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In Vitro Efficacy of Silver Carbene Complexes, SCC1 and SCC22, Against Some Enteric Animal Pathogens

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ABSTRACT

Silver carbene complexes (SCCs), a group of novel silver-based compounds capable of gradually releasing silver ions, have shown significant antimicrobial activity against a wide range of bacterial pathogens mainly isolated from human cases. The antimicrobial activity against animal isolated pathogens has yet been done. The *in vitro* efficacy of two SCCs with different carrier molecules (SCC1 with a methylated caffeine backbone and SCC22 with a dichloroimidazolium backbone) was investigated against three important animal and human pathogen species. SCC1 and SCC22 exhibited bacteriostatic and bactericidal effects against multidrug resistant *Salmonella* Typhimurium (poultry isolate), *E. coli* 843 and *E. coli* 1568 (swine isolates), and the poultry field isolates *Salmonella* Heidelberg, *Salmonella* Enteritidis, and *Salmonella* Montevideo with MICs and MBCs ranged from 16-21 µM (6-8 µg/mL) and 16-32 µM (6-12 µg/mL), respectively. *Clostridium perfringens* type A was sensitive to both SCC1 and SCC22 with the MICs being 11 (4 µg/mL) and 21 µM (8 µg/mL), respectively. These values were comparable to the MICs and MBCs for silver acetate. The MBCs against *C. perfringens* was >85 µM for SCCs and >192 µM for silver acetate (>32 µg/mL for all compounds). Ten hours incubation of *C. perfringens* with 40 µg/mL of all three products showed down regulation of virulence genes *plc* and *netB,* suggesting viable cells and silver can modulate the virulence. Treating the *C. perfringens* with higher concentration (100 µg/mL) of each SCC for 10 hours inhibited more bacteria compared to the untreated bacterial cells, however, no differences in the ultrastructure of lysed bacteria were seen and this concentration might not induce viable but non-culturable (VBNC) state as suggested by transmission electron microscopy findings. SCCs showed a broad antimicrobial activity against all bacterial species tested including multidrug resistant pathogens. Both SCCs demonstrated inhibitory effect against the Gram-positive anaerobic *C. perfringens* type A which could have a high accumulation capacity for silver ion. These data suggest that SCCs may represent a novel class of broad-spectrum antimicrobial agents, which may be used to reduce the burden of pathogenic bacteria in the gastrointestinal tract of poultry.

Keywords: silver ion, SCC1, SCC22, necrotic enteritis, *Clostridium*, foodborne

INTRODUCTION

Enteric diseases caused by opportunistic microorganisms pose a significant threat to animal production, leading to economic losses due to decreased productivity and increased potential for human health risks associated with foodborne illnesses (1). One strategy employed by producers to mitigate enteric bacterial burden and enhance growth performance has been the inclusion of antibiotics in animal feed, often at sub-therapeutic doses.

However, this practice has contributed to the alarming rise of antibiotic-resistant bacteria, posing a major public health concern. Consequently, the use of antibiotic growth promoters (AGPs) has been banned in the European Union since 2006 (2) and is facing increasing restrictions in the United States. This has spurred research into alternative strategies for maintaining gut health and optimizing production efficiency in poultry (3).

Silver has a long history of use as an antimicrobial agent, dating back to ancient times (4). Its broad-spectrum activity against bacteria, fungi, and even viruses, coupled with its relatively low toxicity to host cells, has renewed interest in its potential as an alternative to traditional antibiotics (5). The antimicrobial action of silver ions is multifaceted, involving disruption of cell membrane integrity, inhibition of respiratory enzymes, and generation of reactive oxygen species (ROS) that damage cellular components (**6, 7**). However, the efficacy and safety of silver-based antimicrobials can be limited by the rapid release of silver ions, leading to potential toxicity and reduced bioavailability (8).

To address these limitations, novel silver-based formulations have been developed, including silver carbene complexes (SCCs). These complexes consist of a silver ion coupled with N-heterocyclic carbenes (NHCs) as ancillary ligands. The relatively strong silver-carbon bond in SCCs facilitates a controlled and gradual release of silver ions, potentially enhancing their antimicrobial efficacy while minimizing host toxicity (**9**). Indeed, SCCs have demonstrated exceptional in vitro activity against a wide range of human pathogens, including multidrug-resistant bacteria and even biosafety level 3 organisms, with minimal cytotoxic effects on mammalian cells (**10-18**).

