EVALUATION OF SILVER ION AS A POTENTIAL ANTIBIOTIC ALTERNATIVE IN BROILER CHICKENS

A Dissertation

by

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ABSTRACT

The goal of this dissertation was to evaluate silver ion as a potential antibiotic alternative in broiler chickens. For this purpose, four experiments were conducted to evaluate silver ion in terms of *in vitro* antimicrobial efficacy, *in vivo* potential toxicity, and to evaluate its potential growth promoting effect.

In experiment 1, an *in vitro* antimicrobial efficacy experiment was conducted using two separate silver carbene complexes (SCCs) with different carrier molecules, (SCC1 with a methylated caffeine backbone and SCC22 with a dichloroimidazolium backbone), and silver acetate were investigated against four important animal and human pathogen species. Both 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. Ten hours incubation of CP 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. 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.

In experiment 2, a preliminary *in vivo* study was conducted to investigate the potential acute oral toxicity of SCC1, SCC22, and AgAc on 300 7-day old broiler chickens in 2 independent replicates trials. Compared to the control and SCCs, single administration of silver acetate at a dose 1000 mg/kg BW reduced ($P < 0.05$) BW after 7 and 14 days of administration, although the birds showed normal weight gain compared to the control. No adverse effects of

SCC1 and SCC22, and silver acetate on relative organ weight of vital internal organs, bone mineralization, or plasma enzymes (ALT, ALP, and GGT) and metabolites (blood urea nitrogen, creatinine, and total bilirubin) were noted compared to control group.

Study 3 was a *Clostridium perfringens* challenge study to evaluate the potential effect silver acetate (AgAc) on starter broiler chicken performance and necrotic enteritis development. The *in vivo* efficacy of AgAc delivered either by adding into the feed (as a prophylactic model) or via drinking water (as a treatment model) during necrotic enteritis challenge and Infectious Bursal disease immunization was evaluated in two experiments. Whether AgAc was added into the feed (for 21 d) or in the water (4 d), there were no differences ($P > 0.05$) seen in bird performance, *Clostridium perfringens* enumeration, and lesion score compared to the positive and negative controls. These results suggest limitation effect of silver acetate on performance and reduce intestinal *Clostridium perfringens* colonization in broiler chickens, although the *in vitro* efficacy results showed bacteriostatic and bactericidal different enteric poultry pathogens.

In experiment 4, we further evaluated the potential effect of silver acetate in comparison to antibiotic and its selected alternatives (probiotic and prebiotic) on performance, energy and amino acids digestibility, intestinal histology, total bone mineral content (BMC) and density (BMD), and hepatic glutathione. Dietary supplementation of AgAc at 10 and 50 ppm did not show different effects on performance compared to the Control group during all production phases. Dietary AgAc supplemented at 250 ppm showed reduced BW ($P < 0.05$) compared to the Control, BMD50, and dietary AgAc at 10 and 50 ppm. Overall, using silver acetate in broiler diets could result in adverse effects at concentrations of 250 ppm, and lower concentrations did not show improvement on performance over the control or BMD50.

DEDICATION

I dedicate this work to everyone who supported me during my PhD journey. To my mother, I wished you were here to follow the pursuit of my PhD degree. To my father, you passed away while I was here obtaining this degree, you were the person who put me on this road, I wished you were here, you are missing greatly. To my family members, brothers and sisters, thank you from the bottom of my heart for all your love, patients, support, and faith in me. To my wife and kids, Mohammed and Sama, for their love and patience while I pursued this degree, you were the main reason pushing me not to stop during hard times, God bless you.

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NOMENCLATURE

Ag^+	Silver ion
AgAc	Silver acetate
AGP	Antibiotic growth promotor
ALT	Alanine transaminase
ALP	Alkaline phosphatase
Alb	Albumin
AID	Apparent ileal digestibility
BMD50	Bacitracin methylene disalicylate
BMD	Bone mineral density
BMC	Bone mineral content
BUN	Blood urea nitrogen
BW	Body weight
Creat.	Creatinine
Ca	Calcium
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
DXA	Dual X-ray Absorptiometry
EDX	Energy Dispersive X-Ray Spectroscopy
FCR	Feed conversion ratio
Glo	Globulin

GGT	Gammaglutamyl transpeptidase
GSH	Glutathione (reduced)
GSSG	Glutathione (Oxidized)
HPLC	High performance liquid chromatography
LD50	Median Lethal dose
mL	Milliliter
µM	Micro molar
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MTL	Maximum tolerable level
MOS	Mannan-oligosaccharide
MH	Mueller-Hinton broth
netB	Necrotic Enteritis toxin B gene
NE	Necrotic Enteritis
NHC	N-heterocyclic carbene
OD_{625}	Optical density at 625
plc	Phospholipase c gene
qRT-PCR	quantitative Real Time Polymer Chain Reaction
SCCs	Silver carbene complexes
SCC1	Silver carbene complex 1
SCC22	Silver carbene complex 22
SEM	Scanning electron microscopy

SFP	Shahidi Ferguson Perfringens agar
TEM	Transmission electron microscopy
TP	Total Protein
TB	Total Bilirubin
WG	Weight gain
YCW	Yeast cell wall

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

The use of antibiotics in livestock production is one of the potential contributors to the emergence of antibiotic resistant bacteria. In an effort to improve production efficiency (weight gain and feed conversion ratio), diets have been fortified with antibiotics, often at sub-therapeutic doses. Long periods of exposure to these sub-therapeutic doses have induced bacterial populations to acquire genetic mutations or plasmids that allow them to survive in the presence of the antibiotics. Recognition of the contribution of antibiotics in feed to the emergence of antimicrobial resistance has led to a ban on the use of antibiotic growth promoters (AGP) in the European Union in 2006 (Castanon, 2007) and increased restrictions on the use of AGP in the United States. As of January 1, 2017, a new regulation of the Food and Drug Administration restricts an animal producer from using antibiotics as growth promoters if they are deemed a medically important antimicrobial with respect to human medicine. Banning AGP in European was almost immediately followed by health problems in broiler chickens, and an outbreak of *Clostridium perfringens* (CP) infections was seen (Castanon, 2007). Banning antibiotics from animal feed in the United States will likely cause the same challenge, which has promoted research into alternatives to AGP (Niewold, 2006) such as probiotics and prebiotics (Gardiner et al., 2004), organic acids (Partanen and Mroz, 1999), herbs and their extracts (Burt, 2004).

Silver (Ag^{+}) is a white lustrous metal whose concentration in the Earth's crust is about 0.1 mg/kg (NRC, 2005). Silver is the second element of group 11 in the periodic table, which is

the group that has most if not all essential biologically trace elements, such as copper (Cu), zinc (Zn) and iron (Fe). Silver has no identified essential metabolic function as a trace mineral (Lansdown, 2007). Silver ion possesses antimicrobial properties and has been used as an antibacterial agent since before the discovery of penicillin to treat different human diseases (Klasen, 2000). The antimicrobial activity of silver ions is characterized by a high effectiveness, low toxicity to host cells, and rare development of resistance. Distinct from conventional antibiotics, silver is active under both aerobic and anaerobic conditions against a wide range of microorganisms including multidrug resistant bacteria due to its multifactorial mechanisms as an antimicrobial: inducing cell membrane damage, inhibiting respiratory enzymes, perturbing metal ion homeostasis, impact antioxidant status, and generating reactive oxygen species that eventually lead to damage of cellular components such as lipids and DNA. This could explain the scarce reported cases of resistance (Hindi et al., 2009). However, resistant against silver ion was reported (Silver 2003). Lansdown (2010 b) in his book mentioned six bacterial species that were reported to have resistance against silver, all of these bacteria were isolated from human infections (*Salmonella* Typhymirium, *E. coli*, *Enterobacter* cloacae, *Klebsiella* pneumonia, *Aceniter baumannii*, and *Pseudomonas stutzeri*).

Both antimicrobial activity and toxicity of silver ion depend on bioavailability of silver ion in a biological system, which in turn (bioavailability of silver ion) depends on the delivery method, silver source, ionization, solubility and concentration of the biological ligands that can bind silver ion such as proteins, peptides and anions. Therefore, different silver-based formulations were synthesized based upon constant bioactive metal with a nontoxic carrier (Cannon et al., 2009).

Recently, N-heterocyclic carbene (NHCs) are being used as ancillary ligands to silver ions. NHCs are neutral 2-electron donors, with an ability to bond to both hard and soft metals. Silver N-heterocyclic carbene complexes (SCCs), a novel of silver-based compounds that gradually release silver ion, have gained a substantial amount of attention in human medicine as a result of exceptional antimicrobial efficacy (Melaiye et al., 2004) against a wide range of microorganisms including both Gram-positive and Gram-negative as well as fungi and even biosafety level 3 bacteria, with low toxic effects on mammalian cells (Hindi et al., 2009; Panzner et al., 2009).

The effect of adding silver-based products to poultry in its ionic form has been studied decades ago in broiler chickens. These studies collectively showed that silver from silver acetate or silver nitrate is toxic at high concentrations (900 mg/kg practical diet in both poults and chickens [Jensen et al., 1974; Peterson and Jensen, 1975], 1500 mg/kg drinking water fed purified diet [Bunyan et al., 1968]). Depends on these and other publications, the NRC (2005) stated that the maximum tolerable level of silver in poultry with no adverse effect at 100 mg/kg feed.

Scarce literature exists on the potentially less adverse effects of ionic silver on broiler performance, or potential *in vivo* efficacy using a disease model with chickens. This is mainly attributed to the fact that ionic silver could be more toxic than particulate silver as the dissociation rate of silver ion (Ag^{+}) plays an important role in its biological activity. However, oral exposure to silver nanoparticles was reported to be very similar to exposure to silver salts such as silver acetate (Loeschner et al., 2011) and silver nitrate (van der Zande et al., 2012) in terms of organ distribution and elimination after oral exposure, or similar in terms of *in vivo* antimicrobial activity (Williams et al., 2015; Wilding et al., 2016; Lok et al, 2006; Hadrup et al.,

2012), and *in vitro* efficacy and toxicity (Greulich et al., 2012), and even more so, in terms of the intestinal stress response (Bouwmeester et al., 2011).

More recently, animal-antibiotic alternative researchers have evaluated silver in particulate form as a feed additive in animal production (poultry and swine) or as antimicrobial agent that can reduce *Campylobacter jejuni* infection, coccidiosis, and aflatoxin in broiler chickens (Sawosz et al., 2007, 2009; Fondevila et al., 2009; Pineda et al., 2012; Vadalasetty et al., 2018; Chauke and Siebrits, 2012; Gholami-Ahangaran and Zia-Jahromi, 2014). It has been hypothesized that since silver ion possesses antimicrobial activity, silver could potentially modify the microbiota profile of the gastrointestinal tract (GIT), increase nutrient utilization, and subsequently promote the bird performance in a manner resembling the action of antibiotic growth promotion. Saleh and El-Magd (2018) reported that dietary supplementation with silver nitrate (100 ppm) and particulate silver (50 ppm) for 12 days improved broiler performance body weight gain, feed intake, and feed conversion ratio. Vadalasetty et al. (2018), however, reported that the application of particulate silver via drinking water for 30 days in the concentration of 50 ppm led to decrease the BW and WG (average body weight gain) with no impact on FCR.

The main goal of this research is to evaluate new novel silver-based compounds, silver carbene complexes (SCCs) with different carrier molecules (SCC1 with a methylated caffeine backbone and SCC22 with a dichloroimidazolium backbone) along with silver acetate (AgAc) as a potential alternative therapy for gut infection in broiler chickens. The specific objectives of this research include the following:

1. Evaluate the *in vitro* antimicrobial efficacy of silver carbene complexes, SCC1, SCC22, and silver acetate against some enteric poultry pathogens

2. Evaluate potential *in vivo* toxicity of silver carbene complexes, SCC1 and SCC22, and silver acetate in broiler chickens

3. Evaluation of the effects of silver acetate on performance and *Clostridium perfringens*-induced necrotic enteritis in broiler chickens

4. Comparison study of silver acetate to probiotic, prebiotic, and antibiotic on full term broiler trial in terms of performance, ileal nutrient digestibility, bone mineralization, intestinal morphology, antioxidant status, and cecal microbiota population

Literature Review

Antibiotics as Growth Promoters

Shortly after the introduction of the therapeutic use of antibiotics which overlapped with intensive animal rearing, Moor et al. (1947) described for the first time the beneficial effects of feeding antibiotics at subtherapeutic levels to improve performance in poultry. The United States Food and Drug Administration (FDA) approved in 1951 the use of antibiotics as an animal feed additive without the prescription of a veterinarian (Jones and Ricke, 2003). There are 4 major mechanisms proposed to explain the mode of action of AGP: 1) inhibition of subclinical infections, 2) reduce growth-depression metabolites by the microbe (such as ammonia), 3) reduce microbial use of nutrients which lead to decrease nutrient competition, and 4) enhance uptake of nutrients as a result of improving the intestinal health (Gaskins et al., 2002). All these mechanisms share a common hypothesis that AGP work directly or indirectly on intestinal microflora, whether commensal or pathogenic bacteria. Moreover, other mechanisms have been proposed for AGP, such as anti-inflammatory effects (Niewold, 2006).

In an effort to improve production efficiency (weight gain and feed conversion ratio), diets have been fortified with many introduced antibiotics. However, it was recognized that long

periods of exposure to these sub-therapeutic doses could induced bacterial populations to acquire genetic mutations or plasmids that allow them to survive in the presence of the antibiotics. Recognition of the contribution of antibiotics in feed to the emergence of antimicrobial resistance has led to a ban on the use of antibiotic growth promoters (AGP) in the European Union in 2006 (Castanon, 2007) and increased restrictions on the use of AGP in the United States. As of January 1, 2017, a new FDA regulation prohibits animal producers from using antibiotics as growth promoters if they are deemed to be a medically important antimicrobial with respect to human medicine. Banning AGP in Europe was almost immediately followed by health problems in broiler chickens, and outbreaks of *Clostridium perfringens* (CP) infections were documented (Castanon, 2007). Banning antibiotics from animal feed in the United States will likely cause the same challenges. This has stimulated new research into alternatives to AGP (Niewold, 2006) such as probiotics and prebiotics (Gardiner et al., 2004), organic acids (Partanen and Mroz, 1999), and herbs and their extracts (Burt, 2004).

Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). The products of probiotics usually contain either a single bacteria strain or a mixture of strains of *Bacillus spp*, *Bifidobacterium, Enterococcus, E. coli, Lactobacillus, Lactococcus, Streptococcus* in addition live yeast species. Each has sole features that make them suitable for use as probiotics.

