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RESEARCH ARTICLE

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Evaluation of the antibacterial activity of cLFchimera and its synergistic potential with vancomycin against methicillin-resistant *Staphylococcus aureus*

Sahar Roshanak[a], Fakhri Shahidi[a], Atefeh Soradeghi Toopkanloo[a], Zana Pirkhezranian[b], Mohammad Hadi Sekhavati[b]

[a] Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

Frequent and unlimited use of antibiotics caused the development of antibiotic resistance by microorganisms. Therefore, there is an urgent need to discover novel antibacterial agents or a combination of agents as a safe treatment strategy for various infections. In the present study, the synergistic effects of cLFchimera, an antimicrobial peptide, and the vancomycin antibiotic were evaluated using the checkerboard method against methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria strain. cLFchimera had antimicrobial activity against MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) (MIC: 256 and 512 µg/mL, respectively). A synergistic effect was observed in the combination of cLFchimera with vancomycin (FIC: 0.375). The results showed that at FIC concentrations, the release of cytoplasmic materials from bacterial cells and the number of surviving cells were significantly ($p \leq 0.05$) higher and lower, respectively, than when peptides or antibiotics were used alone. SEM electron microscopic analysis at FIC concentration showed severe membrane damage of bacterial cells. In conclusion, the use of cLFchimera and vancomycin at FIC concentration reduces the consumption of both substances.

Keywords

Synergetic effect, Antimicrobial peptides, Antibiotics, Resistant bacteria

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Abbreviations

MRSA: Methicillin-resistant *Staphylococcus aureus*
MSSA: Methicillin-sensitive *Staphylococcus aureus*
Antimicrobial peptides: AMPs
MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration
FIC: Fractional inhibitory concentration

Corresponding author: *Fakhri Shahidi*
Email: fshahidi@um.ac.ir
Tel: +98 (51)3880-6056

Introduction

Staphylococcus aureus is a dominant pathogen both in the community and within hospitals. *S. aureus* is a Gram-positive bacteria which classified as a member of the family Micrococcaceae [1]. In humans, these bacteria commonly colonize on surfaces of the skin and the upper respiratory tract. Inflammation of lungs (pneumonia), infection of the mammary glands (mastitis), infections of the skin (impetigo), infection of the bone (osteomyelitis), infection of the endothelial lining of the heart and valves (endocarditis), and infection in the blood (bacteremia) are some disorders caused by *S. aureus*. *S. aureus* can also cause food poisoning, the result of enterotoxin production [1].

Administration of benzylpenicillin, β-lactam antibiotic, was the first treatment of *S. aureus* infections before the 1950s [2, 3], but producing a β-lactamase which inactivated the β-lactam, by resistant strains were causing increasing concern in the late 1950s [4]. Efforts were made to synthesize penicillin derivatives, methicillin, that were resistant to β-lactamase hydrolysis. Unfortunately, as soon as methicillin was used clinically, methicillin-resistant *S. aureus* (MRSA) strains were isolated [4]. We are currently in a situation where, in some cases, the glycopeptide antibiotic vancomycin, is the only choice for antimicrobial therapy. However, some reports demonstrated that vancomycin resistance-conferring genes from other bacterial groups can be expressed in *S. aureus* [5]. Therefore, an imperative need to propose new anti-staphylococcal agents to reduce or moderate methicillin resistance in *S. aureus* to an existing antibiotic.

Antimicrobial peptides (AMPs), which since the 1980s have been considered a possible alternative to existing antibiotics [6]. AMPs play an important role in the natural defense mechanism for destroying microbial infections [6]. AMPs have a net positive charge and an amphipathic structure and usually contain 12–50 amino acid residues [7, 8]. As is the case with AMPs, bacterial cells are less likely to develop resistance to AMPs because they disrupt the structure and function of cell membranes [9].

More recently, a chimeric form of peptide named cLFchimera has been expressed and purified in *E. coli* [10] in our lab. The results of in vitro studies showed that this peptide has antibacterial [10-12], antiviral [13], and anticancer [14] properties. Furthermore, the results of an in vivo experiment showed that supplementing *E. coli* challenged broilers with cLFchimera improved villi morphology in the jejunum, restored microbial balance in the ileum, and improved gene expression of cytokines and tight junctions in the jejunum of challenged birds [15]. These results revealed that this peptide could be nominated as an alternative for growth promoter antibiotics.

