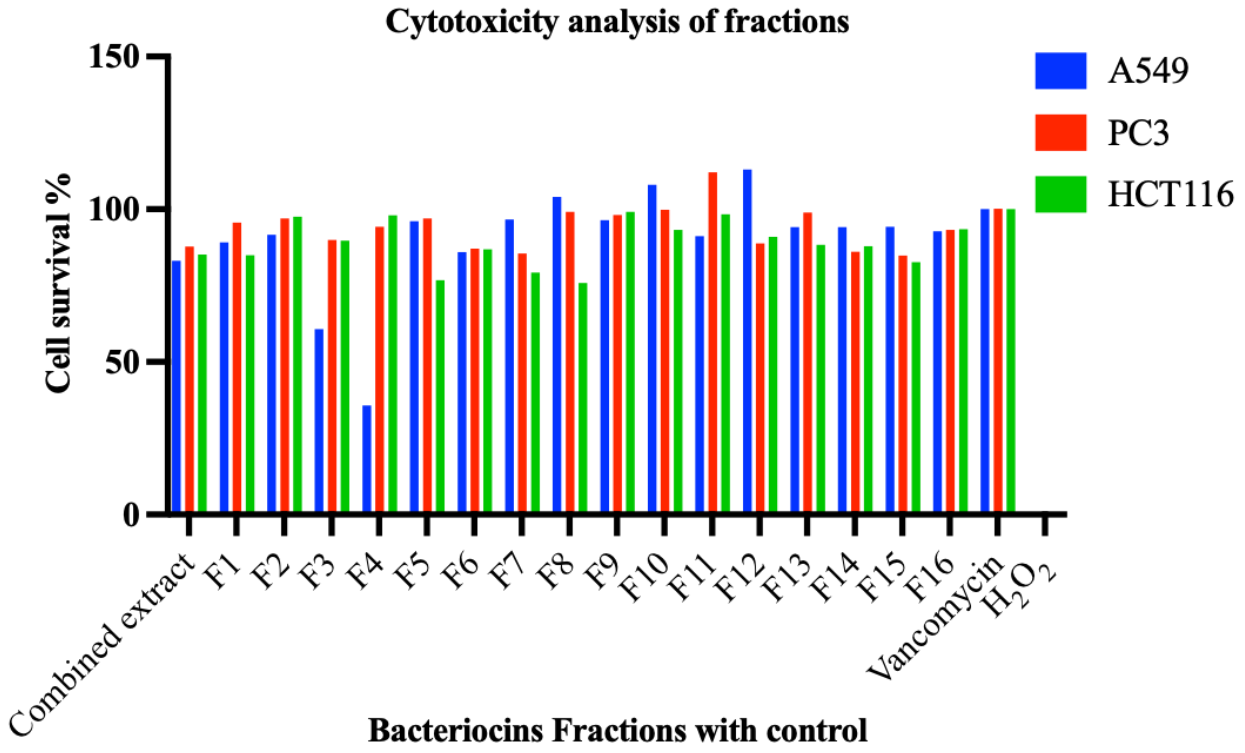



**Figure 8.** Cytotoxicity analysis for fractions with A549, PC3 and HCT 116 Cell lines.

## 4. Discussion

The detection of bacteriocins-producers with activity against MRSA offer vital insights into the potential of the oral and skin microbiota. The oral cavity is provide a complex microbial ecosystem, making it a promising reservoir for the discovery of novel antimicrobial agents.[30,31] This study isolated six *S. aureus* isolates from the microbiota of human skin and oral cavity, which produced bacteriocins with inhibitory activity against MRSA. The production of bacteriocins by these isolates highlights their potential as antimicrobial agents against MRSA. Their ability to produce bacteriocins adds another layer of complexity to their interactions with the skin and oral microbiota.[32]

MRSA causes a wide range of illnesses, such as pneumonia, which can be fatal for elderly people. Indrawattana et al.'s (2013) study on the prevalence of MRSA in Thailand found that 60.9% of the isolates were resistant to methicillin.[33] Another study of skin and soft tissue

infections conducted in European medical centers found that MRSA was the most common pathogen (22.5%).[34] MRSA has also been reported in the nasal cavity of healthy individuals.[35] In this study, the prevalence of molecularly characterized MRSA, isolated from healthy individuals' skin and oral cavity, was 71.3%. Healthcare-associated MRSA infections are frequently seen in cancer patients, while community-acquired infections are commonly observed in outpatients. The prevalence of MRSA infections has been reported to be 77.8% in Japan and 44.4% in France.[36] An Egyptian study found that 41.7% of the total *S. aureus* observed in cancer patients was MRSA.[37] MRSA isolates can present different characteristics in cancer and non-cancer patients.[38]