However, the antimicrobial efficacy of SCCs against pathogens specifically isolated from animals, particularly under anaerobic conditions relevant to the poultry gut environment, remains largely unexplored. Therefore, this study aimed to investigate the *in vitro* activity of two SCC formulations (SCC1 and SCC22) against important poultry pathogens, including *Salmonella* spp., *E.* coli, and *Clostridium perfringens* type A. Additionally, the study sought to explore the potential of SCCs to modulate virulence gene expression and induce morphological changes in *C. perfringens*, providing further insights into their mode of action against this critical poultry pathogen.

MATERIALS AND METHODS

Silver Carbene Complexes (SCCs)

Two distinct SCCs with different carrier molecules were used in this study: SCC1, an organic compound with a methylated caffeine backbone, and SCC22, which contains a dichloroimidazolium backbone. Both SCCs were provided in pure formulations by the Department of Microbial Pathogenesis and Immunology at Texas A&M Health Science Center and have been previously synthesized and characterized (11, 14, 17). SCC1 has a molecular weight of 375.13 g/mol and exhibits solubility in water at 11 mg/mL, while SCC22, with a molecular weight of 375.99 g/mol, is soluble up to 110 mg/mL in water. Additionally, silver acetate (AgAc) with a purity of 99.9% and a molecular weight of 166.92 g/mol was sourced from Sigma-Aldrich for comparative analyses.

Bacterial Species and Growth Conditions

A selection of bacterial species, provided by the USDA-ARS facility in College Station, TX, was tested, encompassing both aerobic and anaerobic pathogens with known zoonotic potentials and varying resistance profiles. *Clostridium perfringens* type A, isolated from various geographical regions (two from Texas and Virginia, two from Georgia) and implicated in necrotic enteritis (19), was the primary anaerobic bacterium under investigation. These isolates were previously screened and confirmed positive for the *plc* gene (20) and were further screened for the presence of the *netB* gene in this study. Five poultry field isolates of *Salmonella* enterica were tested, including a multidrug-resistant *Salmonella* Typhimurium, resistant to 14 antibiotics, *S. Heidelberg*, *S.* Enteritidis, *S.* Kentucky, and *S.* Montevideo. Three strains of *E. coli* were tested, including two multidrug-resistant isolates from swine (*E. coli* 843 resistant to 5 antibiotics, and *E. coli* 1568 resistant to 6 antibiotics) and one poultry field isolate. One poultry field isolate of *Staphylococcus aureus* was evaluated.

The bacteria were cultivated from glycerol stocks on blood agar (Difco Laboratories, USA) plates and incubated at 37°C overnight. Following this, bacteria were inoculated into either standard Mueller-Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ) or Brucella broth (Becton Dickinson and Company, Franklin Lakes, NJ) for *C. perfringens*, with subsequent incubation until the mid-log phase was reached. Optical density measurements were utilized to standardize the bacterial concentrations for the assays.

Antibacterial Activity

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of SCC1, SCC22, and AgAc were determined using a broth microdilution method, following the standard protocols outlined by the Clinical and Laboratory Standards Institute (CLSI) for aerobic and anaerobic bacteria. Bacterial strains were initially revived from glycerol stock and cultured on blood agar plates (Difco Laboratories, USA) followed by overnight incubation at 37°C. Subsequently, colonies from these plates were transferred into Mueller-Hinton broth (Becton Dickinson and Company, USA) or Brucella broth (Becton Dickinson and Company, USA) for Clostridium perfringens, aiming for an initial optical density at 650 nm (OD_{650}) of 0.25. The cultures were then agitated at 200 rpm (excluding *C. perfringens*) until they reached an OD_{650} (or OD_{625} for *C. perfringens*) of 0.4, correlating with approximately 2×10^8 Colony-Forming Units per milliliter (CFU/mL) for aerobic bacteria and approximately 108 CFU/mL for Clostridium perfringens, as verified by serial dilution plating. The bacterial suspensions were diluted to 10^5 CFU/mL (10^6 CFU/mL for *C. perfringens*) in a volume of 100 µL. This preparation was introduced into a 96-well