Prebiotics are defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of intestinal bacteria (Gibson and Roberfroid, 1995). A variety of oligosaccharides and non-starch polysaccharides (NSP) have been consider as prebiotics, including fructooligosaccharide (FOS), mannanoligosaccharide (MOS), inulin, galactooligosaccharide, maltooligosaccharide, lactulose,

lactitol, xylooligosaccharide, glucooligosaccaride, soya-oligosaccharide, isomaltooligosaccharide (ISO), and pyrodextrins (Patterson and Burkholder, 2003).

Silver Ion, Brief Historical Review

Silver ions (Ag^{+}) have an atomic number of 47 and an atomic mass of 107.868 g/mole. The name of the element derives from the Anglo-Saxon "seolfor" and the Latin argentum. Silver ions are dissociated from different salts and from particulate silver. Silver is the second element of group 11 in the periodic table which includes many transition metals that are biologically important such as copper and zinc as trace minerals, and it was the third metal recognized after gold and copper to be used by the Ancients (Alexander, 2008). However, silver has no nutritional values in animal and human tissues (Lansdown, 2006). Silver occurs naturally as two isotopes Ag^{107} and Ag ^{109} in about similar proportions. Silver exhibits three oxidation states Ag (I), Ag (II) and Ag (III) but only compounds of the Ag (I) state are effective and stable to be as antimicrobial (Lansdown, 2006). Silver has been used as an antimicrobial agent in a variety of ways to control infections since ancient times (Russel and Hugo, 1994). There is no date reported for silver discovery, and no one has the honor to discover this metal as it is known since ancient time. Metallic silver in earliest time was used as a disinfectant to store and purify water, and it was reported that Alexander the Great (335 BC) stored and used water in silver vessels during his travels (White, 2002). Forms of silver such as silver nitrate and silver sulfadiazine have been therapeutically well-known since the 18^{th} century (Klasen, 2000). The reputation of silver nitrate continued into the early 19^{th} century, which led to its utilization as a treatment of different cases such as chronic skin ulcers, open wounds (Silver et al., 2006). German obstetrician, C.F. Crede, introduced a prophylactic 2% silver nitrate ($AgNO_3$) eye solution to prevent ophthalmia neonatorum in newborns in 1880 (Klasen, 2000) and then he reduced the concentration to 1% as

because of the irritation the higher concentration affected. This was a highly effective therapy, reducing the incidence of ophthalmia neonatorium from 7.8% to 0.13% in 13 years (Alexander, 2002). As a result of this effective therapy of silver, the use of silver nitrate as eye drops in newborn infants was broadly established throughout the world, this therapy was mandated by law and continued even after the introduction the antibiotics in several countries (Alexander, 2002).

However, many of these inorganic forms rapidly lose effectiveness since the silver cations are released rapidly at the infection site (Napoli et. al., 2013). Colloidal silver (metallic silver particles with >10% ionized silver ion) was introduced in the late 1800's as alternative for the rapid dissociation from the silver anion and irritation that occur from the anion after dissociation (Gibbs, 1999). Other approaches were introduced in early 1970 based on complexing the silver ion with ancillary ligands for more stabilization (for example: silver sulfadiazine (Fox, 1968). After World War II, silver-based antimicrobials were abandoned with the discovery of penicillin and other antibiotics. The emergence of resistant organisms such as *Pseudomonas aeruginosa* occurred later and led to revival of the use of silver nitrate by Moyer et al. (1965). Advances in technology of silver chemistry during the last two decades was as race to synthesis new, safe silver-based products that able to sustain release bioactive silver ion with minimizing the potential hazard effect.

N-heterocyclic Carbene

NHCs are neutral 2-electron donors, with an ability to bond to both hard and soft metals. Silver N-heterocyclic carbene complexes (SCCs), a novel of silver-based compounds that gradually release silver ion, have gained a substantial amount of attention in human medicine as a result of exceptional antimicrobial efficacy (Melaiye et al., 2004) against a wide range of

microorganisms including both Gram-positive and Gram-negative as well as fungi and even biosafety level 3 bacteria, with low toxic effects on mammalian cells (Hindi et al., 2009, Panzner et al., 2009).

Silver as an Antimicrobial

Metallic silver is not reactive with tissue or a microorganism unless it ionized, and it is very well known that Ag^+ ion is the bioactive form of silver. The antimicrobial activity of silver ions is characterized by a high effectiveness, low toxicity to host cells, and rare development of resistance. Distinct from conventional antibiotics, silver is active under aerobic and anaerobic conditions against a wide range of microorganisms including multidrug resistant bacteria due to its multifactorial mechanisms as an antimicrobial: inducing cell membrane damage, inhibiting respiratory enzymes, perturbing metal ion homeostasis, impact antioxidant status, and generating reactive oxygen species that eventually lead to damage of cellular components such as lipids and DNA. This could explain the scarce reported cases of resistance (Hindi et al., 2009).

The cytoplasmic membrane of the bacteria functions as a mechanical barrier protecting from outside adverse environment and has a biological function by exchanging nutrients with internal cytoplasm. Silver ion can readily interact with sulfdryl groups in thiol-containing compounds. The interaction of silver ions with bacterial inner and outer membranes reported as an important mechanism of silver ion to initiate its toxicity (Percival et al., 2005; Jung et al., 2008; Randall et al., 2013). Jung et al. (2008) showed that the accumulation of silver ion in the bacterial cell wall is followed by the separation of the cytoplasmic membrane from the cell wall in both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*E. coli*), and carboxyl groups in glutamic acid and phosphate groups in teichoic acid are mostly responsible for binding of silver ions.

It was showed also that *E. coli* exposed to 10 µg/mL particulate silver on agar plates inhibited the bacterial growth by 70%, severe pitting in the cell membrane, and silver deposited as particles in the cell membrane with increase in cell wall permeability which led to reduce the uptake and exchange of essential nutrients such as phosphates, succinate, mannitol, and the amino acids glutamine and proline (Lansdown, 2010 b; Sondi and Salpopek-Sondi, 2004; Scheurs and Rosenberg, 1982). Kascatan-Nebioglu et al. (2006) treated *Burkholderia dolosa* with 5 µg/mL of silver carbene complex 4, bacteria demonstrated disruption of the bacterial cell morphology characterized by cell "ghosts" devoid of cytoplasm, the "ghost" cell membranes were studded with numerous electron dense clusters which likely representing outer membrane deposition of silver salts. It has been demonstrated that after silver ions pass the bacterial cell membrane can be accumulated inside the cytoplasm and bind to different cytoplasmic components such as proteins, enzymes, and nucleic acids (Lansdown, 2006), and disturb the electron transport chain by uncoupling from the oxidative phosphorylation leading to deplete the ATP (Hidalgo and Domínguez, 1998). Silver from silver nitrate deposited as particles in *E. coli* and *Pseudomonas aeruginos*a induced irreversible DNA damage, degenerating cytoplasmic proteins, and loss the ability to replicate (Lansdown, 2010 b).

Strictly anaerobic microorganisms have no electron respiratory chain which is different from aerobic bacteria (Thauer, 2015) consequently they cannot generate reactive products from reduction of O_2, such as H_2O_2. Therefore, the Fenton reaction would not occur without H_2O_2, which affects the formation of hydroxyl free radical via Fenton reaction (Pesakhov et al., 2007).
In aerobe and facultative anaerobe microorganisms, protection against free radicals can be provided by antioxidative enzymatic systems, which are responsible for the removal of ROS. Microorganisms with durable antioxidant defense system are thought to have a moderate or high

tolerance to the oxidative stress compared to strictly anaerobes which lack an antioxidative enzymes or because of antioxidative enzymes displaying low activity (Benov and Fridovich, 1995).

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 as same as its activity under aerobic, yet it caused reduction in bacterial growth (Park et al., 2009). Bactericidal activities of silver zeolite and silver nitrate in anaerobic condition was examined against *E. coli* strain OW6 showed that under anaerobic conditions more cells were viable than in the presence of oxygen (Matsumura et al., 2003). Sütterlin et al. (2012) showed that a minimal bactericidal concentration (MBC) of silver ion for Gram-positive bacteria was more than 32 times higher than the MBC values for the Gram-negative bacterial cells.

Bacterial Resistance to Silver

Metallic Silver and its products such as, silver nitrate and silver sulphadiazine have been practiced as broad-spectrum antibiotics to control infections usually associated with burns and chronic dermal wounds (Klasen, 2000). Although there is doubting that bacteria can not develop resistance against silver ions, silver-resistant bacteria can occur (Gupta et al, 1999; Gupta and Silver, 1998) in a range of circumstances such as in chronic wounds, burns, dentistry, occupational exposure and water systems (Davis et al, 2005; Pruitt et al, 1998; Modak and Fox, 1981). In fact, silver- resistant bacteria have been documented (Slawson et al., 1992) and molecular genetic basis for bacterial resistance to silver is mentioned in many published reports. However, only a few of these reports include data that support clarify resistance mechanisms against silver. The region of silver-resistant plasmid (pMG101) of *Salmonella* Typhymirium (isolate from burn ward at Massachusetts General Hospital) which determines the resistance to

silver (McHugh, 1975) was cloned and sequenced by Gupta, et al. (1999). This plasmid system contains 9 genes; seven genes were recognized and named, while the rest two genes are less recognized (unknown function). Resistance to silver initiate with silE gene which encodes for protein SilE. This protein is periplasmic protein that binds silver ions specifically at the cell surface. Therefore, it will work to protect the cell from taking up the silver into cytoplasm. The next 2 genes are silR (transcriptional responder) and silS (membrane kinase sensor). These genes encode proteins to direct or signal mRNA transcription in silver resistance. The rest 4 genes silCBAP are transcribed to form two parallel efflux pumps that protect the bacteria from silver ions that escaped from binding to SilE protein. These 2 efflux pumps actually consist of silCBA encode for a three-protein, membrane potential-dependent (SilCBA), cation/proton antiporter, and SilP protein which is function as ATPases (Silver, 2003). Although most heavy metal resistances result from either efflux pumping of the metal from the cytoplasm or by chelating it with metal-binding protein, silver resistance applied both binding and efflux. This complicated mechanisms to detoxify silver was first seen in silver resistant bacteria (Silver, 2003). Moreover, this mechanism could be alternative to another possible one which is proposed by Gadd et al (1989). Where the silver ion could be detoxified from the cell by binding to netallothionein and when this mechanism is fully occupied with silver ions, the bacterium may use the energy-dependent ion efflux as a mechanism of resistance. The plasmid pMG101 has been shown to confer resistance to several heavy metals including mercury and silver, and when presented into *E. coli* allows for growth in more than 0.6 mM solution of silver ions (Gupta, 1999). Moreover, this plasmid confers resistance to several antibiotics, such as ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamides. This suggests that the resistance against silver could be plasmid-mediator, and may be the activation of silver resistance system could be

developed from previous mercury and antibiotic resistance systems (cross-resistance). The protein SilE is 47% identical to PcoE protein that found in *E. coli* plasmid copper resistance system (Silver, 2003) and it synthesized only when there is silver in the medium of growth (Gupta, 1999). Same analogues have noticed for the SilRS proteins, where they are homologous to sensor-responder proteins (PcoRS) that found in *E. coli* chromosome. These evidences suggest that silver resistant system may be evolved from preceding copper resistant system. In addition, there is controversial between some studies in terms of the true mechanism of resistance that bacteria possess against silver. Li et al. (1997) reported that efflux of silver was enhanced in mutants of *E. coli* which indicate that the resistance against silver could be as a result of bacterial mutation. However, this contrasts with both plasmid mediator route and with Maple et al. (1992) who reported that there were no resistant mutants of *Staphylococcus aureus* to silver.

In poultry, particulate silver has been introduced as antiseptic agent, feed additive, and possible development resistant is critical. Interestingly, in a study to determine the carriage rate of silver resistance genes and their products (SilE, SilP, and SilS) in different *E. coli* populations (human vs birds) showed that 12% of human E. coli isolates but none of the avian isolates harbored silver resistance genes (Sütterlin et al., 2012). This is not surprisingly, since the wide use of silver products nowadays as an effective chemotherapeutic antibacterial agent in wound care products, medical devices, and purification of drinking water and many other silver-based applications. Treatment with Ag-NPs or silver acetate for long period at the doses below the minimum inhibitory concentration did not provide a sufficient selection pressure for evolving resistance through up-regulation of silver resistance genes silRS, silP and silCBA (Hadrup et al., 2012).

Even though silver resistant bacteria have been reported, present evidences suggest that clinical threat is low and the true bacterial resistance is rare and erratic (Landsdown and Williams, 2007; Chopra, 2007; Wright et al., 2012). This is may be because the most studies for silver resistant derived from either burn wound infections that treated with silver nitrate or covered with a dressing has silver in it and from environments where the toxicity of silver is highly possible.

Silver Absorption and Distribution

Silver is not a recognized trace metal but can accumulate in the human body at low concentrations (< 2.3 µg/L) by ingestion with food or drinking water, inhalation and occupational exposures (Lansdown, 2006). The skin, eye, brain, liver, kidney spleen, intestine, and bone marrow were recorded as main target tissues for silver deposition after absorption (U.S. EPA, 1985). Silver absorbed into the body as ionized form (Ag^{+}) and it readily binds to intracellular proteins, especially serum albumins and macro-globulins for metabolism and distribution to different tissues. It was mentioned that the acute toxicity of silver to animals characterized by effects on the central nervous system leading mainly to respiratory paralysis. However, some studies mentioned silver is not absorbed into the brain and central or peripheral nervous systems in any species (Lansdown, 2007, Zheng et al., 2003). The effect of chronic exposure of animals to silver is limited to the argyria which is characterized by bluish discoloration of skin, eye and internal organs (U.S. EPA, 1985; Lansdown, 2010 a). In the dermis, silver deposition was proved to be as of inert precipitates of silver selenide and/or silver sulphide in the connective tissue surrounding the vascular tissue and glands of the papillary layer of the dermis but not epidermis (Tanita et. al., 1985; Sato et. al., 1999). These precipitates distribute as intracellular which is usually bound to lysosome or intercellular which precipitated

either as soluble or insoluble form. In the bone, no evidence was noticed that the deposition of silver in the bone caused osteoporosis although it was approved *in vitro* study that the silver binds to the hydroxyapatite complex and can displace calcium and magnesium ions (Gould et al., 1987; Lansdown, 2009). Silver deposition in kidney (glomerular basement membranes, arteriolar endothelia and elastic laminae), without obvious structural damage also were reported (Berry, et. al. 1995; Walker, 1972). Creasey and Moffat (1973) reported the deposition of silver from silver nitrate in rat kidney as granules when silver was administered in the drinking water of 26 weanling rats in the form of a 0.15% aqueous silver nitrate solution for periods of from 4 to 15 weeks. The density of the silver deposits as granules increased towards the tip of the papilla of the rat kidney and could be recognized easily by naked-eye examination. Although *in vitro* toxicity studies mentioned the cytotoxic effects of metallic silver and silver compounds in terms of carcinogenicity, *in vivo* studies indicate that silver is not carcinogenic in any tissue and must be placed in a "No Risk" category (U.S. Department of Health and Human Resources, 2010). The liver is considered the major tissue for silver deposition and elimination without hepatic damage except elevation in some hepatic enzymes (Trop et al., 2006). However, many other studies reported alteration in some plasma biochemical indicators for hepatotoxicity (ALT, ALP, AST, and GGT).