Enhancing the efficacy, restoring the sensitivity, and reducing the minimum effective dose of antibiotics, combined with other antimicrobial agents is one of the promising strategies[16, 17]. It has also been demonstrated that the combined use of APMs and antibiotics increase the bacterial killing of antibiotics regardless of the antibiotics' mode of action [18-20]. Membrane perturbation in or pore formation on the bacterial cell wall may enhance the uptake of antibiotics and increase their antibacterial effect [21].

The present study aimed to evaluate the combined effects of cLFchimera and vancomycin antibiotic compared with peptide alone, against methicillin-resistant *S. aureus* bacteria. The final goal of the present study was to determine the synergistic effects of these compounds to decrease the effective dose of antibiotics, thereby minimizing the potentially toxic side effects of these drugs and reducing the chance of antibiotic resistance.

Results

MIC determination and Check board assay

The MIC and MBC of vancomycin and cLFchimera were determined using the broth microdilution method before the synergy examination, and the highest concentration obtained was 512 µg/mL (Table 1). cLFchimera showed weak activity against *S. aureus* and MRSA in the present study (256 and 512 µg/mL, respectively). We chose commercially available vancomycin to investigate whether the combination of the cLFchimera with this antibiotic provided a synergistic effect. In combination with a low peptide concentration equivalent to 8-16 of its MIC (Fig. 1, A), vancomycin had improved antimicrobial activity, with 2–8-fold reduced MIC values (Figure 1B).

The microdilution checkboard method was carried out to evaluate the synergistic effects of the antibiotics combined with the cLFchimera, which were determined as FICIs (Table 1). The combination of vancomycin and cLFchimera had a synergic effect against both *S. aureus* and MRSA giving total synergism (FIC= 0.375), (Table 1).

Release of cytoplasmic material absorbing at 260 nm

Figure 2 shows that, for both bacteria, the release of cellular content, absorbing at 260 nm, is higher in vancomycin-treated bacteria than in cLFchimera -treated bacteria ($p \leq 0.05$) (Figure 2A). This release of cytoplasmic material was associated with total cellular mortality (Figure 2B). As shown in Figures 2A and 2B, the release of cytoplasmic materials and sur-

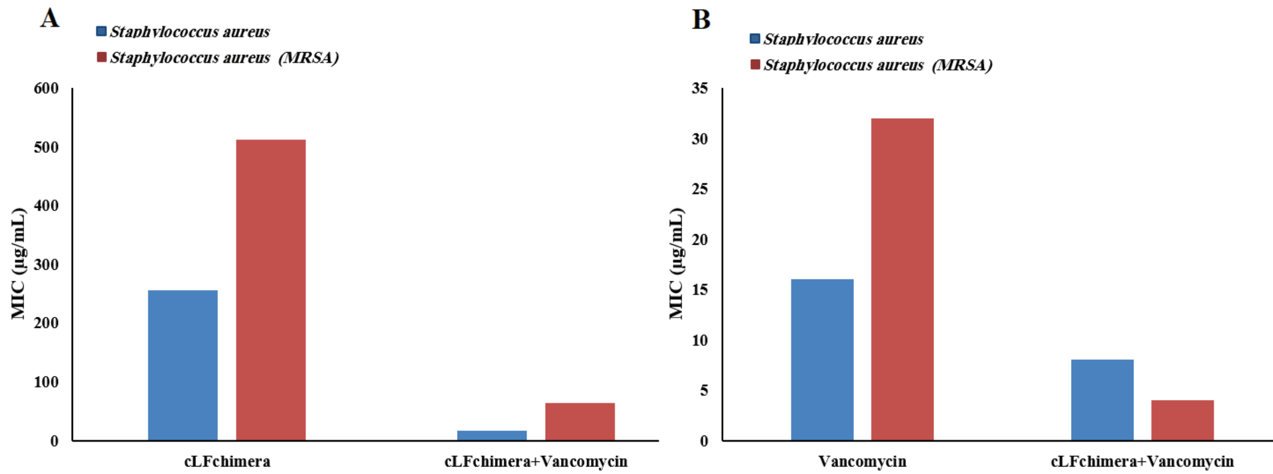

Figure 1.
Antibacterial activity of vancomycin in combination with cLFchimera. Bacterial cultures were treated with a series of concentrations of antibiotics in the presence of a low peptide concentration equivalent to 8-16 of its MIC at 37° C overnight (A). Vancomycin had improved antimicrobial activity, with 2–8-fold reduced MIC values (B). The OD600 was recorded using a microtiter plate reader. The MIC was defined as the lowest antibiotics concentration that inhibited the bacterial growth.