microplate (Becton Dickinson and Company, USA), each well receiving 100 µL of SCC1, SCC22, or AgAc solutions. These solutions were initially prepared in deionized distilled water using a Milli-Q biocel system (Millipore, Billerica, MA), from stock concentrations of 10 mg/mL. SCC solutions were further diluted from 1 mg/mL and 0.1 mg/mL stocks in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) resulting in a mixture of 95% sterile water and 5% DMSO. Control wells contained the same percentage of DMSO without the antimicrobial agents. The range of final concentrations examined were 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, and 32 µg/mL. The MIC was determined as the lowest concentration showing no visible bacterial growth across three wells after 18–20 h of incubation at 37°C (or 24-48 h for *C. perfringens*). For the MBC assessment, a 100 µL sample from each clear well was cultured on blood agar and incubated for an additional 18-20 h, with the MBC defined as the lowest concentration showing no bacterial growth. All MIC and MBC measurements for each bacterium were performed at least in duplicate. For *C. perfringens*, MIC and MBC evaluations were performed under strictly anaerobic conditions facilitated by an anaerobic chamber (Koy Laboratory Products INC, USA) maintaining an atmosphere of 5% CO_2, 5% H_2, and 90% N_2 at 80% relative humidity. Assays involving *Salmonella* spp, *E. coli* strains, and *Staphylococcus aureus* were conducted under aerobic conditions. Stock solutions for each antimicrobial agent were prepared by dissolving them in deionized distilled water, with subsequent 1:10 dilutions to achieve the working concentrations, ensuring each dilution was double the final desired concentration.

C. perfringens Gene Expression of Virulence Genes

C. perfringens genes involved in toxin production (*plc* and *netB*) were selected for a gene expression study. To prepare total cellular RNA for gene expression analysis, 0.5 mL of *C. perfringens* (10^8 CFU/mL) at the mid-log phase of bacterial growth was treated with SCC1, SCC22, or AgAc (40 µg/mL, slightly higher concentration than MBC at 32 µg/mL) for 10 h. RNA stabilization was achieved using RNAprotect Bacteria Reagent (Qiagen, Germany), and total RNA was extracted using the RNeasy® Protect Bacteria Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription of total RNA to cDNA was performed using the EasyScript Plus™ cDNA Synthesis kit (Lamda Biotech, Carlsbad, CA, USA). Primers for real-time PCR (Table 1) that produced PCR amplicons of 100–150 bp in length were designed based on the *C. perfringens* strain 13 sequence (NCBI accession number: BA000016.3) using the Primer 3 plus Input software (21). Real-time PCR was carried out using 1× iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 100 ng of the cDNA template, and 300 nM each of the forward and reverse primers. Thermal cycling conditions were as follows: 95°C for 5 min to activate the AmpliTaq DNA Polymerase, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extending at 60°C for 1 min. The dissociation curve was run following the real time reaction to determine if the primers used in each reaction generated a specific product. The relative expression levels of the virulence genes were quantified using the $2^{-\Delta\Delta Ct}$ method, with *16S rRNA* as the reference gene. The results expressed as $\log_2$ fold changes to allow for easier visualization and interpretation of gene expression levels.

Table 1. Primers used in this study for *plc* and *netB* gene expression

Target	Primer	Sequence (5' – 3')
plc	Forward	TGACACAGGGGAATCACAAA
	Reverse	CGCTATCAACGGCAGTAACA
netB	Forward	GGAAAAATGAAATGGCCTGA
	Reverse	GCACCAGCAGTTTTTCCTTC
16S rRNA	Forward	TGCACCAGGAACTAAAGCAA
	Reverse	TTCCAAGTCCTGAGCAAGGT

Ultrastructural Analysis

The combined *C. perfringens* strains, at a final concentration of approximately 10^6 CFU/mL, were treated with 100 µg/mL of SCC1 or SCC22 for 10 h at 37°C under anaerobic conditions. The bacterial suspensions were then prepared for ultrastructural examination using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Fixation was performed using a glutaraldehyde fixative containing 4% glutaraldehyde in 100 mM phosphate buffer (pH 7.3) with 100 mM sucrose buffer (Electron Microscopy Sciences, Hatfield, PA, USA). Post-fixation with 1% osmium tetroxide was omitted for one set of samples to facilitate the detection of silver precipitates by Energy Dispersive X-Ray Spectroscopy (EDS). The samples were dehydrated through a series of ethanol solutions and embedded in epoxy resin (Electron Microscopy Sciences) for thin sectioning and TEM examination using an FEI Tecnai G2 F20-TEM (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Oxford Instruments EDS detector (Abingdon, UK).