The majority of silver is excreted in the feces (via biliary route) rather than in the urine regardless of the type of silver compound and the rout of administration (U.S. EPA, 1985). However, experimental studies in animal models have shown distinctions in biliary excretion of silver. For example, intravenous injection of silver nitrate was associated with biliary excretion patterns of 0.25, 0.05, 0.005 μg/kg/min in rats, rabbits, and dogs, respectively (U.S. EPA, 1992). The cumulative recovery of silver in feces after oral exposure was 98%, 99%, and 94% in rats,

mice, and monkeys, respectively. In human studies, the main route of silver excretion is detected via bile in to feces, although the urinary concentrations could be used as indicator of exposure (Landsman, 2010).

Silver retention in liver, kidney, spleen, muscles, eggshells, and intestine has also been reported in previous *in vivo* studies where Ag-NPs were administered to hens or broilers (Chauke and Siebrits, 2012; Ahmadi and Rahimi, 2011; Gallocchio et al., 2017; Chmielowiec-Korzeniowska et al., 2015; Kulak et al., 2018 a,b). Kulak et al. (2018) reported that the administration of Ag-NPs in the amount of 2.87 to 63.74 mg/bird for different time points does not cause silver to accumulate in the breast muscle. The ingestion of 2.87 mg/bird was found to result in the accumulation of this element in the wall of the small intestine and in the liver in dose dependent manner, while accumulation of silver in the heart of the chickens was not observed until the dose reached 22.5 mg/bird. Ognik et al. (2017) showed that broiler chickens received silver nanoparticles (0.5, 1.0, or 1.5 mg/kg body weight/d) via a tube into the crop in 3-d periods (d 8–10, 22–24, and 36–38) or three 7-d periods (d 8–14, 22–28, and 36–42) particulate silver supplied via ingestion led to dose-dependent accumulation of Ag in the intestinal walls.

In hens, administration of particulate silver could transfer to the liver and yolk. Gallocchio et al. (2017) used a 22-day *in vivo* study carried out by oral administration of 20 nm coated Ag-NPs to hens, with total dose received approximately 6 mg/kg of Ag-NPs, and atomic absorption spectroscopy was used for quantitative determination of residual total silver in different organs and matrices. The results showed that silver accumulated in livers (concentration ranging from 141 to 269 μg/kg) and yolks (concentration ranging from 20 to 49 μg/kg) but not in muscles, kidneys, and albumen.

Silver Toxicity

Silver is not a recognized trace metal but can accumulate in the human body at low concentrations (< 2.3 µg/L) by ingestion with food or drinking water, inhalation and occupational exposures (Lansdown, 2006). Toxicity of silver to human and mammals is still under examination (Cannon et al., 2009). Silver is known to be one of the least toxic metals. Use in high doses by humans can cause a rare irreversible pigmentation of the skin (argyria) and/or the eyes (argyrosis) (Lansdown, 2006). The toxicity of silver compounds can be often linked to the carrier molecules (Gear et al., 1997), or partially to the shape and size of particulate silver (Kim et al., 2010).

The toxicity of silver in animal reported controversially both *in vitro* and *in vivo* studies. (Lansdown, 2006). Some studies related to silver toxicity and its compounds evidenced that silver has adverse effect on different tissues and cells (Fraser et al., 2004; Hollinger, 1996; Hidalgo and Dominguez, 1998; Hussain, et al., 1992; Hassanpour et al., 2015). However, other studies have reported that silver was not observed as a toxic metal inside the cell (Ghosh and Banthia, 2004, Alt et al., 2004, Lansdown, 2006). From *in vivo* and *in vitro* studies, there is a general agreement that the mitochondria are the main target of silver ions after absorption from the cell membrane (Stensberg, et al., 2011). This is because the mitochondria are susceptible to the permeability transition pathway. This pathway is characterized by the formation of proteinaceous pores in mitochondrial membranes, which results in mitochondrial swelling with abnormal metabolism and ultimately cellular apoptosis. The most common description of the events of toxicity before apoptosis can be generalized as oxidative stress. After uptake of silver ion in the mitochondria, it can stimulate the production of reactive oxygen species (ROS), because of disruption of the influx of ions and electrons across the mitochondrial membrane (as

the bacteria lack the mitochondria, these events accorded in the lipid bilayer cytoplasmic membrane). Silver can bind and modulate the glutathione (GSH) function as non-enzymatic antioxidant by an oligodynamic effect. This will increase ROS because of depleting GSH, and eventually lead to damage the cellular components such as proteins, lipids, RNA and DNA as ROS increase in the cell.

Effect on Performance

Saleh and El-Magd (2018) reported that dietary supplementation with silver nitrate (100 ppm) and particulate silver (50 ppm) for 12 days improved broiler performance body weight gain, feed intake, and feed conversion ratio. Vadalasetty et al. (2018), however, reported that the application of particulate silver via drinking water for 30 days in the concentration of 50 ppm led to decrease the BW and WG (average body weight gain) with no impact on FCR. Song et al. (2017) reported that the intestinal and plasma oxidative stress resulted in a reduction in body weight and feed intake with no effect on FCR, and dietary treatment with Zn, vitamin E as antioxidants, or their combination at different inclusion rates failed to alleviate the negative effect of dietary particulate silver (at 1000 mg/L drinking water for 42 days) on the body weight and feed intake of broiler chickens.

Feeding a diet containing graded concentrations 10, 25, 50 and 100 and 200 mg/kg silver sulfate for 3 weeks did not affect growth performance and mortality of broiler chickens 100 mg Ag/kg as silver sulfate to one-day-old chicks did not adversely affect growth, mortality, hemoglobin concentration, and elastin content of the aorta (Hill et al., 1964).

Felehgari et al. (2013) showed that silver nanoparticles fed to broiler chickens at 25 and 50 ppm for 21 d did not affect the bird performance parameters BW, WG, and FCR. Similarly, Pineda et al. (2012) reported that providing silver nanoparticles to broiler chickens at 10 and 20

mg/kg in drinking water from d 7 to 36 did not affect bird performance BW, FCR and mortalities.

Kulak et al. (2018) and Ognik et al. (2016 a) demonstrated that oral administration of particulate silver to chickens effects the morphology and functioning of the gastrointestinal tract, as well as the parameters of immune and redox status accompanied with intestinal wall accumulation of silver, regardless the size and doses used. However, growth performance parameters of the chickens (body weight gain, feed conversion, and weight gain) did not affect significantly compared to the control.

Higher dietary concentrations of silver induced signs associated with Cu and Se deficiency including depressed growth, hemoglobin and aortic elastin, increased mortality and heart weight, and exudative diathesis in chicks (Hill et al., 1964; Bunyan et al., 1968; Petersen and Jensen, 1975a, b). Peterson and Jensen (1974) showed that adding 900 ppm silver nitrate for 4 weeks to a practical diet for chicks significantly depressed growth, reduced the copper content of blood, spleen, brain, liver and 50 ppm Cu supplementation only partially corrected the growth depression.

In turkeys, 100, and 300 mg Ag/kg diet did not affect the growth, and 900 mg Ag/kg diet induced gizzard musculature dystrophy, enlarged hearts, and decreased packed red blood cell volume, in addition to depressed growth performance (Jensen et al., 1974).

Kulthong et al. (2012) orally supplemented 0, 50 100, 250, 500 and 1000 mg/kg/day for 14 days did not significantly change in the rat body weight, liver weight and relative liver weight in all treatment groups. Felehgari et al. (2013) showed that silver nanoparticles fed to broiler chickens at 25 and 50 ppm for 21 d did not affect the bird performance parameters BW, WG, and FCR. Similarly, Pineda et al. (2012) reported that providing silver nanoparticles to broiler

chickens at 10 and 20 mg/kg in drinking water from d 7 to 36 did not affect bird performance BW, FCR and mortalities.

Lethal Dose

Silver from different compounds showed different LD_{50} values which mainly depend on animal species sensitivity for different silver-based products, rout and duration of administration. Venugopal and Luckey (1978) reported oral LD_{50} values for mice using colloidal silver and silver nitrate 100 mg/kg and 129 mg/kg, respectively. While for silver cyanide, the LD_{50} for rats was 125 mg/kg, and silver oxide with LD_{50} of 2820 mg/kg for rats. In a single oral acute toxicity study in 7-week-old rats, administration of silver ion from particulate silver and $AgNO_3$ with 2 and 20 mg/kg BW did not cause death or changing in BW (Park, 2013). Haque et al. (2013) reported no signs of toxicity were observed and animals remained alive, healthy, and agile when administered single oral dosages of 300 and 2000 mg/kg BW of dinuclear Ag(I)-NHC complex. Tamimi et al. (1998) worked on silver nitrate as the active ingredient in an anti-smoking mouthwash, the author reported: in rats, following oral administration, the LD_{50} was found to be 280 mg of silver/kg of BW, while in rabbits the LD_{50} was found to be 800 mg of silver/kg of BW/day. Walker also identified silver nitrate at concentrations 308 mg of silver/kg BW (rat) in the drinking water induces death over the course of a few days (Walker, 1971). Maneewattanapinyo et al. (2011) reported at acute doses of up to 5000 mg/kg of BW/day, silver was not toxic when orally administered nanoparticulate to guinea pigs. There is no LD_{50} value available in the literature for chicken.

Effect on Relative Organ Weights

Vadalasetty et al. (2018) reported that the application of particulate silver via drinking water in the concentration of 50 mg/kg had no effect on relative organ weight of liver and heart, but significant interactions between age and treatment were observed for the relative weight of bursa and spleen, where a lower relative bursa at age 15 day, and for spleen at age 30 day were noticed for AgNP group. Hadrup et al (2012) examined the effect of administration particulate silver and silver acetate at 9 and 14 mg/kg BW, respectively, and no differences in relative organ weight of liver, kidney, spleen, and heart was noticed

Raieszadeh et al. (2013) evaluated the impact of adding 10, 20, 30, 50, and 70 ppm of particulate silver in drinking water for 26 days on broiler chicken heart and the echocardiographic assessment results showed that high dosage of particulate silver led to cardiovascular problems with decrease in myocardial contractility and increase in the internal diameter of left ventricle. Kim et al. (2008, 2010) found no histopathological changes in the heart after up to 13 weeks of treatment with 56–60 nm silver nanoparticles at doses up to 1000 mg/kg of BW/day.

Effect on Serum Biochemicals

Ag-NPs fed orally to rats at doses of 0, 50 100, 250, 500 and 1000 mg/kg/day for 2 weeks did not significantly alter the serum ALT or AST levels, suggesting no major loss of the parenchymal and other liver cell integrity, while the absence of any significant change in the ALP blood levels in all treated groups also suggested no significant damage to the biliary tract and so overall no serious liver dysfunction (Kulthong et al., 2012). Effect of silver on ALT and ALP serum or plasma enzymes in broiler chickens was studies in many studies using different rout of administration (feed or water). Administration of silver nanoparticles 5 mg/kg body

weight per day for first 3 days of weeks 2, 4, and 6 of life via a tube into the broiler chicken crop, particularly 5 nm lipid-coated nanoparticles led to a disturbance in protein catabolism in the organism, which was evidenced by the decrease in the activity of the liver enzymes AST and ALT and the decreased concentration of the main protein metabolism products (creatinine and urea) (Ognik et al., 2016). Ahmadi (2012) reported application 20, 40, 60 ppm Ag-NP diet for 42 days reduced serum ALT and ALP. Elkloub et al. (2015) reported that administration of Ag-NPs at 2, 4, 6, 8 and 10 ppm/kg feed throughout broiler chicken's growth trial period (7-35 days) did not affect the serum ALT levels significantly when compared to control group. Ahmadi (2009) reported administration of 300, 600, 900 ppm particulate silver in broiler feed for 42 days did not affect the liver enzymes ALT, ALP, AST. Sawosz et al. (2009) observed no effect of administration of nanosilver to chickens on the activity of AST, ALT or ALP.

In a single oral dose, Park (2013) reported significant increases in the activities of AST and ALT were observed in the 20 mg/kg Ag^+ ($AgNO_3$)-treated group, but no effect on ALT with same dose of Ag-NP. However, the author suggested serum activity levels of both AST and ALT, which are indicators of liver damage, increased in rats treated with the high dose of Ag^+ (20 mg/kg). The increased AST and ALT activities may be correlated with liver necrosis observed in rats treated with the high dose of Ag^+ (20 mg/kg). However, the elevated activity levels of AST and ALT were not suggestive of severe damage.

Effect on Histology

In rodent toxicity studies, it has been reported that oral ingestion of particulate silver at 10 and 20 mg/kg for 28 days could lead to destruction of intestinal microvilli, reduce absorption capacity of nutrients and subsequently reduce growth performance (Shahera and Young, 2013). Another pathological change reported in a rodent stud by Jeong et al. (2010) were increased

numbers of goblet cells in the intestine that had released abnormal composition of mucus granules following the oral administration of 30 mg/kg of BW/day of nanoparticles for 28 days, but no adverse effect on performance was noticed.