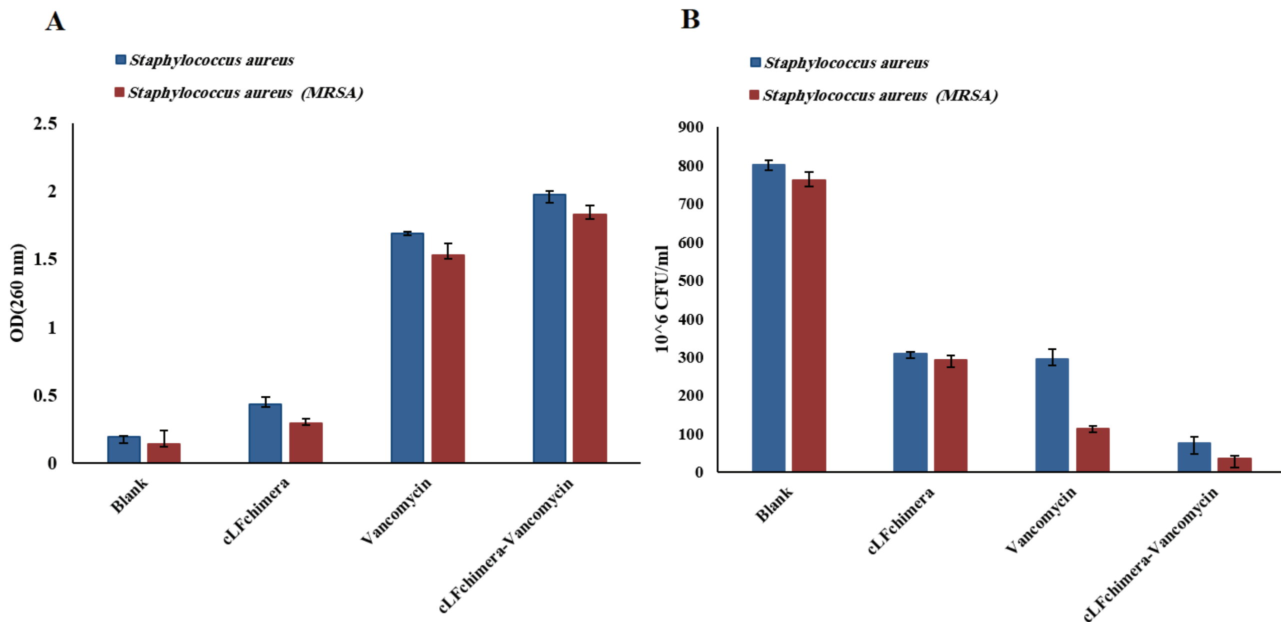

Figure 2.
Leakage of cytoplasmic material (OD at 260 nm) (A) and viable cell concentration in CFU/ml after incubation (B). Blank: without any treatment.

Table 1.
The MIC/MBC, FIC, and FIC Index of peptide and vancomycin against bacterial strains.

Microorganism	cLFchimera (µg/ml)		vancomycin (µg/ml)		FIC (µg/ml)		FIC index cLFchimera + Vancomycin
	MIC	MBC	MIC	MBC	MIC	MBC	
S. aureus (MSSA)	256	> 512	16	32	16	8	0.375[a]
S. aureus (MRSA)	512	> 512	32	64	64	4	0.375[a]

MSSA: Methicillin-sensitive *Staphylococcus aureus;* MRSA: Methicillin-resistant *Staphylococcus aureus*; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; FIC: Fractional inhibitory concentration ; [a]: Total synergism

vival of bacterial cells at MIC concentration of vancomycin and/or cLFchimera in comparison with FIC concentration of the combination of vancomycin and cLFchimera were higher and lower, respectively ($p \leq 0.05$).