Four *C. perfringens* bacterial strains were adjusted to a concentration of approximately 10^6 colony-forming units per milliliter (CFU/mL) and treated with 100 µg/mL of either SCC1 or SCC22. This incubation was conducted for 10 h at 37°C under anaerobic conditions. For the purpose of ultrastructural studies, the bacterial suspensions were mixed with an equal volume of glutaraldehyde fixative, comprising 4% glutaraldehyde in a 100 mM phosphate buffer (pH 7.3) supplemented with 100 mM sucrose buffer (Electron Microscopy Sciences, Hatfield, PA, USA). Following the initial fixation, the samples underwent a single buffer rinse before being consolidated in 2.5% agar. After solidification, the samples were divided into two groups; one underwent further fixation in 1% osmium tetroxide, while the secondary fixation step was bypassed for the other group. Subsequently, the samples were separated again for analysis: some were prepared for TEM by being embedded in epoxy resin (Electron Microscopy Sciences) following a methodical dehydration and infiltration process, and others were set up for SEM

observation. The decision to omit post-fixation with osmium tetroxide aimed to facilitate the detection of nickel metal deposits, both visually and through Energy Dispersive X-Ray Spectroscopy (EDS) analysis, using an FEI Tecnai G2 F20-TEM equipped with an Oxford Instruments EDS detector. The presence of nickel was confirmed via EDS in both control and treated samples. The fixation protocol's components were selected to prevent X-ray signal interference from any silver atoms potentially present in the samples (22).

RESULTS AND DISCUSSION

MIC and MBC

Results of the MICs and the MBCs of SCC1, SCC22, and AgAc against the tested bacterial species are shown in Table 2. The results showed that the combined four wild isolates of *C. perfringens* type A were sensitive to both SCCs treatment with the MICs for SCC1, SCC22, and AgAc using Brucella broth at 11, 21, and 48 µM/mL, respectively. *C. perfringens* seems to be more sensitive to SCCs, in particular SCC1, than AgAc. Similarly, SCC1 and SCC22 exhibited bacteriostatic and bactericidal effects at lower concentrations than AgAc against multidrug resistant *Salmonella* Typhimurium, *E. coli* 843 and *E. coli* 1568, and the poultry field isolates *S.* Heidelberg, Salmonella Enteritidis, and *Salmonella* Montevideo with MICs and MBCs ranging from 16-21 µM (6-8 µg/mL) and 16-32 µM (6-12 µg/mL), respectively. This could be credited to the fact the ligands act to stabilize corresponding silver complexes to a certain extent, thus controlling the release of the silver ion in the culture medium. On the other hand, silver in a salt form such as silver nitrate could precipitate as insoluble AgCl salt, thus reducing the concentration of the biologically active silver ion in the media (23). However, MBC against *Salmonella* Kentucky was >85 µM for SCC1. This *Salmonella* serovar also showed the highest bactericidal effect values among gram negative bacteria for SCC22 (63 µM =24 µg/mL), and AgAc (73 µM=12 µg/mL). The MIC and MBC against the poultry field isolate *E. coli* for SCC1 and SCC22 were 21 and 42 µM, respectively. This suggests the functionality is for the silver moiety. The MICs against the poultry field isolate *Staphylococcus aureus* was 43, 43, and 24 µM for SCC1, SCC22, and AgAc, respectively. Similar to *C. perfringens*, bactericidal effect against *Staphylococcus aureus* was > 32 µg/mL for the 3 products.

Table 2. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) in µM of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc)

	SCC1		SCC22		AgAc	
Bacterial spp.	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
C. perfringens [1]	11	>85	21	>85	48	>192
Salmonella Typhimurium [2]	16	21	16	21	24	24
Salmonella Enteritidis [3]	16	16	16	16	24	24
Salmonella Kentucky [3]	32	>85	32	63	24	73
Salmonella Heidelberg [3]	21	21	16	32	24	34
Salmonella Montevideo [3]	16	16	16	32	24	34
Staphylococcus aureus [3]	43	>85	43	>85	24	>192
E. coli [3]	21	42	21	42	24	34
E. coli 843 [4]	16	16	16	16	24	24
E. coli 1568 [5]	16	16	16	16	24	24