Other studies reveled no adverse effect on intestinal histology when applying particulate silver with different doses, exposure duration, and rout of administration in quails (Sawosz et al., 2007), broiler chickens (Ahmadi et al., 2009), weaned pigs (Fondevila et al., 2009), and mice (van den Brule et al., 2016). Hadrup et al. (2012) reported that histological examination of the liver, kidneys, ileum and heart from six animals of both sexes from the control and the high-dose Ag-NP groups (9 mg/kg BW/day for 28 days) and of six females from the silver acetate group did not exhibit any differences compared with the vehicle control group. Ahmadi and Mehrdad (2009) have observed slight necrotic changes in liver of high dose Ag-NP-treated chickens. However, Ognik et al. (2016) have not noticed deviations from the normal structure of liver when using Ag-NPs with larger size with lower dose. Ahmadi et al. (2009) showed that 300, 600, 900 ppm particulate silver in drinking water did not affect the liver histological structure. Loghman et al. (2012) evaluated the toxicity of particulate silver and observed pathological and morphological changes in the liver of broiler chickens administered at 4, 8, 12 ppm in drinking water, the author reported infrequent accumulations in the hepatocytes (cell swelling) and hyperemia at 4 ppm, while at 8 and 12 ppm showed dilated central vein, hyperemia with severe vacuolation fatty change. Similarly, in sub chronic toxicity (Amin et al., 2015) and acute toxicity (Cho et al., 2015) reported moderate to severe fatty changes of hepatocytes and cytoplasmic vacuolization when silver particulate administered to rats and mice.

Hadrup et al. (2012) in the kidney, no differences to the control group in absolute and relative kidney weights histological changes and apoptosis levels indicated no adverse effects of

Ag-NPs or silver acetate on kidneys at 9 mg/kg BW. In rabbit, after a single intravenous injection of 0.5 and 5 mg/kg BW Ag-NP the accumulation of silver was observed in all the tested organs including liver, kidney, spleen, lung, brain, testis, and thymus at 1 day, 7 day, and 28 day of measurement. The author mentioned that the liver and spleen seemed to be the major targets because of high accumulation of silver. However, histopathological changes in spleen were not significant when compared to liver and kidney which shoed mild histopathological changes, while spleen seemed to be the major target of Ag-NPs (Lee et al., 2015).

Effect on Bone

In broiler chickens, Peterson and Jensen (1974) noticed that among mortalities weak bones were a noticeable clinical sign after feeding an industry-type diet supplemented with 900 ppm silver nitrate for 4 weeks, while Jensen et al. (1974) did not mentioned such effect on young turkeys when fed the same concentration of 900 ppm, neither at lower concentration 100 and 300 ppm. In a recent study done by Ognik et al. (2017), it has been found that administration of silver nanoparticles to intestine of chickens did not interfere with calcium (Ca) absorption but there was a decrease in the absorption of K and Fe. For the Ca ion, in rodent and broiler studies that have measured serum or plasma Ca (Kim et al., 2008, 2010; Park, 2013; Wen et al., 2017; Ognik et al, 2016; Sikorska et al., 2010), no difference was seen in the Ca levels after administration of silver nanoparticles. Ji et al, (2007), however, reported higher Ca level with higher silver dose in an inhalation study. While, Lee et al. (2013) reported decrease in serum Ca levels compared to control group after a single intravenous injection of AgNPs at 0.5 and 5 mg/kg BW. Sikorska et al. (2010) show that there was a tendency towards increasing mineral content of Ca, Cu, and iron in thigh bone of chicken embryo when administered 50 ppm particulate silver, indicating that

particulate silver may influence bone mineralization, and it could be speculated that particulate silver has the ability to stimulate the hydroxyapatite formation.

In Vivo Efficacy of Silver

It has been hypothesized that since silver ion possesses antimicrobial activity, silver could potentially modify the microbiota profile of the gastrointestinal tract (GIT), increase nutrient utilization, and subsequently promote the bird performance in a manner resembling the action of antibiotic growth promotion. Sawosz et al. (2007) reported no major effect of colloidal silver on bacterial population in the digestive tract of quails, however, only a significant increase in lactic acid bacteria was observed with 25 mg/kg in drinking water. It has been observed *in vitro* that the proportion of coliforms in pigs' ileal contents was linearly reduced with doses 0, 25, 50, and 100 ppm colloidal silver, whereas no effect was observed on *Lactobacilli* proportion with same concentrations (Fondevila et al., 2009). In their *in vivo* study, the author reported no significant coliform reduction in ileal contents when 20 and 40 ppm of metallic silver nanoparticles were given to weaned piglets as metallic silver, however, the concentration of the pathogen *Clostridium perfringens* group was reduced with 20 ppm silver. The latter two authors hypothesized selectivity of silver ion over bacterial spp. could be possible. The selectivity of silver ion is documented that cell membrane is a key target of ionic silver making Gram-positive bacteria are in general to be less sensitive to ionic silver than Gram-negative bacteria due to differences in the structure of the cell wall.

No effect of silver in particulate form on microbial population including *Clostridium perfringens* when broiler chicken supplemented 10 and 20 mg/kg drinking water for 22 days with no positive effect on bird performance BW and FCR (Pineda et al., 2012). Vadalasetty et al. (2018) reported that the application of particulate silver via drinking water in the concentration

of 50 mg/kg had no antibacterial effect on different intestinal bacterial groups including *Clostridium perfringens*, and had no effect on colonization of *Campylobacter jejuni* experimentally infected in broiler chickens although the *in vitro* results showed bactericidal at the same concentration used *in vivo* but this concentration led to decrease the BW. Hadrup et al. (2012) showed neither silver nanoparticle (9 mg /kg BW/day) nor silver acetate (14 mg/kg BW/day) affected the balance between the two main phyla of gastrointestinal tract bacteria in GIT of rates, Firmicutes and Bacteriodetes. The author suggested that silver nanoparticles and silver acetate in the applied doses did not disturb the microbiological balance of the gastrointestinal environment at the phyla level.

Effect on Nutrient Utilization

To the best of our knowledge, there are scarce studies regarding effect of silver on nutrient utilization, such as effect on energy and amino acids digestibility's. A study conducted by Saleh and El-Maged (2018) evaluated dietary silver nitrate (100 mg/kg feed) and particulate silver (50 mg/kg feed) on protein digestibility and the author reported a tendency to increase protein digestibility, and the nitrogen (N) content in the muscle tissue increased significantly in the chicks fed particulate silver and silver nitrate compared with that in the control group. Pienda et al. (2012) reported high N intake and more N retention per kg metabolic body size of broiler chickens with supplementation 10 ppm particulate silver for 4 weeks.

Silver ion possesses antimicrobial properties and has been used as an antibacterial agent since before the discovery of penicillin and distinct from conventional antibiotics, silver is active against a wide range of microorganisms due to its multifactorial mechanisms as an antimicrobial.

From the literature, researchers hypothesized that particulate silver could be employed as an alternative to antibiotics in animal production (Sawosz et al., 2007, 2009; Fondevila et al.,

2009; Pineda et al., 2012; Vadalasetty et al., 2018; Ahmadi and Kurdestani, 2010, Ahmadi, 2012; Saleh and El-Magd, 2018) did show very slight change in bird performance, and *in vivo* efficacy and that toxicity could be accorded at low doses preventing the silver being antibiotic alternative (Sawosz et al., 2007). However, research in to find a silver-based product that could be apply as antibiotic alternative are continuing, as it need further research in practical (Fondevila, 2010).

The main goal of this research is to evaluate new novel silver-based compounds, silver carbene complexes (SCCs) with different carrier molecules (SCC1 with a methylated caffeine backbone and SCC22 with a dichloroimidazolium backbone) along with silver acetate (AgAc) as a potential alternative antibiotic in broiler chickens. The specific objectives of this research evaluate these products in terms of *in vitro* antimicrobial efficacy, *in vivo* potential toxicity, *in vivo* prophylactic and therapeutic efficacy against *Clostridium perfringens*-experimentally induced, and to evaluate silver acetate as a potential growth promoter (dietary silver supplementation) and compared it to antibiotic and selected alternatives, probiotic (*Bacillus* spp) and prebiotic (yeast cell wall fractions from *Saccharomyces cerevisiae*) in terms of performance, nutrient digestibility, gastrointestinal histology, bone mineralization, and hepatic glutathione and related amino acids cysteine and cystine.

CHAPTER II

IN VITRO ANTIMICROBIAL EFFICACY OF SILVER CARBENE COMPLEXES, SCC1 AND SCC22, AGAINST SOME ENTERIC POULTRY PATHOGENS, AND EVALUATION POTENTIAL MODULATION OF VIRULENCE GENES AND MORPHOLOGY OF CLOSTRIDIUM PERFRINGENS TYPE A UNDER ANAEROBIC CONDITIONS

Introduction

Enteric diseases caused by opportunistic microorganisms are detrimental to animal production. This is because of the loss of productivity, increase potential human health risks related with foodborne diseases (Patterson and Burkholder, 2003) and increased prevalence of multiple drug resistance. One strategy followed by animal producers to reduce the enteric bacterial burden and subsequently promote the growth performance is to fortify diets with antibiotics, often at subtherapeutic doses. Long periods of exposure to these subtherapeutic doses have induced bacterial populations to acquire genetic mutations or plasmids that allow them to survive in the presence of the antibiotics. Recognition of the contribution of antibiotics in feed to the emergence of antimicrobial resistance has led to a ban on the use of antibiotic growth promoters (AGP) in European Union in 2006 (Castanon, 2007) and increased restrictions on the use of AGP in the United States. This has promoted research into alternatives to AGP (Niewold, 2006).

Silver has been used as an antimicrobial agent in a variety of ways to control infections since ancient times. Formulations of silver such as silver nitrate have been therapeutically well-known since the 18^{th} century (Klasen, 2000). However, many of these inorganic forms rapidly lose effectiveness since the silver cations are released rapidly at the infection site (Napoli et al.,

2013). Colloidal silver (metallic silver particles with >10% ionized silver ion) was introduced in the late 1800's as alternative for the rapid dissociation from the silver anion (Gibbs, 1999). Other approaches were introduced in early 1970 based on complexing the silver ion with ancillary ligands for more stabilization (for example: silver sulfadiazine (Fox, 1968)).

Recently, N-heterocyclic carbenes (NHCs) are being used as ancillary ligands to silver ion. NHCs are neutral 2-electron donors, with an ability to bond to silver forming more stable silver-based compounds because of the relatively strong silver-carbon bond (Johnson et al., 2017) which leads to slow release of the cation by which systemic toxicity decrease and cation availability for antimicrobial purposes increase. Silver carbene complexes (SCCs), a novel of silver-based compounds that gradually release silver ion, have gained a substantial amount of attention in human medicine recently as a result of exceptional antimicrobial efficacy (Melaiye et al., 2004) against a wide range of microorganisms mainly isolated human including both Gram-positive and Gram-negative as well as fungi and even biosafety level 3 bacteria, with low toxic effect on mammalian cell (Melaiye et al., 2004; Kascatan-Nebioglu et al., 2006; Leid et al., 2012; Patil et al., 2011; Hindi et al., 2008, 2009; Panzner et al., 2009 ab; Cannon et al., 2009).

As a general role that a substance to introduce as feed additive, effectiveness of its antimicrobial activity, safety to the host with excreting potential positive effect should be evaluated as a first steps. In addition, the wide range antibacterial of SCCs scarcely reported their efficacy against animal-isolated pathogens, in particular under anaerobic conditions.

Therefore, using a micro-dilution method, 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 the pathogenic bacteria that can cause illness in humans and that have been reported in the chicken gut microbiota (Oakley 2014), 5

Salmonella enterica, *Escherichia coli*, *Clostridium perfringens*, in addition to *Staphylococcus aureus*. These bacteria species are all known to cause significant numbers of foodborne diseases in humans (Scallan et al., 2011), in addition *E. coil* and *Clostridium perfringens* are pathogenic bacteria that can cause disease in animals and impact the general performance. In addition to growth inhibition of *Clostridium perfringens*, potential modulation of virulence and morphological changes were further investigated using RT-qPCR and transmission and scanning electron microscopies.

Materials and Methods

Silver Sources

Two separate SCCs in pure formulations with different carrier molecules, SCC1 and SCC22 (Figure 1) provided by the Department of Microbial Pathogenesis and Immunology (Texas A&M Health Science Center). These compounds have been previously synthesized and characterized (Panzner et al., 2009; Kascatan-Nebioglu et al., 2006; Hindi et al., 2008). SCC1 is an organic complex with a molecular weight of 375.13 g/mol with a solubility of 11 mg/mL in water, and SCC22 has a molecular weight of 375.99 g/mol with a solubility of 110 mg/mL in water. Silver acetate (AgAc) was purchased from Sigma-Aldrich with 99.9% purity and molecular weight 166.92 g/mol.

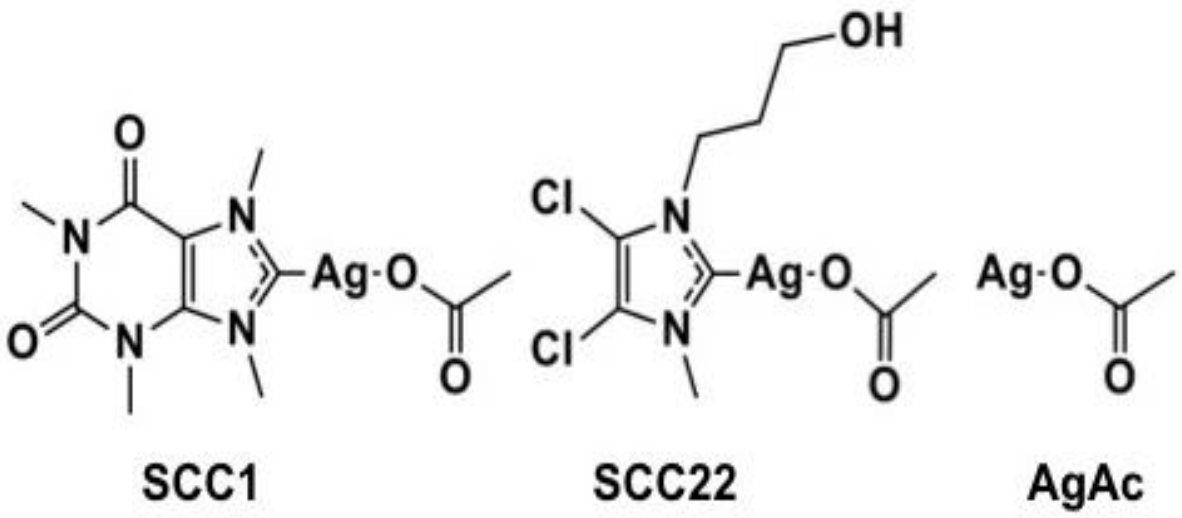


Figure 1 Chemical structures of silver carbene complex 1 (SCC1, molecular weight 375.13 g/mol), silver carbene complex 22 (SCC22, molecular weight 375.99) and silver acetate (AgAc, molecular weight 166.92 g/mol)
(Leid et al., 2012)

Bacterial Species and Growth Conditions

The bacteria used in this study are listed in Table 2. All bacteria were provided by the USDA-ARS facility in College Station, TX. A mixed culture of four field isolates of *Clostridium perfringens* type A from different geographical locations confirmed with necrotic enteritis infection (2 isolates from Texas and Virginia and 2 isolates from Georgia) was prepared by combining equal volumes of each culture growing at the mid-log phase and tested in combined. To the author knowledge, almost no literature document silver activity facing geographically different strains of same species in combined. These isolates will be used further for future *in vivo* studies to induce necrotic enteritis. Thus, we tested in combination to closely mimic the *in vivo* antimicrobial examination. A *Salmonella* Typhimurium resistant to 14 antibiotics plus four

Salmonella spp. field isolates (5 strains in total), two resistant strains of *E. coli* (*E. coli* 843 resistant to 5 antibiotics, *E. coli* 1568 resistant to 6 antibiotics, wild swine isolates) and a poultry field isolates *E. coli*, and *Staphylococcus aureus* (**SA**) tested in this study. These chosen bacteria are either important as animal or human pathogens with different biochemical properties that are obtained zoonotically from animal to test the hypothesis of wide range activity of SCCs.