Morphological study of S. aureus treated with peptide and vancomycin

For a better understanding of the effect of cLFchimera, vancomycin, and their combination (cLFchimera + vancomycin) on the bacterial morphology, the treated bacteria were observed using SEM (Figure 3). *S. aureus* was treated with cLFchimera (256 µg /mL), vancomycin (32 µg/mL), and their combination (4 and 64 µg/mL for antibiotic and peptide, respectively), and any damaging effects were observed with SEM (Figure 3). The shape of the bacteria treated with vancomycin, cLFchimera, and their combination did not differ greatly from the control group (blank, Figure 2A), suggesting that perhaps some molecular-level mechanisms that interact with peptidoglycan precursors, may disrupt efflux pumps, and cause irreversible changes in the flip-flop of membrane phospholipids [22] and may play important roles in the synergistic action of cLFchimera-vancomycin.

Discussion

Increasing the treatment difficulty and complexity regarding MRSE strains due to incorrect antibiotic use is currently a global issue in human health. AMPs are one of the newest and most promising classes of potent antibacterial drugs that can be considered as an alternative to antibiotics because they have useful features such as broad-spectrum antimicrobial activity and distinct membrane action mechanisms and are less likely to induce drug resistance in comparison to current antibiotics [23-25]. Furthermore, the effects of AMPs combined with antibiotics as a combination therapy often exceed those of the individual drugs, reduce the dose of drugs to minimize adverse effects, and thus be a way to overcome problems with toxicity and the development of resistance [26]. AMPs facilitate the passage of conventional small-molecule antibiotics through the membrane by disrupting the cell membrane, increasing access to these components to the cell, and exerting synergistic effects [27, 28].

The present study confirmed the synergistic effect between cLFchimera and vancomycin as the currently used antibiotic, against *S. aureus* and MRSA strains. When used as monotherapy, vancomycin showed moderate antibacterial activity against *S. aureus* and MRSA strain, with MIC values of 16 and 32 µg/mL, respectively. However, these antibacterial activities were significantly improved (2–8-fold reduced MIC values) in combination with the cLFchimera at a low concentration equivalent to 8-16 of the MICs. In addition, a total synergistic effect was also observed for the combination of the peptides and vancomycin against both strains in this study by using checkerboard assays. Similar to our results, Wu et al., (2017) showed that vancomycin had a high synergistic activity with a combination of DP7 peptides against *S. aureus* isolates [29].

The release of cellular content in treated samples with cLFchimera was significantly lower than in the antibiotic-treated groups. These results led us to hypothesize that cLFchimera exerts its antibacterial activity from other pathways instead of membrane disruption. Moreover, the results of SEM analysis showed that cLFchimera had no visible damaging effect on the outer layer of *S. aureus* as a model of Gram-positive bacteria, suggesting that maybe a molecular-level mechanism plays an important role in the synergistic action of cLFchimera. In this regard, Reyes-Cortes et al. (2016) showed that this chimeric peptide mediates its antibacterial activity by entering the cytoplasm through translocation across the bacterial membrane and possibly by interacting with internal organelles [30]. Consistent with these results, Pirkhezranian et al., using molecular simulation analysis showed that cLFchimera and its derivatives had a higher affinity for DNA interaction and hypothesized this chimeric peptide mediates its activity by intramolecular mechanisms which is the interference of DNA related pathways such as DNA replication [31, 32]. Moreover, treatment of both strains with vancomycin and its combination (vancomycin + peptide) resulted in significantly increased release of cellular contents. Vancomycin inhibits cell wall biosynthesis in Gram-positive bacteria by binding to D-alanine residues in the glycopeptide chain and thereby inhibiting cell wall synthesis [33]. Therefore, our results regarding the release of cellular content fit well with the mode of action of vancomycin.

Our results suggest that the cLfchimera may be used as promising synergistic agents to improve the antibacterial effectiveness of vancomycin against *S. aureus* and MRSA strains and to reduce the therapeutic dose of antibiotics, thus minimizing their toxic side effects. Overall, our results may suggest that cLFchimera mediates its synergistic activity independent of antibiotics by disrupting the cell membrane and intramolecular mechanisms which requires more investigation in future studies.