The discovery of bacteriocin-producing *S. aureus* is intriguing. Bacteriocin production by *S. aureus* strains has been reported as potentially useful for targeting MRSA infections. Our study explored six *S. aureus* isolates that produced bacteriocins, which were effective against two of the clinical isolates of MRSA with a considerable zone of inhibition. Therefore, this finding suggests that some *S. aureus* strains may possess antimicrobial properties that can target some MRSA strains, indicating the possibility of novel bacteriocins with broad-spectrum activity as reported from Brazil.[39] MRSA was recently found to be a source of bacteriocins that target the same species as those already reported in previous studies. The bacteriocin pentocin JL-1 has broad-spectrum antibacterial activity. In contrast, other studies have suggested that bacteriocins may target the cell membrane of MRSA, which may lead to the bacteriostatic mechanism or may result in bactericidal activity. Most bacteriocins have a molecular mass range of 10 kDa to 90 kDa, similar to proteins.[40] Bacteriocins have shown efficacy against MRSA strains, making them potential candidates for novel therapeutic approaches.[41]

A study from China demonstrated that bacteriocins are not only resistant to high temperatures and diverse protease enzymes but also possess significant antibacterial properties against MRSA. Stability against these factors is a promising option for enhancing bacteriocin production and providing better yields.[42,43] The bacteriostatic activity of bacteriocin peptides is influenced by factors such as pH, temperature, surfactants, and enzymes. Variation in temperature affects the molecular structure of bacteriocins, and a wide pH range also influences bacteriocin activity. Odah et al. reported that isolated bacteriocins are stable at a wide temperature range from 37 °C to 75 °C and are also up to 70% stable at 121 °C for 15 minutes.[44] In this study, all extracts were treated with different pH and temperature ranges, as

well as several enzymes; the optimum pH range was 6.5–7 and temperature range was 25–30 °C for bacteriocins from the specific isolates tested. Therefore, the stability of bacteriocins at different temperature ranges and acid–base environments and against protease degradation broadens their application range to different sectors. The bacteriocin-producing isolates identified in this study provide a foundation for further characterization, purification, and optimization of these antimicrobial peptides for potential clinical applications.

A study described two bacteriocins, Garvicin KS (GarKS) and Micrococcin P1 (MP1), with therapeutic potential against MRSA.[45] In our study, the statistically significant MICs of the characterized bacteriocins were observed against MRSA isolates. The lowest concentration of the bacteriocin that resulted in no visible growth or a significant reduction in OD compared to the control wells was considered the MIC, which provided valuable information about their inhibitory potential and aided in the development of potential therapeutic applications. In this study, the analysis of cytotoxicity of bacteriocin extracts at concentrations of 2 µL and 8 µL against the cell lines PC3, A549, and HCT 116 indicated non-cytotoxicity to cells.

Understanding the genetic and regulatory factors involved in bacteriocin synthesis will provide insights into the biosynthetic pathways and facilitate the development of strategies to enhance bacteriocin production. In addition, investigations of the dynamics of the interaction between bacteriocin-producing strains and the surrounding human microbiota are essential to assess its potential impact on the overall microbial ecology. Future studies should focus on purification and characterization of bacteriocins to determine their mechanisms of action, stability, safety profiles, and implications for the clinical sector. A limitation of the present study is that the effectiveness of bacteriocins was not evaluated through in vivo studies. These bacteriocins should be further assessed and described through in vivo studies using different infection models.

## 5. Conclusions

The isolation of bacteriocin-producing bacteria from the skin and oral cavity has important implications for the advancement of alternative antimicrobial treatments. We report six bacteriocin extracts that demonstrated strong activity against two clinically isolated MRSA strains that were resistant to multiple antibiotics. The extracted bacteriocins showed stability in

pH ranges of 6.5 – 7 and temperatures of 25 – 30°C. Enzymatic treatment showed significant results, with less activity compared to controls after protease enzyme treatment. Moreover, the cytotoxicity analysis of bacteriocins demonstrated no toxicity to prostate adenocarcinoma cells PC3, lung carcinoma epithelial cells A549, and colorectal carcinoma cells HCT116 at 2 µL bacteriocin extracts. Their use could significantly reduce the dependency on traditional antibiotics, thereby mitigating the growing issue of antibiotic resistance. However, further studies and clinical trials are needed to fully understand the therapeutic potential of bacteriocins and their mechanisms of action, stability, and safety profiles, paving the way for innovative and effective treatment for MRSA and other MDR infections.

**Acknowledgments**

We are thankful to the Jouf University Saudi Arabia, the Higher Education Commission of Pakistan, and Mr. Awais Malik, SSP, Punjab Police, for their support in conducting this study. We are also grateful to Professor Rehan Ahmad Khan, the University of the Punjab, for validating our statistical analysis.

**Funding**

Not applicable.

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**Competing interest**

The authors declare no competing interests.

## Data availability

All data generated or analyzed during this study are included in this published article.

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