Minimum Inhibitory Concentration, Minimum Bactericidal Concentration (MIC and MBC)

The MICs of SCC1, SCC22, and AgAc were determined by a broth microdilution as described by Hindi et al. (2008) and Kascatan-Nebioglu et al. (2006) by using standard Clinical and Laboratory Standards Institute (CLSI) protocols for aerobic and anaerobic bacteria. Briefly, bacteria were streaked from glycerol frozen stocks onto blood agar plates and incubated overnight at 37°C. Bacteria from fresh overnight plates were suspended in standard Mueller-Hinton broth, or Brucella broth for CP, to an optical density at 650 nm (OD_{650}) of 0.25 and were grown in a shaking incubator, 200 rpm (not for CP), to an OD_{650} (OD_{625} for CP) of 0.4, which corresponds to $\sim 2 \times 10^8$ CFU/mL ($\sim 10^8$ CFU/mL for CP), confirmed by plating serial dilutions. The bacteria were diluted in the broth to a concentration of 10^5 (10^6 for CP) in 100 µL, which was added to 3 wells of flat bottomed 96-well microplate containing 100 µL of either SCC1, SCC22, or AgAc diluted in deionized distilled water to various concentrations from 10 mg/mL stocks. The dilutions for SCCs were prepared from 1 mg/mL stocks in DMSO with the final solution composition being 95% sterile water and 5% DMSO by volume. Solutions containing 95% sterile water and 5% DMSO but no SCCs were used as the appropriate control. The final concentrations tested 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 at which each of the 3 wells were clear in two sets of plates after incubation for 18–20 h (24-48 h for CP) at 37°C. To determine the minimal

bactericidal concentrations (MBCs), 100 µL from each clear well was plated on blood agar, incubated for 20-24 h, and MBC was recorded as the lowest concentration with no growth. All MIC and MBC measurements for each bacterium were performed at least in duplicate. MICs and MBCs for CP were conducted under anaerobic conditions (Koy Laboratory Products INC, USA), in which 5% CO_2, 5% H_2, 90% N_2, at 80% relative humidity was maintained, while *Salmonella* spp, *E. coli* strains and *Staphylococcus aureus* were tested under aerobic conditions. A stock solution was made by dissolving 10 mg of each compound in 1 mL of deionized distilled water (EASYpure® UV/UF, Barnstead). Then, 1:10 dilutions were made to prepare working stocks (1 and 0.1 mg/mL). All dilutions were prepared at a concentration twice the desired final concentrations.

Clostridium Perfringens Gene Expression of Virulence Genes

Clostridium 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 CP (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 was stabilized by adding 2 volumes of RNAprotect Bacteria Reagent (Qiagen). Cells were harvested by centrifugation (13,000 x g for 10 min) and bacterial cells were disrupted by resuspending the pellets in 200 µL of Tris EDTA buffer (10 mM Tris, 1 mM EDTA) containing 5 mg/mL Lysozyme (Sigma). Total RNA was extracted by RNeasy® Protect Bacteria Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity and quality of total RNA was determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE). Total RNA was then reverse-transcribed to first-strand cDNA using EasyScript PlusTM cDNA Synthesis kit (Lamda Biotech, Carlsbad, CA).

The sequence of CP strain 13 (http://www.ncbi.nlm.nih.gov/nuccore/BA000016.3) was used to design the primers (Table 1) for this study that produced PCR amplicons of 100–150 bp in length using the default setting of Primer 3 plus Input software. Each PCR reaction mixtures contained 1X iQTM SYBR Green Supermix (Bio-Rad), 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 minutes to activate the AmpliTaq DNA Polymerase, followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing and extending at 60°C for 1 minute. The dissociation curve was run following the real time reaction to determine if the primers used in each reaction generated a specific product. 16S rRNA gene was amplified as a reference RNA of equivalent size for normalization. Data obtained were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression.

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

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

Detection of netB Gene in Clostridium perfringens Type A

Detection of the *netB* gene (encoding necrotic enteritis toxin B, NetB) of each of the four *Clostridium perfringens* isolates was performed using specific primers as previously described by Keyburn et al. (2008) with minor modifications. Briefly, DNA was isolated from each strain using UltraClean™ Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc., USA) following the instruction of the manufacturer. PCR was performed in a 25 µL reaction mixture containing: 1X PCR buffer (Bio-Rad); 2.5 mM $MgCl_2$; 0.2 mM dNTP mixture; 2.5 units of Taq DNA polymerase (Bio-Rad); 50 pM of primers AKP78 (5´-GCTGGTGCTGGAATAAATGC-3´) and AKP79 (5´-TCGCCATTGAGTAGTTTCCC-3´) and 5 µL of template. Thermal cycling conditions were as follows: denaturation at 94ºC for 2 min; 35 cycles of denaturation at 94ºC for 30 sec; annealing at 55ºC for 30 sec; and extension at 72ºC for 1 min; with the final extension step at 72ºC for 12 min. PCR products were electrophoresed and visualized on 1 % agarose gels. Positive *netB* CP strain was kindly provided by Dr. S. Pillai's lab which was run along with 100 bp DNA ladder (BioLabs).

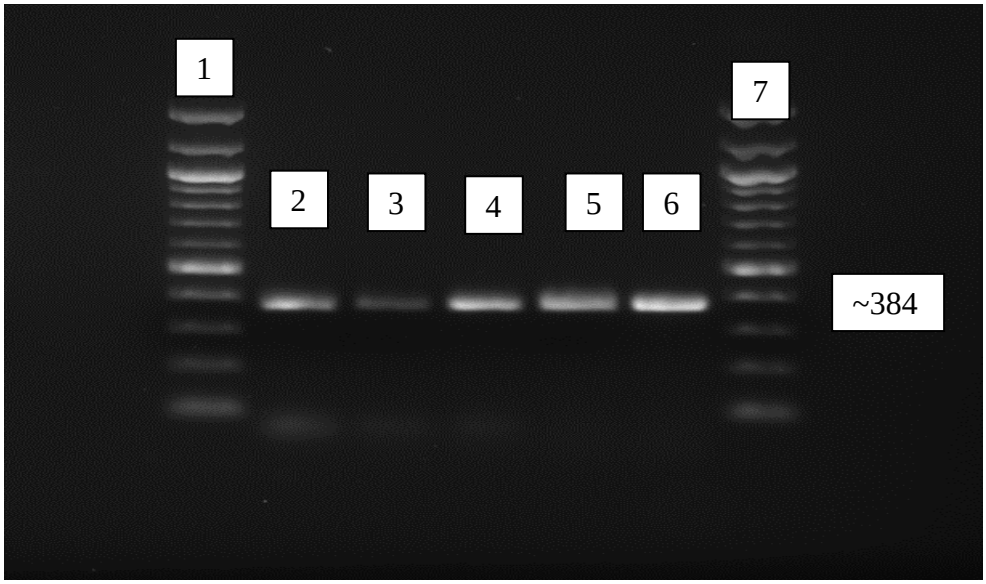


Figure 2 Gel electrophoresis of Clostridium perfringens netB gene in four strains

Lane 1: 100 bp standard, Lane2: Strain1, Line 3: starin2, Line 4: strain 3, Lane 5: strain 4, Lane 6: netB positive provided by Dr. Pillai's lab, Lane 7: 100 bp stranded.

Transmission and Scanning Electron Microscopy (TEM and SEM)

The four CP bacterial strains with a final concentration of ~10^6 CFU/mL were subjected in combined to 100 µg/mL of SCC1 or SCC22 for 10 h at 37°C under anaerobic conditions. Bacterial suspensions were preserved for ultrastructural examination by the addition of an equal volume of a glutaraldehyde fixative containing 4% glutaraldehyde prepared in a 100 mM phosphate buffer, pH 7.3 with 100 mM sucrose buffer. Following primary fixation, suspensions were rinsed once in buffer then pelleted into 2.5% agar. Subsequent to agar solidification, samples were split into two groups. One group was post-fixed in 1% osmium tetroxide while the other group of samples had this post-fixation step omitted. The samples were further subdivided

again into sets; one that was embedded into epoxy resin through a step-wise dehydration and infiltration protocol for thin sectioning and examination via TEM and anther that was prepared for observation via SEM. Post-fixation in osmium tetroxide was omitted in the hopes that its absence for allow for the easier detection of nickel metal precipitates both visually and by Energy Dispersive X-Ray Spectroscopy (EDS, FEI Tecnai G2 F20-TEM with Oxford Instruments EDS detector). Nickel was detected in EDS for control and treated groups. All components of the fixation protocol used in this experiment were selected to eliminate the possibility X-ray overlap with those produced by silver atoms that might be present in the samples (Mollenhauer, 1964).

Results and Discussion

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

Results of the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentration (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 *Clostridium 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. *Clostridium 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* Typhymirium, *E. coli* 843 and *E. coli* 1568, and the poultry field isolates *Salmonella* 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 (Clement and Jarrett 1994). 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, SC22, and AgAc, respectively. Similar to CP, bactericidal effect against SA was > 32 µg/mL for the 3 products.

Table 2 Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) 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	Note
Clostridium perfringens	11	>85	21	>85	48	>192	4 wild isolates, type A
Salmonella Typhimurium	16	21	16	21	24	24	Poultry isolate, resistant,14 antibiotics[1]
Salmonella Enteritidis	16	16	16	16	24	24	Poultry field isolate
Salmonella Kentucky	32	>85	32	63	24	72	Poultry field isolate
Salmonella Heidelberg	21	21	16	32	24	34	Poultry field isolate
Salmonella Montevideo	16	16	16	32	24	34	Poultry field isolate
Staphylococcus aureus	43	>85	43	>85	24	>192	Poultry field isolate
E. coli	21	42	21	42	24	34	Poultry field isolate
E. coli 843	16	16	16	16	24	24	Swine isolate, resistant, 5 antibiotics[2]
E. coli 1568	16	16	16	16	24	24	Swine isolate, resistant, 6 antibiotics[3]

* Minimum inhibitory concentration µM/ml

** Minimum bactericidal concentration µM/ml

[1] Resistant to: clindamycin, cloxacillin, erythromycin, lincomycin, vancomycin, methicillin, nalidixic acid, novobiocin, penicillin G, rifampin, streptomycin, chlortetracycline, tetracycline, and sulfizoxazole (sulfisoxazole); [2] Resistant to: ampicillin, tetracycline, chloramphenicol, kanamycin, and sulfizoxazole; [3] Resistant to: 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 *Clostridium perfringens*, and foodborne pathogens isolated from animals, with MICs values constant as previously reported for different bacterial species. Leid et al (2012) reported MICs for SCC1 and SCC22 against methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from human septum and blood were 45.4 mg/mL. while MICs against the *Staphylococcus aureus* isolated from blood was 22.7 mg/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 mg/mL, and SCC22 were 6 and 10 mg/mL. Panzner et al. (2009) 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 MIC90s of SCC1 against strains of *Pseudomonas aeruginosa* and multidrug-resistant organisms from the *Burkholderia cepacia* complex was 6 µg/mL (Kascatan-Nebioglu et al., 2006).

MBCs against *Clostridium 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. (2012) 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 (Messner and Imlay, 1999), silver ions are known to inhibit thiol-containing enzymes (Lansdown, 2002) such as NADH dehydrogenase II. *Clostridium perfringens* is a Gram-positive, obligate anaerobe that uses nitrate (NO_3) as the final electron acceptor in anaerobic respiration (Hasan and Hall, 1975). Additionally, the *Clostridium 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) (Shimizu et al., 2002). Thus, *Clostridium perfringens* cannot generate ROS as they do not reduce the O_2 and subsequently generating reactive products such as hydrogen peroxide (H_2O_2) which is required for Fenton reaction to generate hydroxyl free radicals (Pesakhov et al., 2007). 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 (Park et al., 2009). 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 (Matsumura et al., 2003). Moreover, Chen et al. (2017) reported that the 93% of the total cells (*Pseudomonas aeruginosa*) which were viable in suspensions treated with silver nanoparticles (5µg/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 (Oliver, 2010). 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 (Quirós et al.,

2015), which is a survival strategy of many bacteria in response to adverse environmental conditions.

Clostridium Perfringens Gene Expression of Virulence Genes

As the 4 tested isolates of combined CP 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 (Alifano et al., 1994). Detection of mRNA might therefore be a good indicator of living cells or those only recently dead at the time of sampling (Sheridan et al., 1998). Thus, the expression of virulence genes of CP after silver treatment could give an indicator of silver ion interacting with viable cells. CP genes involved in toxin production (*plc* and *netB*) were thus selected for a gene expression study.

RT-qPCR revealed that the expression levels of *plc* and *netB* genes were down-regulated 8.8- and 315-fold, respectively, in response to treatment with 40 µg/mL of SCC1 for 10 h at the mid-log phase of bacterial growth (Figure 3). Similarly, testing CP with 40 µg/mL depressed *plc* and *netB* gene expression by 1.86- and 48-fold for SCC22, 47- and 36-fold for AgAc.

The results suggest decreased the pathogenicity of CP after being subjected to silver ion, and silver from all 3 products interact with viable CP. 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 (Selby and Sancar, 1990; Osman and McCready, 1998).