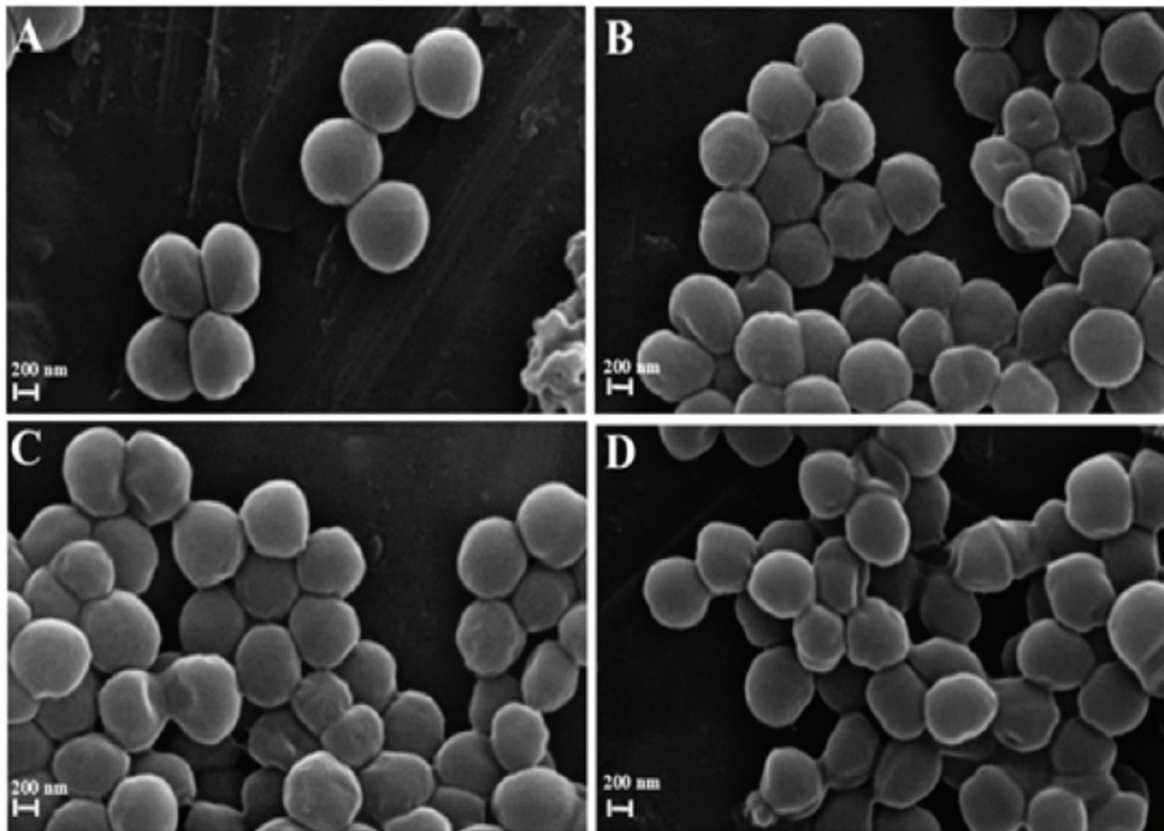

Figure 3.
SEM of *S. aureus* without vancomycin (A) and with 32 µg/mL vancomycin (B), with 256 µg /mL cLFchimera (C), and with the combination of 4 µg/mL vancomycin and 64 µg/mL cLFchimera (D).

Materials & Methods

Preparation of cLFchimera and antibiotic concentrations

The cLFchimera recombinant peptide developed in our previous study [10], was prepared n a sterile culture medium at 1000 mg/ml concentration and was filtered with a 0.22µm filter. This stock solution was used to prepare other dilutions [34]. Vancomycin was purchased from Jaber Ebne Hayyan Pharmaceutical Company, Tehran, Iran, and prepared according to CLSI [35].

Preparation of inoculum

The bacterial strains were obtained from the microbial collection of the Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad. Strains were *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* (MRSA) ATCC 33591. The microbial strains were cultured in Muller Hinton Broth (MHB) (Sigma-Aldrich) for 24 hours at 37 ºC, standardized with the 0.5 McFarland standard, and antimicrobial tests were performed according to the instructions [36], equivalent to 1.5×10^8 CFU/mL of microorganism.

Determination of minimum inhibitory concentration

MIC was performed using the micro broth dilution as suggested by the Clinical and Laboratory Standards Institution [34]. The cLFchimera dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/mL) were prepared in sterile MHB. 20 µL of microbial suspensions with an optical density of 630 nm (OD630) equal to 0.08-0.13, was added to 190 µL of each dilution in 96-well plates. The microwell plates were incubated at 37 ˚C for 24 h. ELISA reader model BioTek ELx808 was used to consider absorbance at 630 nm to determine the MICs. The protocol was repeated at the same concentrations for each microorganism using vancomycin. Growth medium without inoculum was used as negative control and MIC was defined as the lowest concentration with no growth of microorganism [37]. The experiments were repeated three times to confirm the results.