[1]Four geographically distinct *C. perfringens* type A field isolates, confirmed as associated with necrotic enteritis infection, was prepared by combining equal volumes of each isolate at mid-log phase. These isolates originated from Texas (1 isolate), Virginia (1 isolate), and Georgia (2 isolates). [2]Poultry field isolat, resistant to 14 antibiotics: clindamycin, cloxacillin, erythromycin, lincomycin, vancomycin, methicillin, nalidixic acid, novobiocin, penicillin G, rifampin, streptomycin, chlortetracycline, tetracycline, and sulfizoxazole (sulfisoxazole); [3]Poultry field isolats. [4]Swine field isolate, resistant to five antibiotcs: ampicillin, tetracycline, chloramphenicol, kanamycin, and sulfizoxazole; 5 [4]Swine field isolate, resistant to six antibiotcs: ampicillin, tetracycline, kanamycin, streptomycin, gentamycin, and sulfizoxazol

It was previously reported that SCCs exhibit unique and broad-spectrum activity against both Gram-positive and Gram-negative bacteria, fungi, methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis* and biosafety level-3 bacteria such as *Bacillus anthracis* and Yersinia pestis. The present study demonstrates that SCCs can be effective as an antimicrobial even in the case of anaerobic Gram-positive *C. perfringens*, and foodborne pathogens isolated from animals, with MICs values constant as previously reported for different bacterial species. Leid et al. (12) reported MICs for SCC1 and SCC22 against methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from human septum and blood were 45.4 µg/mL. while MICs against the *Staphylococcus aureus* isolated from blood was 22.7 µg/mL for both SCC1 and SCC22. The same author reported the MICs and MBCs of human clinically isolates of MRSA for SCC1 were 6 and 8 µg/mL, and SCC22 were 6 and 10 µg/mL. Panzner et al. (17) reported that the minimum inhibitory concentration at which 90% of the strains tested fail to grow (MIC_{90s}) of SCC1 and SCC22 against *Burkholderia pseudomallei* were 8 and 6 µg/mL, respectively. The MIC_{90s} of SCC1 against strains of *Pseudomonas aeruginosa* and multidrug-resistant organisms from the *Burkholderia cepacia* complex was 6 µg/mL (11).

MBCs against *C. perfringens* type A were >85 µM for SCCs, and >192 µM for AgAc (>32 µg/mL for all compounds). It is well known that Gram positive bacteria have higher MBC values than gram negative as the structure of cell wall is different. Sütterlin et al. (24) showed that MBC of silver ion for Gram-positive bacteria was more than 32 times higher than the MBC values for the Gram-negative bacterial cells. On the other hand, under anaerobic condition silver ion could lose a major mechanism as antimicrobial, generating ROS. The oligodynamic effect of

silver ion is well known as it can bind the thiol group when present inside the cell and thus inhibit the activity of several enzymes. In the aerobic respiratory chain, which is identified as a primary site of ROS generation (**25**), silver ions are known to inhibit thiol-containing enzymes (26) such as NADH dehydrogenase II. *C. perfringens* is a Gram-positive, obligatory anaerobe that uses nitrate (NO_3) as the final electron acceptor in anaerobic respiration (27). Additionally, the *C. perfringens* genome does not contain enzymes for the tricarboxylic acid cycle or respiratory chain but contains anaerobic fermentation enzymes leading to gas production (CO_2 and H_2) (28). Thus, *C. perfringens* cannot generate ROS as they do not reduce the O_2 and subsequently generate reactive products such as hydrogen peroxide (H_2O_2) which is required for Fenton reaction to generate hydroxyl free radicals (29). Additionally, there is no literature available evaluating SCCs against Gram positive restricted anaerobic bacteria. Previous studies using silver nitrate under anaerobic and aerobic condition, against Staphylococcus aureus and E. coli showed that the effect of silver ion under anaerobic conditions is not the same as its activity under aerobic conditions, yet it caused reduction in bacterial growth (30). Bactericidal activities of silver zeolite and silver nitrate was examined against *E. coli* strain OW6 and showed that under anaerobic conditions more cells were viable than in the presence of oxygen (31). Moreover, (32) reported that the 93% of the total cells (Pseudomonas aeruginosa) which were viable in suspensions treated with silver nanoparticles (5 µg/mL) did not correspond to an increase in cell death ratio but accelerated the transition to viable-but nonculturable (VBNC) status which plays a significant role in the survival of bacteria (33). Under VBNC status the transport, biosynthesis and the ability to utilize substrates are still continued but accompanied by a reduction in metabolic activity levels to minimize cellular energetic requirements (34), which is a survival strategy of many bacteria in response to adverse environmental conditions.