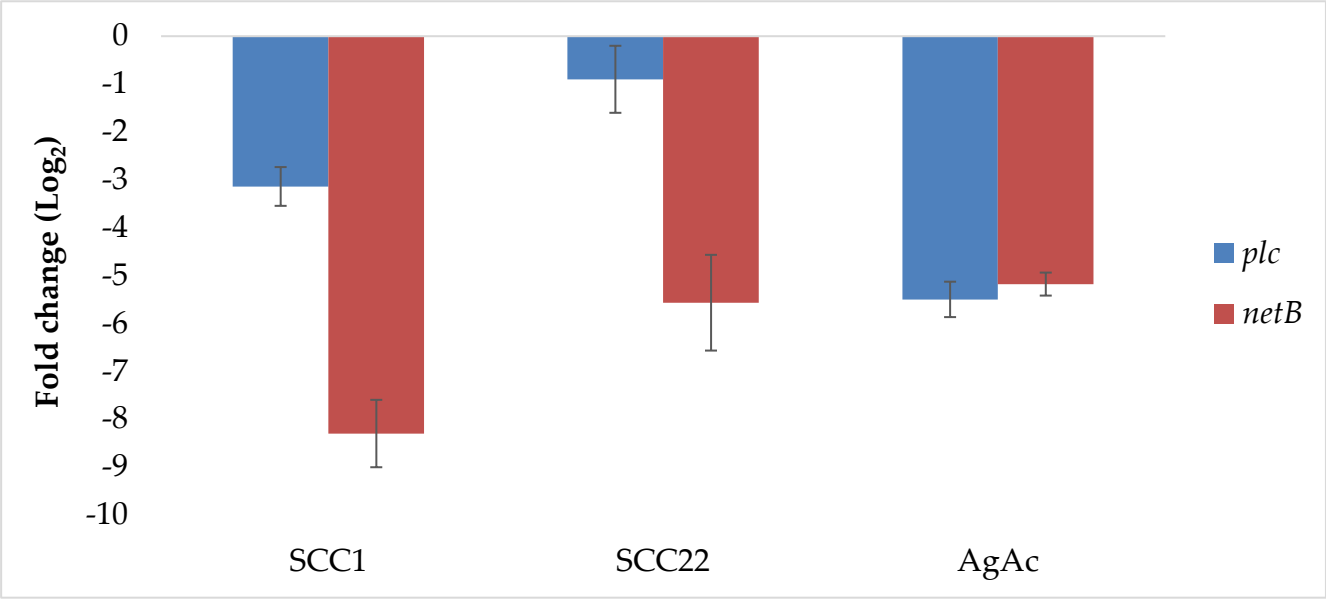


Figure 3 Relative Gene expression of *plc* and *netB* of *Clostridium perfringens* treated with 40 µg/mL of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc) for 10 h

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.

Morphological Changes

Transmission and scanning electron microscopy (TEM and SEM) along with Energy Dispersive X-Ray Spectroscopy (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-postifxed samples by TEM revealed bacteria with similar ultrastructural characteristics (Figure 4, 1 to 3).

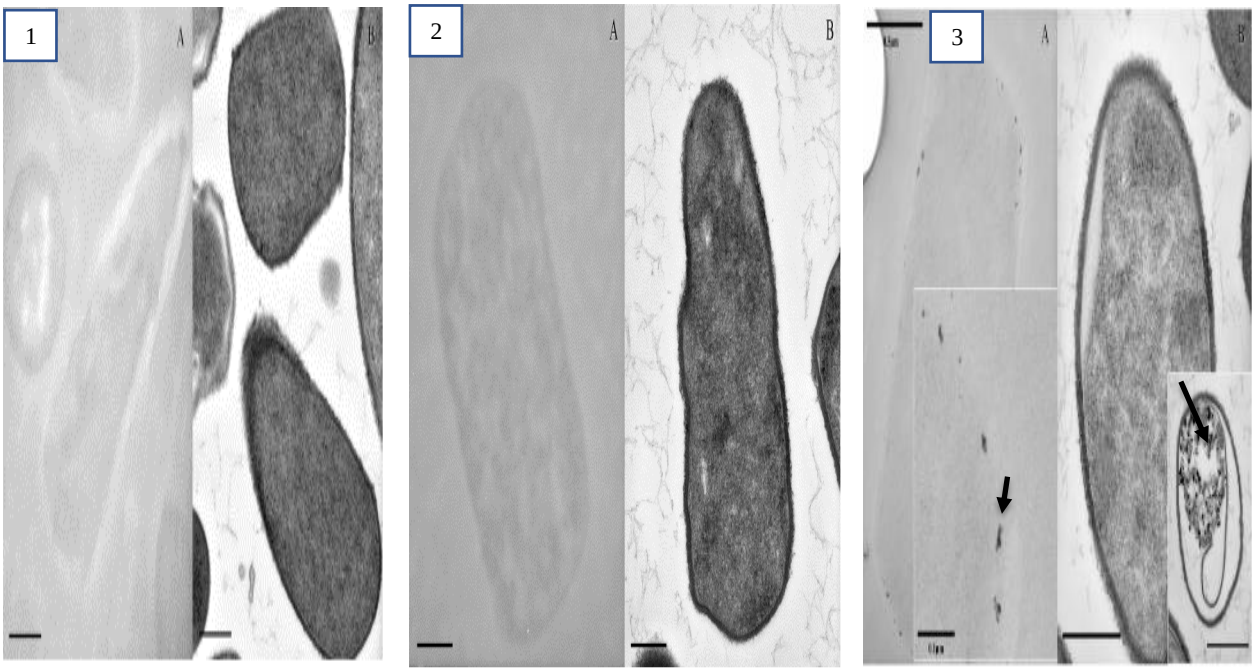


Figure 4 Micrograph of external morphology of *Clostridium perfringens* type A treated with 100 µg/mL of silver carbene complex 1(SCC1) and silver carben complex 22 (SCC22) for 10 h observed by transmission electron microscopy (TEM)

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 0.5µm

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 (Diekert and Ritter, 1982). However, it is not clear if the nickel detected by EDS could be influx or efflux of the metal.

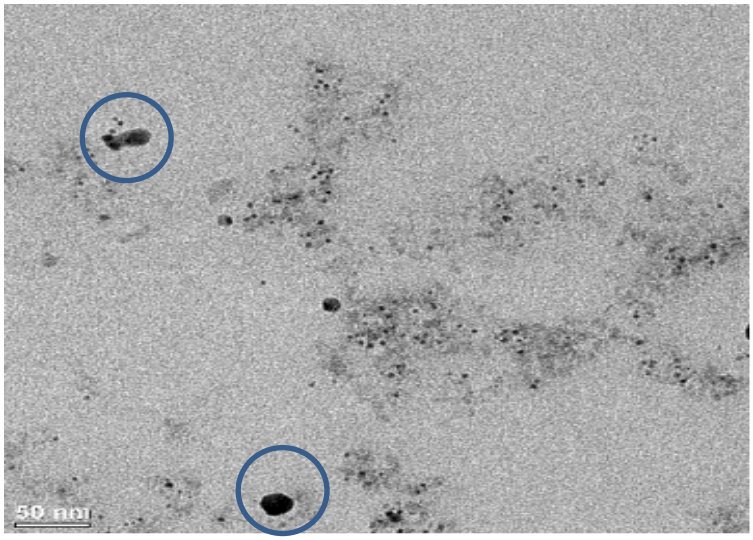
SCC22-treated bacterial cells showed small dense staining aggregate clusters on the bacterial cells when bacteria were viewed using TEM. These structures were located on the along the exterior cell wall of live bacteria (Figure 4, 3A) and within the interior of lysed bacteria (Figure 4, 3 B inset). It seems that CP 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 (Percival et al., 2005). Jung et al. (2008) 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 5) 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* Typhymirium and *E. coli* isolated from poultry and swine, respectively. Both SCCs demonstrated inhibitory effects and virulence modulation against the Gram-positive anaerobic *Clostridium 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.

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



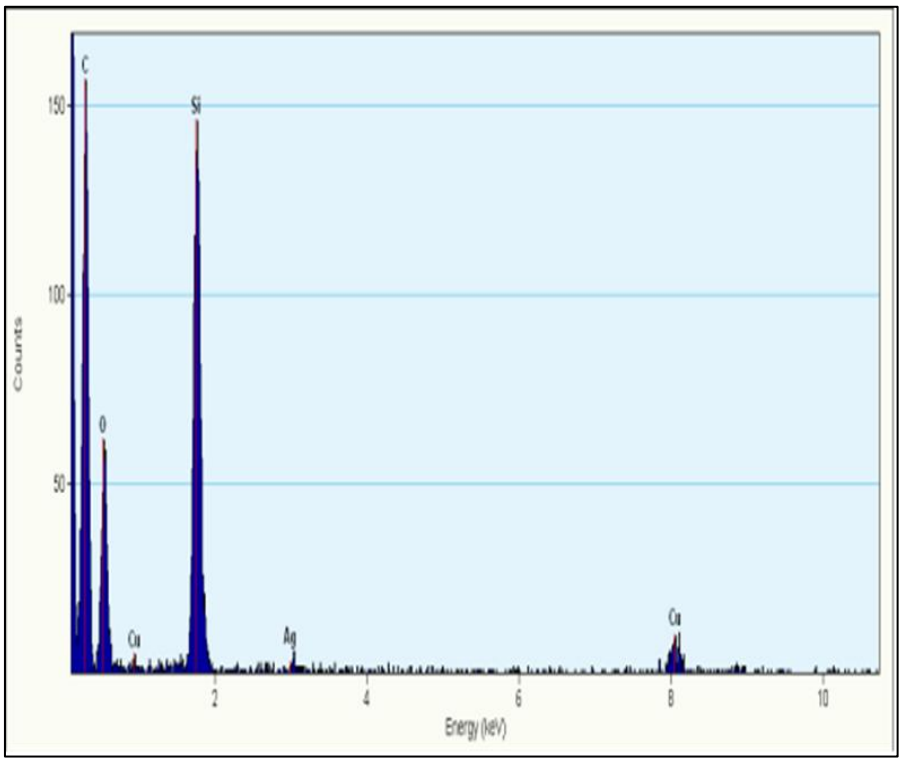


Figure 5 Transmission electron microscopy image of electron dense particles seen within samples of silver carbene complex 22 (SCC22) treated bacteria and the accompanying EDS data for the particles observed in that area of the sample

CHAPTER III

EVALUATION OF POTENTIAL IN VIVO TOXICITY OF SILVER CARBENE COMPLEXES, SCC1 and SCC22, AND SILVER ACETATE IN BROILER CHICKENS

Introduction

Silver has been used as antimicrobial agent in a verity of ways to control infections since ancient times (Russell and Hugo, 1994). The antimicrobial activity of silver ions is characterized by high effectiveness, low toxicity to host cells and rare resistance of microorganisms to the presence of this metal. These features led to extensive use of silver-based compounds as unique antimicrobial agents and attracted animal-antibiotic alternative researchers (e.g. Sawosz et al., 2007,2009; Fondevila, 2009; Pineda et al., 2012; Vadalasetty et al., 2018; Saleh and El-Magd, 2018; Chiao et al., 2012) to use different silver formulations, mainly particulate silver, as alternative to antibiotics in animal production. The challenge of using silver as antimicrobial agent is the effective therapy with low toxic effect on host cells, which requires the therapeutic window being safe. Toxicity of silver to mammals is still under examination (Cannon et al., 2009). Silver is known to be one of the least toxic metals. Use in high doses or misusing by humans can cause a rare irreversible pigmentation of the skin (argyria) and/or the eyes (argyrosis) (Lansdown, 2010 a). Silver is deposited in the basement membranes of various internal organs, but no adverse health effects occur (EPA, US, 1985).

The toxicity of silver ion occasionally attributed either to the silver carrier molecules (Gear et al., 1997) or, in part, particle size in particulate silver form (Kim et al., 2008, 2010; McShan et al., 2013) which in both the dissociation rate of ionic silver (Ag^{+}) plays a role determining silver bioactivities. The inorganic form of silver such as silver nitrate and silver

sulfadiazine is therapeutically well-known. However, this form rapidly loses its effectiveness since the silver cations will be released rapidly at the infection-site (Napoli et. al., 2013). In contrast, metallic silver in form of colloidal solution with size at nanodiameter scale is less toxic to eukaryotic cells owning to low absorbability, which lead to increase ion availability and extend antimicrobial effect (Choi et al., 2008).

Therefore, different silver-based formulations were synthesized based upon constant bioactive metal with a nontoxic carrier (Cannon et al., 2009). Silver-N-heterocyclic carbene complexes or shortly silver carbene complexes (SCCs), a group of novel silver-based compounds capable of gradually releasing silver ions, possess two major features: significant wide antimicrobial activity and low toxicity on host cells.

In previous chapter (chapter 2), *in vitro* efficacy of two formulations of SCCs, silver carbene complex 1 (SCC1, with a methylated caffeine backbone) and silver carbene complex 22 (SCC22, with a dichloroimidazolium backbone) in addition to silver acetate (AgAc) showed promising antimicrobial activity against different poultry enteric pathogens (aerobic, anaerobic, and multiantibiotic resistant strains). In terms of the latter (toxicity to host and safety) the study this chapter was conducted to evaluate the potential acute *in vivo* toxicity of these compounds prior including them in chicken feed or water to evaluate their *in vivo* antimicrobial efficacy.

Materials and Methods

All experiments were conducted under the Texas A&M University guidelines approved by the Institutional Animal Care and Use Committee (IACUC No. 2016015). All procedures were reviewed and approved by the USDA-ARS animal care committee (IACUC 2016015).

Birds and General Management

A total of 300 Ross-308 (mixed sex) broiler chickens were obtained on day of hatch and caged in Petersime Battery Brooders (48 pens, 5 birds pen, 2 sq ft per pen) located in an environmentally-controlled rearing room at the USDA-ARS facility (College Station, TX). The room was thermostatically controlled, 24-hour lighting provided with relative humidity kept between 60-70%. Each brooder pen contained supplemental heat as required. Birds were fed ad libitum a commercial-type with a balanced minerals and electrolytes corn-soy broiler starter diet as a crumbled pellet with 22% protein and 3050 AME kcal/kg for a 21-day rearing period (Table 4). Chicks received the standard vaccinations used by the hatchery. At the time of arrival to the experimental facility, all chickens were weighed in groups of 20 birds and an average body weight was calculated to distribute the birds.