Determination of minimum bactericidal concentration

100 µl of each well, in which microbial growth was not observed according to the previous section, was cultured on Müller Hinton agar (Sigma-Aldrich). Then, the plates were incubated at 37 °C for 24 h and MBC was defined as the lowest concentration with no observable colony of microorganism [38]. The experiments were repeated three times to confirm the results.

Checkerboard assay to analyze the synergistic interaction between cLFchimera and antibiotic

The synergistic interactions between cLFchimera peptide and vancomycin were measured by the checkerboard method [17, 39]. Seven numerals of two-fold serial dilutions (from 2MIC to MIC/32) of the cLFchimera and vancomycin according to obtained MIC in the previous section for each microorganism were prepared. An equal amount (25 µl) of each dilution was poured into 96-well microplates to obtain a fixed amount of both antimi-

crobial peptides. Therefore, each row (and column) contained a fixed amount of the first agent and increased amounts of the second one. A total of 50 µl of fresh bacteria suspension (108 CFU/ml) were added to each well and cultured at 37˚C. The Fraction Inhibitory Concentration Index (FICI) was calculated using the following formula:

$FIC_I = MIC_{A/B} / MIC_A + MIC_{B/A} / MIC_B$

In the above formula, MIC_A and MIC_B belong to compounds A and B, respectively. $MIC_{A/B}$ belongs to the MIC of compound A in combination with B. Total synergism ($FIC_I \leq 0.5$), partial synergism ($0.5 < FIC_I \leq 0.75$), Indifference ($0.75 < FIC_I \leq 2$) or antagonism ($FIC_I > 2$) between the two compounds were obtained using FIC_I [40].

Survival Curve

The effect of cLFchimera and Vancomycin were evaluated and combination on the growth of microbial strains through the construction of a survival curve was illustrated [41]. The final concentration of suspension of the strains (adjusted to 10^6-10^8 CFU/ml) was added to the wells of 96-well microplates, and 50µl of the antimicrobial agent (at MICs or FICs concentrations), was added to each well. The bacterial strains were cultured at 37˚C for 24 h. After incubating, a 50 µl liquid from each dilution was spread on the surface of the agar plates and were incubated at 37 °C for 24 h, eventually, the number of CFU/ml was counted. It is worth noting that 50µl of the microbial suspensions without antimicrobial agents were used as a control group. Finally, survival curves were constructed using plotting the log number of CFU/ml against time (h).

Release of cytoplasmic material absorbing at 260 nm

The suggested method by Fadli et al. (2012) was used to measure the release of cytoplasmic material at 260 nm. Viable cells in their exponential phase were collected using centrifugation (4000 rpm for 15 min.), washed three times, and resuspended in a saline buffer solution. Three milliliters of cell suspension of approximately 108 UFC/ml were incubated, under agitation, for 1 h at 37 °C in the presence of the antimicrobial agent (at MICs or FICs concentrations). After incubation, cells were centrifuged at 4000 rpm for 20 min, and the absorbance (260 nm) of the supernatant was determined using a WPA Lightwave S2000 UV/Vis Spectrophotometer (Richmond Scientific Ltd, England). The untreated cells (control) were corrected with buffer saline [16].

Scanning electron microscopy

The bacterial strains were cultured to the logarithmic phase in 100 ml of MHB at 37 °C. The suspension was divided into four portions. Antimicrobials were added to three of the portions at MICs or FICs concentrations. The remaining portion was left untreated as a control. The resuspension was incubated at 37 °C for 3 h, and subsequently, the cells from all four tubes were harvested through centrifugation and fixed with 2.5% glutaraldehyde overnight at 4 °C. Subsequently, the cells were dehydrated using sequential ethanol concentrations ranging from 30 to 100%. The samples were gold-covered through cathodic spraying. The morphology of the bacterial cells was observed through scanning electron microscopy (SEM, LAO-1450VP, Germany) [17] .

Authors' Contributions

Conceptualization, SR and FS; methodology, SR and AS; software, ZP; validation, FS and MHS; investigation, MHS; original draft preparation, SR; review and editing, SR and ZP; supervision, FS; funding acquisition, FS. All authors have read and confirmed the final version of the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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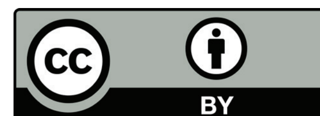

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