Gene Expression Modulation by SCCs

As the 4 tested isolates of combined *C. perfringens* showed no detectable cidal effect at up to 32 µg/mL after 24 h incubation for all products, which clearly indicates bacteria were not introduced to a viable but non-culturable (VBNC) state. We further investigated silver ion effects during the first 10 h of treatment initiation by treating the bacterial cells with a slightly higher than MBC (40 µg/mL) to see if silver ion from different carrier molecules could induce an earlier VBNC state with this higher concentration and potentially induce virulence modulation. The microorganism gene expression after a stress treatment could give an indicator of viable cells. mRNA is turned over rapidly in living bacterial cells, with most mRNA species having a half-life of only a few minutes (35). Detection of mRNA might therefore be a good indicator of living cells or those only recently dead at the time of sampling (36). Thus, the expression of virulence genes of *C. perfringens* after silver treatment could give an indicator of silver ion interacting with viable cells. *C. perfringens* genes involved in toxin production (*plc* and *netB*) were thus selected for a gene expression study.

RT-qPCR analysis showed that the expression levels of *plc* and *netB* genes were down-regulated by 8.8-fold (log_2 fold change of ~-3.14) and 315-fold (log_2 fold change of ~-8.303), respectively, after treatment with 40 µg/mL of SCC1 for 10 h at the mid-log phase of bacterial growth (Figure 1). Similarly, treatment with 40 µg/mL of SCC22 led to a down-regulation of *plc* and *netB* gene expression by 1.86-fold (log_2 fold change of ~-0.901) and 48-fold (log_2 fold change of ~-5.569), respectively, while AgAc treatment resulted in a down-regulation of 47-fold (log_2 fold change of ~-5.5) and 36-fold (log_2 fold change of ~-5.18) for *plc* and *netB*, respectively.

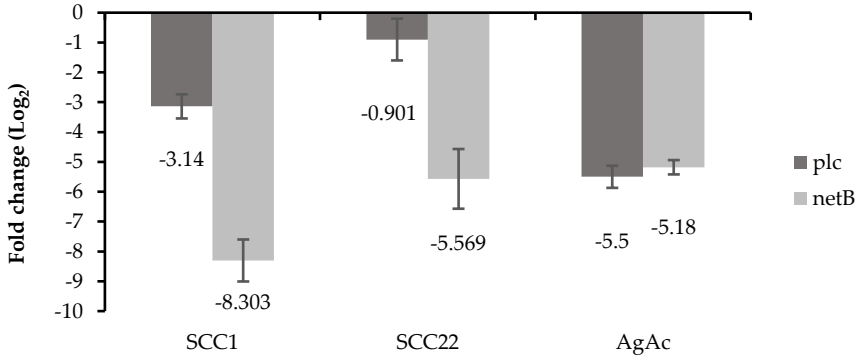

Figure 1. Relative gene expression of *plc* and *netB* of *Clostridium perfringens* type A treated with 40 µg/mL of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc) for 10 h. Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression. The relative expression ratio for each gene is presented as a log_2. A ratio more or less than 0 indicates up/down regulation of gene expression. Error bars indicate standard deviation for 6 replicates

The results suggest decreased the pathogenicity of *C. perfringens* after being subjected to silver ion, and silver from all 3 products interact with viable *C. perfringens*. SCC1 and SCC22 induced more depression of netB compared to plc, and this could be attributed to netB being a plasmid gene, while plc is a chromosomal gene. In addition, the carrier molecules, caffeine in SCC1, is known to induce mutations in bacteria and fungi by binding to DNA and interfering with normal cell cycle checkpoint functions (37, 38).

Morphological Changes

TEM and SEM along with EDS were performed to investigate possible structural changes and silver deposition that could appear when treating the bacterial cell with higher concentration (100 µg/mL) for 10 h. Examination of postfixed and non-postfixed samples by TEM revealed bacteria with similar ultrastructural characteristics (**Figure 2, 1 to 3**). Post-fixation in osmium tetroxide was omitted to enhance the observation of nickel particles by both visual and EDS observation. Examination of whole block faces of embedded bacteria using the combination of SEM and EDS did reveal the presence of silver at a higher percent in SCC22 treated bacteria (0.52 Wt%, Table 3) compared with control and SCC1 treated bacteria. The very small amount of silver detected in control group that has not been treated by silver could be a minor contamination or it could reflect that the host could also subjected to silver ion. Nickel is an essential trace element for the genus Clostridia to synthesis carbon monoxide dehydrogenase which catalyzes the reversible oxidation of carbon monoxide to carbon dioxide as a source of energy (39). However, it is not clear if the nickel detected by EDS could be influx or efflux of the metal.