Table 4 Feed composition of the basal diet

Ingredients	%
Yellow corn, grain	62.0
Soybean meal (CP, 48%)	32.0
Soybean oil	2.2
Limestone	1.4
Sodium chloride (salt)	0.50
Monocalcium phosphate	1.6
DL-methionine	0.23
L-Lysine·HCl	0.18
Vitamin premix*	0.25
Mineral Premix**	0.05
Calculated composition	
ME (kcal/kg)	3050
Crude protein	22
Crude fat	3.92
Calcium	0.95
Available phosphorus	0.45
Lysine	1.18
Methionine	0.53

* Provided the following per Kg of diet: vitamin A, 11 IU; vitamin D3, 3,850 IU; vitamin E, 45.8 IU; menadione, 1.5 mg; B12, 0.017 mg; biotin, 0.55 mg; thiamine, 2.93 mg; riboflavin, 5.96 mg; d-pantothenic acid, 20.17 mg; B6, 7.15 mg; niacin,45.8 mg; folic acid, 1.74 mg; choline, 130.3 mg.

**Trace minerals premix added at this rate yields (mg/kg): zinc, 60.0; manganese, 60.0; iron, 60.0; copper,7.0; iodine,0.4.

Experimental Design

The birds were used in two independent replicates over time. In each replicate trial, a total of 9 treatment groups (3 replicates/5 birds) arranged as 3 x 3 factorial (3 products and 3 doses) with separate positive control pens. After an acclimation period (1 week), birds were weighed again, and doses were calculated based on body weight and acute oral toxicity using the limit dose procedure described by the Organization for Economic Cooperation and Development guideline No. 223, with minor modification regarding the limit does used. Ascending doses (10,

100, 1000 mg/kg body weight [BW]) of SCC1, SCC22, and AgAc were dissolved in deionized distilled water (EASYpure® UV/UF, Barnstead) and orally administered.

The doses were chosen based on maximum tolerable level (MTL) of silver determined by NRC (2005) which is 100 mg/kg feed, thus, as we intended to use these silver-based compounds in later *in vivo* antimicrobial efficacy, we chose 10-fold higher and lower than MLT. In addition, there is no initial LD_{50} value using silver-based products in broiler chickens. This will give general initial insight of their tolerated toxicity to apply for further future studies regarding *in vivo* antimicrobial efficacy.

Birds were fasted for 6 h prior gavage. A dose of distilled water served as a control. The dosing volume was 1.5 mL/100 g BW. For dose 1000 mg/kg BW (for all products SCC1, SCC22, and AgAc) administered within less than 1 h by about 1.5 ml/100 g BW. To eliminate stress factor that could be generated from catching the birds at dose 1000, other treatment groups including the control group were cached only as same as 1000 group. Birds were observed periodically for any mortality. Time of death was recorded for mortalities which then weighed and necropsied by a licensed poultry veterinarian. The observation continued for 2 weeks to investigate any potential prolonged effects of the treatments on bird performance parameters, some plasma enzymes and metabolites, relative organ weight, bone ash, and histological examination for various tissues as indicators.

Data Collection

Body weight and feed consumption per pen were recorded to calculate weight gain (WG) and feed conversion ratio (FCR) at days 7, 14 and 21. One week after the administration of the silver treatment (d 14 of age), blood samples were taken from 6 birds via jugular vein (2-3 birds/pen) and collected in lithium heparinized tubes. The 5 mL blood samples were centrifuged

(3000 × g for 20 min) and plasma stored at -20°C and examined for selected plasma indices: blood urea nitrogen (BU), creatinine (Creat.), total bilirubin (TB), total protein (TP), calcium (Ca), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gammaglutamyl transferase (GGT) on an automatic blood chemical analyzer (Heska, Element DC™ Veterinary Chemical Analyzer, USA), using a commercially available kit (Heska). On day 21, chicks were weighed, euthanized using CO_2, necropsied, and the weight of the liver, kidney, heart, and spleen were recorded for all birds, and portions of each organ (plus portions of the jejunum and ileum from six birds/treatment/replicate trial) were harvested and fixed in 10% neutral buffered formalin for histological examination (only six organs were chosen). Hematoxylin and eosin (H&E) were used for histological staining. Also, the right tibia was removed for total ash determination to evaluate bone mineralization. After tibias were dissected from the birds, muscle and cartilage were removed before being placed in 4 liters of petroleum ether for 48 h at room temperature. Following this, tibias were dried for 48 h in a forced draft oven at 105°C. Finally, tibias were ashed at 650°C for 23 h. Percent bone ash was calculated based on the dry bone weight and remaining ash.

Statistical Analysis

SCC1, SCC22, and AgAc data were pooled from the 2 replicates into a combined data set and analyzed as 3X3 factorial. All data were analyzed using the GLM procedure of JMP Pro 12 software (SAS, Institute Inc., Cary NC). Significant means were separated using Tukey's HSD test at $P < 0.05$. Individual pens served as the experimental unit for production variables as well as plasma biochemistry, whereas individual birds were the experimental unit for the organ weights, histology, and bone ash. The control served as a reference treatment and was compared with the silver treatments by Dunnett's test ($P < 0.05$). For median lethal dose (LD_{50}), a logistic

regression analysis was performed using JMP Pro 12 software where the SCC and AgAc doses were set as continuous variable (x) and the nominal variable (y) set as killed (succeed) or not killed (failed) proportion. Using the inverse prediction option with a probability of P = 0.50; the predicted value of LD_{50} was calculated for each product.

Results and Discussion

The goal of this research was to evaluate organic sources of silver ions with different carrier molecules (SCC1, SCC22, and AgAc) across a range of doses in terms of potential host toxicity using *in vivo* assay prior including them in broiler feed or water to evaluate their *in vivo* antimicrobial efficacy. Acute toxicity is the observation of adverse effects taking place in an animal within a short time of administration of either a single dose or multiple doses of a chemical given to the animal (Walum, 1998), and it can give more information about the biologic properties of a chemical compound than any other single test (Paget, 1983). In current study, acute oral toxicity using the limit dose test procedure described by OECD test No. 223 guideline with minor modification regarding the limit dose used.

Short-Term Observation

LD50 and Mortalities. The SCC1 did not cause any mortality among the birds at any of the doses given up to 24 h and two weeks after exposure. In contrast, there were 4 mortalities (2 in each replicate) in SCC22 and AgAc treatment groups at the 1000 mg/kg BW. SCC22 also had a 3% mortality in the dose 10 and 100 mg/kg BW in replicate 1 (Table 5). The computed LD_{50} of SCC22 and AgAc for 7-day-old chick in current study were calculated as 1925 and 1079 mg/kg BW, respectively. The SCC1 result suggests that the LD_{50} is greater than 1000 mg/kg BW.

Table 5 Acute oral toxicity of Ross 308 broiler chickens subjected to 10, 100, 1000 mg/kg BW of SCC1, SCC22 and AgAc at day 7 of age. The LD_{50} values were obtained as results of a logistic regression‡

	Replicate 1					Replicate 2					Total			
	No. birds	No. rep	BW (g/dose mg)	Mortality %/No.	TD (h)†	No. birds	No. rep.	BW (g/dose mg)	Mortality %/No.	TD (h)	No. birds	No. rep.	Mortality %	LD_{50}*
Control	15	3	180/0	0/0	-	15	3	147/0	0/0	-	30	6	0	-
SCC1														
10	15	3	173/1.73	0/0	-	15	3	147/1.47	0/0	-	30	6	0	
100	15	3	174/17.4	0/0	-	15	3	148/14.8	0/0	-	30	6	0	>1000
1000	15	3	176/176	0/0	-	15	3	142/142	0/0	-	30	6	0	
SCC22														
10	15	3	175/1.75	3/1	12	15	3	146/1.46	0/0	-	30	6	3	
100	15	3	176/17.6	3/1	10	15	3	143/14.3	0/0	-	30	6	3	1925
1000	15	3	182/182	13/2	8, 23	15	3	137/137	13/2	3, 20	30	6	13	
AgAc														
10	15	3	170/1.7	0/0	-	15	3	148/1.48	0/0	-	30	6	0	
100	15	3	174/17.4	0/0	-	15	3	143/14.3	0/0	-	30	6	0	1079
1000	15	3	174/174	13/2	1, 20	15	3	137/137	13/2	3, 24	30	6	13	

* mg/kg body weight

† TD=Time death in hours

‡Logistic regression analysis was performed using JMP Pro 12 software where the SCC doses were set as continuous variable (x) and the nominal variable (y) set as killed (succeed) or not killed (failed) proportion. Using the inverse prediction option with a probability of P= 0.5; the LD_{50} was calculated for each product

In vivo oral acute toxicity studies of silver carbene complexes are rare (Haque et al., 2013). SCC1 has previously been shown to have a low toxicity to host cells in mice when administered via nebulization twice daily for a total of 5 days with 10 mg/mL concentration (Cannon et al., 2009). *In vitro* cytotoxicity of SCC1 was evaluated on primary cell culture of the respiratory epithelium by same author and 50% lethal dose was 289 µg/mL which was greater by 48-fold than minimum inhibitory concentration of *Pseudomonas aeruginosa* suggesting wide therapeutic index of this silver formulation. SCC1 is formed from coupling the silver ion from silver acetate to methylated caffeine (Kascatan-Nebioglu et al., 2006). These derivatives are naturally occurring and are not toxic after the silver ion has disassociated. Preliminary toxicity studies showed the intravenous LD_{50} of the xanthinium iodide salt of methylated caffeine to be 1.068 g/kg in rats (Panzner et al., 2009). *In vivo* oral acute toxicity study (sighting study) carried out by Haque et al. (2013) showed that 2000 mg/kg dose of dinuclear silver-NHC complex was an appropriate and safe dose for conducting main study to treat cancer cells in rats.

Similarly, there is scarce literatures investigated oral *in vivo* toxicity of SCC22 (Panzner et al., 2009). SCC22 are derivatives of 4, 5-dichloroimidazole. Anti-cancer studies using an ovarian cancer xenograft model in thymic nude mice has demonstrated that SCC5 (another dichloroimidazolium silver acetate derivative) can be administered in doses of 333 mg/kg subcutaneously with no acute toxicity observed (Medvetz et al., 2008). In a comparison study of SCC1 and SCC5 to treat pulmonary infection, SCC5 showed unexpected toxicity by increasing lung inflammation compared to SCC1 which was safe to deliver by nebulizing at 5 doses of 50 mg within 12 h with minimal systemic toxic effect (Taylor et al., 2009).

Silver ion from different compounds showed different LD_{50} values which mainly depend on animal species sensitivity for different silver-based products (with different carrier

molecules), rout and duration of administration. In the literature, there is no LD_{50} value available of sliver-based compounds in broiler chickens. Venugopal and Luckey (1978) reported oral LD_{50} values for mice using colloidal silver and silver nitrate 100 mg/kg and 129 mg/kg, respectively. While for silver cyanide, the LD^{50} for rats was 125 mg/kg, and silver oxide with LD_{50} of 2820 mg/kg for rats. In a single oral acute toxicity study in 7-week-old rats, administration of silver ion from particulate silver and $AgNO_3$ with 2 and 20 mg/kg BW did not cause death or changing in BW (Park, 2013). Haque et al. (2013) reported no signs of toxicity were observed and animals remained alive, healthy, and agile when administered single oral dosages of 300 and 2000 mg/kg BW of dinuclear Ag (I)-NHC complex. Tamimi et al. (1998) worked on silver nitrate as the active ingredient in an anti-smoking mouthwash, the author reported: in rats, following oral administration, the LD_{50} was found to be 280 mg of silver/kg of BW, while in rabbits the LD_{50} was found to be 800 mg of silver/kg of BW/day. Walker also identified silver nitrate at concentrations 308 mg of silver/kg BW (rat) in the drinking water induces death over the course of a few days (Walker, 1971). Maneewattanapinyo et al. (2011) reported at acute doses of up to 5000 mg/kg of BW/day, silver was not toxic when orally administered nanoparticulate to guinea pigs. The LD_{50} of SCC22 1925 mg/kg BW is relatively higher than AgAc LD_{50}. The oral acute toxicity of silver carbene compounds and silver acetate in broiler chickens appears to be higher in some instances than it is in laboratory animals.

Gross Necropsy and Clinical Signs. In replicate 1, upon gross necropsy of mortalities, the most common lesions we noticed were black-gray material found mainly in lung, eye, esophagus, trachea, increase mucus secretion in GIT, and very week bone to break.

SCC22 mortalities following the administration of 10 and 100 mg/kg BW showed moderate congested liver, kidney, intestine, and gizzard which was filled with feed. The lungs

were congested and surrounded by black-gray material and blood spots. Increase accumulation of eye fluids which had significant amount of that described material. Weak bone was observed in the mortality at 10 mg/kg BW, and normal break bone in 100 mg/kg BW. No prior to death clinical signs were observed for these mortalities.

SCC22 early and late-mortalities in replicate 1 (following 1000 mg/kg BW administration) showed moderate congestion in internal vital organs, and excessive black-gray material found mainly in lung, eye, esophagus, and trachea. Soft and hard to break bones, and abnormal broken bone at the hip joint (which suggests sever bacterial infection) were also noted. Early-and late-mortalities of SCC22 showed excessive mucous which was fulling the entire GIT. Gizzard contained foamy fluid with slight congestion in proventriculous. For AgAc mortalities at 1000 mg/kg BW, similar weak bone noticed, the mucus excretion was noted only in the duodenum with empty intestine, and more congestion was noticed in the lung and kidney of AgAc mortalities, and no black-gray material was observed.

In replicate 2, the most common lesions of SCC22 and AgAc mortalities (at 1000 mg/kg BW) shared with replicate 1 observed were increase mucous secretion in the GIT and very weak bone to break. No black-gray material was observed in these mortalities. Late mortality (after 20 h) of SCC22 at 1000 mg/kg BW showed white pseudo membrane-like structure, lining the interior surface of the crop; easy to remove, and the interior esophagus was covered with white cheesy material similar to (or suggesting) candidiasis lesion. Similarly, the late mortality of AgAc (24 h) showed similar lesion in the crop which appeared degenerated, but not in the esophagus (Figure 6).