SCC22-treated bacterial cells showed small dense staining aggregate clusters on the bacterial cells when bacteria were viewed using TEM. These structures were located on the along the exterior cell wall of live bacteria (**Figure 2, 3A**) and within the interior of lysed bacteria (Figure 4, 3 B inset). It seems that *C. perfringens* can be exposed to high concentrations of (accumulate) more silver with keeping integrated cell wall with no lysis. The lysed bacteria showed detachment the cell wall from cytoplasm membrane. The interaction of silver ions with bacterial inner membrane is an important mechanism of silver ion toxicity (40). Jung et al. (41) demonstrated that the accumulation of silver ion in the bacterial cell envelope is followed by detachment of the cytoplasmic membrane from the cell wall in both Gram-positive and Gram-negative bacteria. These structures (electron dense particles) were further examined in detail by TEM and EDS (Figure 3) which failed to demonstrate the presence of nickel above background levels recorded in un-osmicated control bacterial samples, instead the silver ion was probably incorporated in relatively small amount. These results would indicate that growth inhibition of bacterial cells can be accomplished without the accumulation of large concentrations of silver ions with the bacteria themselves.

SCCs showed broad antimicrobial activity against all bacterial species tested including multidrug resistant pathogens *Salmonella* Typhimurium and *E. coli* isolated from poultry and swine, respectively. Both SCCs demonstrated inhibitory effects and virulence modulation against the Gram-positive anaerobic *C. perfringens* type A which could have a high accumulation capacity for silver ion. These data suggest that SCCs may represent a novel class of broad-spectrum antimicrobial agents, which may be used to reduce the burden of pathogenic bacteria in the gastrointestinal tract of poultry.

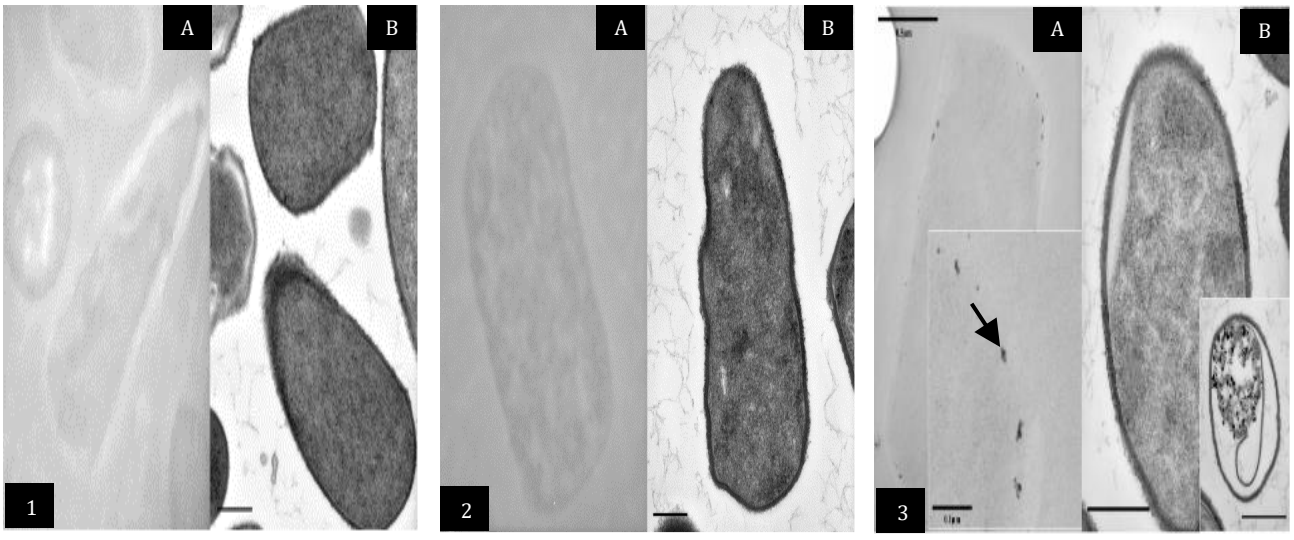

Figure 2. Micrograph of external morphology of *Clostridium perfringens* type A treated with 100 µg/mL of SCC1 and SCC22 for 10 h observed by TEM. Untreated bacteria **(1)**, unosmicated **(A)** and osmicated **(B)**, SCC1-treated bacteria **(2)**, unosmicated **(A)** and osmicated **(B)**, SCC22-treated bacteria **(3)**, unosmicated **(A)** and osmicated **(B)** samples with electron dense granular material (arrows) on the outside of bacterial cell walls and within what appeared to be lysed bacteria **(B Inset)**. Micron bars in **B** represent 0.5 µm

Table 3. Element composition and relative weight percentages (Wt%) of each element when whole block faces of epoxy embedded bacteria examined by scanning electron microscopy (SEM) and energy dispersive X-Ray Spectroscopy (EDS), Wt% sigma represents standard deviation

	Control		SCC1		SCC22	
Element	**Wt%**	**Wt% sigma**	**Wt%**	**Wt% sigma**	**Wt%**	**Wt% sigma**
C	72.23	1.33	84.73	3.72	84.69	0.42
O	27.54	1.33	12.30	3.78	14.11	0.42
Al	0.04	0.06	2.63	0.33	0.25	0.02
Si	0.10	0.05	0.29	0.16	0.41	0.02
K	0.04	0.04	0.00	0.00	0.00	0.00
Ag	0.04	0.11	0.05	0.34	0.52	0.05

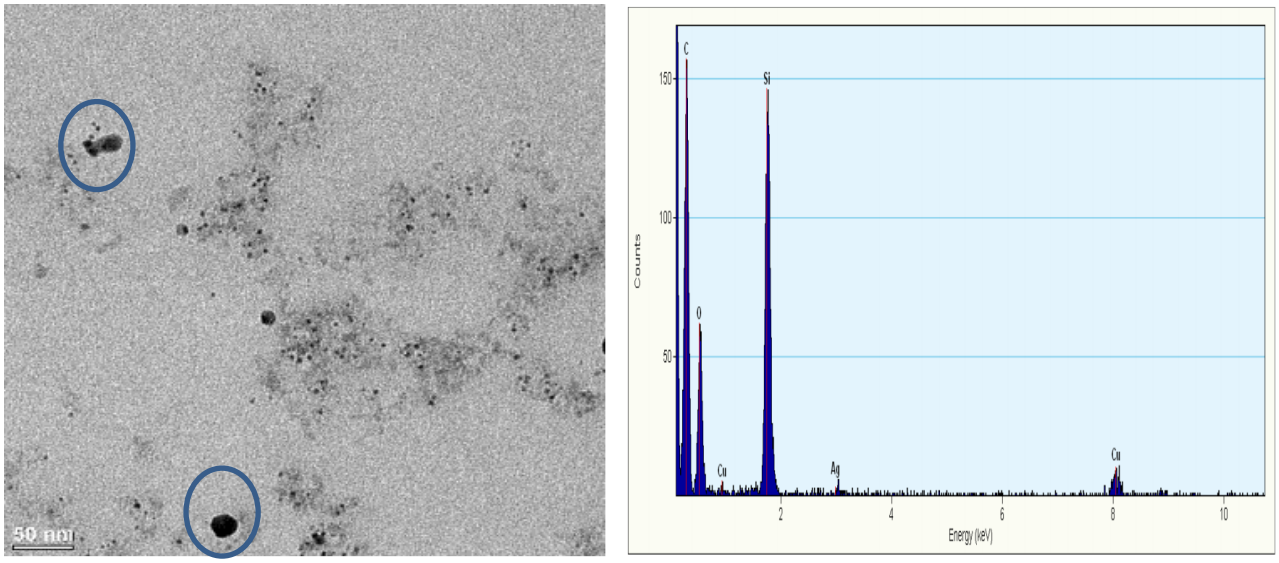

Figure 3. Transmission electron microscopy image of electron dense particles seen within samples of silver carbene complex 22 (SCC22) treated bacteria and the accompanying EDS data for the particles observed in that area of the sample

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N/A

CONFLICT OF INTEREST

The authors declare no conflict of interest.

EDITORIAL TRANSPARENCY

Akhil M Alsadwi serves as a member of the editorial board for The Iraqi Journal of Veterinary Medicine. Despite this role, the peer review process and the final publication decision were made independently and impartially, ensuring no influence from the author's editorial position

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