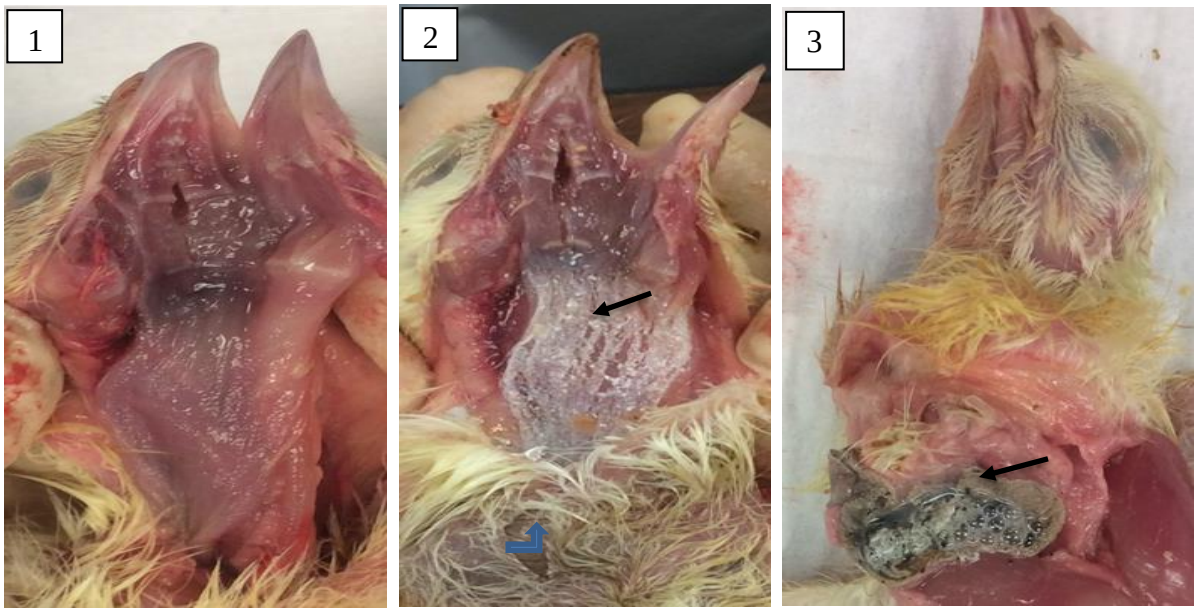


Figure 6 Mortalities of silver carbene complex 22 (SCC22) and silver acetate (AgAc) at 1000 mg/kg BW in replicate 2

(1) SCC22 mortality after 3 hours of administration, (2) SCC22 mortality after 20 hours of administration; white cheesy material in the esophagus (arrow) and the crop looked dilated and degenerated (blue arrow), (3) AgAc mortality after 24 hours of administration; crop was contained white cheesy material (arrow) easy to remove but no lesion in the esophagus

Ionic and nanoparticulate silver have been described in terms of tissue deposition following oral administration to be deposited in a wide range of organs including GIT, stomach, liver, kidneys, eye, lungs, muscle, brain, plasma (Olcott, 1947, 1948; Chang et al., 2006; Loeschner et al., 2011; Matuk et al., 1981). This deposition was described in microscopic studies following oral exposure [to both ionic and nanoparticulate silver] to be deposited as fine black granules or particles. These particles have been described to be 12 nm in diameter in the rat intestines and to contain sulphur and selenium in addition to silver (Loeschner et al., 2011). Particle deposition was described to increase both in number and size in rat eyes during the first weeks of silver ion administration, and the particles decreased in number and size when silver was withdrawn, although the particles were still present one year after the silver was withdrawn (Matuk et al., 1981).

In current study, the gross black-gray material noticed in SCC22 mortalities in only replicate 1, is puzzling as no further histological examination was performed. However, it is not unlikely that silver could be deposited from blood vessels after entering the circulation. Creasey and Moffat (1973) reported the deposition of silver from silver nitrate in rat kidney as granules when silver was administered in the drinking water of 26 weanling rats in the form of a 0.15% aqueous silver nitrate solution for periods of from 4 to 15 weeks. The density of the silver deposits as granules increased towards the tip of the papilla and could be recognized easily by naked-eye examination.

The most that gross lesions that could be related to silver toxicity are the increase mucus secretion in the GIT and weak bones. Jeong et al. (2010) found oral administration of silver nanoparticles induced the discharge of mucus from goblet cells, mostly in the ileum. Leg weakness immediately prior to death was a common observation when broiler chickens fed 900

ppm silver nitrate for 4 weeks, and the mortalities (35%) constantly revealed blood in the abdominal cavity, mouth, trachea, and esophagus (Peterson and Jensen, 1975).

The prior to death clinical symptoms of birds in SCC22 and AgAc at 1000 mg/kg BW in both replicates were similar: apparent lack of anxiety, dyspnea, tachypnea, close eyes gradually and continually with abdominal recumbency. Moderate uncontrolled vent pasty-diarrhea (similar to *Salmonella pullorum* infection) was also noted. This could be evidence along with the abnormal broken bone at the hip joint, (in addition to suggested candidiasis in replicate 2) which suggest high infection could be occurred as a result of immunosuppression induced by silver. Moreover, there was one chick died after 10 days of exposure on dose 1000 mg/kg of SCC22 and apparently it had a severe internal infection with normal eye fluids as evidenced by necropsy. Van der Zande et al. (2012) observed no immunotoxicity following the oral administration of 90 mg/kg BW of silver nanoparticles or 9 mg/kg BW/day of ionic silver for 28 days. In addition, a decreased thymus weight was recorded following the administration of 9 mg/kg of BW/day (28 d) of ionic silver (Hadrup et al., 2012) and it was suggested that the lower relative thymus weight in ionic silver (as silver acetate) treated group could indicate a stress-related immune alteration (Descotes, 2006).

Prior to death, birds showed very slow, irregular, labored respiration, along with uncontrolled-shivering like movement in the legs, wings and heads (continuous muscle contraction [noticed immediately after gavage, and was gradually decreased in severity prior to death as the bird looked exhausted]). Effects on the nervous system were reported in early studies including weakness, rigidity of legs, loss of voluntary movement, and respiratory paralysis following intravenous administration of high doses of silver compounds to dogs, guinea pigs, and rats (U.S. EPA, 1985). Rungby and Danscher (1984) found that 0.015% silver nitrate in

the drinking water for 125 days (14 mg/kg BW/day) induced hypoactivity in mice after a 10 day silver withdrawal period. It was previously reported that silver-induced neurotoxic effects may occur via secondary molecules that are released from the periphery (Hadrup and Lam, 2014).

Long-Term Observation

Clinical Signs and Performance. Clinical signs and mortalities were not observed for the rest of the birds up to trials termination. Necropsy after 14 days of administration did not show significant gross pathological lesion for all treatment groups.

After 7 days of administration, dose and product main effects were observed on bird performance. Weight gain was reduced ($P < 0.05$) in a dose dependent manner for all products (Table 6). However, no differences were observed ($P > 0.05$) in the bird performance of SCC1 and SCC22 compared to control group. Compared to the control and SCCs, AgAc at dose 1000 mg/kg BW reduced ($P < 0.05$) BW and WG with non-significant decrease in FCR ($P > 0.05$). At d 21, the decrease product effect on BW of AgAc was significant ($P < 0.05$) compared to SCCs formulations, although the birds gained weight similar to that in control group with no effect on FCR. These results suggest that oral administration of a single dose of SCC1 and SCC22 at doses 10, 100, and 1000 mg/kg BW did not change the BW, WG, and FCR compared to control group after 7 and 14 days of administration, demonstrating that SCCs did not negatively affect broiler growth when dosed acutely for short period.

Cannon et al (2009) reported with repeated dosing of nebulized SCC1 (5 mg/dose SCC1, 5 min exposure, twice daily) over a period of 5 days the animals exhibited no weight loss. Haque et al. (2013) reported no changes in body weight when administered single oral dosages of 300 and 2000 mg/kg BW of dinuclear Ag (I)-NHC complex. Park et al. (2013) in male Sprague-

Dawley rats up to 24 h after a single oral administration of silver nanoparticles (AgNP) and silver ions from silver nitrate (AgNO3) body weight changes were not observed.

Although there are numerous oral toxicity investigations who reported no effect on body weight (Jeong et al., 2010; Kim et al., 2008, 2010; Van der Zande et al., 2012), some other investigation reported detrimental effects. Matuk et al. (1981) found that growth rates were retarded in rats administered 81 mg/kg of BW/ day silver nitrate in the drinking water for more than 8 months. The author also reported when silver was withdrawn, the body weight became normalized over the course of 10 weeks. Body weight gain decreased following the oral administration of silver acetate (9 mg/ kg BW/day) (Hadrup et al., 2012). Decreased body weight was observed in male rats only in a 13-week study of the oral administration of 500 mg of silver nanoparticles/kg BW/day (Kim et al., 2010).

Table 6 Performance (body weight, weight gain, and feed conversion ratio) of broiler chickens at d 14 and d 21 dosed with 10, 100, 1000 mg/kg BW of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc)[1]

		d 14			d 21		
		BW (g/bird)	WG (g/bird)	FCR (g:g)	BW (g/bird)	WG (g/bird)	FCR (g:g)
Product	Dose						
Control	0	438	277	1.03	842	404	1.32
SCC1	10	447	287	1.00	876	428	1.25
SCC22	10	447	287	1.02	863	416	1.29
AgAc	10	403	253	1.00	792	389	1.26
SCC1	100	438	277	1.00	847	409	1.26
SCC22	100	432	272	1.03	845	414	1.31
AgAc	100	377	224	1.00	784	408	1.28
SCC1	1000	420	260	1.01	838	419	1.29
SCC22	1000	411	251	1.00	826	415	1.30
AgAc	1000	358*	215 *	0.85	758	400	1.28
SEM[2]		18.09	12.55	0.08	32.84	17.26	0.03
Main Effects							
Product							
SCC1		435 [a]	274 [a]	1.01	854 [a]	419	1.27
SCC22		430 [a]	270 [a]	1.02	845 [a]	415	1.30
AgAc		379 [b]	231 [b]	0.94	778 [b]	399	1.28
Dose							
10		432	276 [a]	1.00	844	411	1.27
100		416	258 [ab]	1.01	826	410	1.29
1000		396	243 [b]	0.96	807	411	1.29
P-Value							
Product		0.001	0.001	0.522	0.013	0.333	0.448
Dose		0.059	0.009	0.704	0.408	0.996	0.801
Product X Dose		0.988	0.943	0.883	0.997	0.858	0.980

[a–b] Means within a column for each product lacking a common superscript differ significantly ($P < 0.05$).[1] Each value is the average of 3 replicates/trial with 5 chicks per replicate (n=6).

[2]SEM=standard error mean.

*Indicates comparing to control which served as a reference treatment and was compared with the silver treatments by Dunnett's test ($P < 0.05$)

Relative Organ Weight. Results of relative organ weights are presented in Table 7. Analysis of organ weight to body weight ratio is one dependable indicator for drug assessment in terms of toxicity (Michael et al., 2007). Compared to the control group, administration SCC1, SCC22 up to 1000 mg/kg BW did not affect the relative organ weights ($P > 0.05$) of liver, kidney, heart, spleen, [except, the relative organ weight of SCC1 at 100 mg/kg BW which showed lower relative weight, this could be attributed to the mild histological changes of 3 birds observed (discussed in histology section). These results agree with previous publication showed no adverse effect on elative organ weigh suggesting tolerated to acute exposure. Product main effect was found in relative weight of the kidney ($P < 0.001$) and liver ($P < 0.009$).

The relative weight of the kidney in AgAc-treated birds was higher than SCC1 and SCC22 treatments. SCC1 also had lower kidney weight than SCC22. The relative weight of liver in AgAc was lower comparing to SCCs. This difference could be attributed to gender effect, as showed by Kim et al. (2008, 2010) that the toxicity on the kidney was gender dependent being more effect in females' rats. In the kidney, it was reported that orally administered silver was found to be deposited in the glomerular basement membrane with no adverse effect (Walker, 1972). Sardari et al. (2012) reported histopathological findings in kidneys (necrosis in Bowman's capsule and proximal tubular cells in the kidney which potentially affect body fluid homeostasis) in rats when orally administered 1 or 2 mg/ kg of BW/day of particulate silver for 30 days. Park et al. (2013) reported with single oral administration of silver nanoparticles and silver ions (Ag^{+}), with 2 and 20 mg/kg the absolute and relative weights of kidney and liver were not evidently different from control group.

Table 7 Effects of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc) on the relative organ weights (as a percent of live body weight) of broiler chickens[1]

Product	Dose	Liver (%)	Kidney (%)	Heart (%)	Spleen (%)
Control	0	2.88	0.54	0.64	0.10
SCC1	10	3.06	0.43	0.58	0.11
SCC22	10	2.94	0.52	0.59	0.11
AgAc	10	2.71	0.61	0.58	0.11
SCC1	100	3.04	0.40 *	0.58	0.09
SCC22	100	2.95	0.53	0.61	0.11
AgAc	100	2.49	0.61	0.59	0.09
SCC1	1000	2.94	0.48	0.60	0.10
SCC22	1000	3.03	0.55	0.64	0.10
AgAc	1000	2.67	0.64	0.63	0.12
SEM[2]		0.123	0.046	0.028	0.01
Main Effects					
Product					
SCC1		3.01 [a]	0.44 [c]	0.61	0.10
SCC22		2.98 [a]	0.53 [b]	0.63	0.11
AgAc		2.63 [b]	0.62 [a]	0.59	0.10
Dose					
10		2.91	0.52	0.59	0.11
100		2.83	0.51	0.59	0.10
1000		2.89	0.56	0.63	0.10
P-Value					
Product		0.009	<0.001	0.486	0.756
Dose		0.771	0.317	0.054	0.503
Product X Dose		0.838	0.885	0.919	0.223

[a–c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1]Each value is the average of 3 replicates/trial with 5 chicks per replicate (n=30). 2SEM=standard error mean

There was tendency with (*P* value 0.059) dose dependent effect on relative weight of heart. Hadrup et al. (2012) investigated the relative weight of heart but found no histological

changes neither relative weight change following the oral administration of 9 mg/kg BW of ionic or 14 nm nanoparticulate silver. Kim et al. (2008, 2010) found no histopathological changes in the heart after up to 13 weeks of treatment that silver nanoparticles at doses up to 1000 mg/kg BW/day administered.

Blood Biochemistry. The results of some blood biochemistry are presented in Table 8. After 7 days of administration, some blood biochemistry parameters were measured as indicator for liver and kidney damage. Across the doses of all products, there were no differences in the concentrations of plasma metabolites which indicate kidney damage BUN and Creat.

Plasma Ca levels were affected in a product (P = 0.002) and dose (P = 0.003) manner, where AgAc caused increase plasma Ca levels compare to SCC1. Plasma Ca levels tended to increase for all products at doses 100 and 1000 mg/kg BW compared to low dose 10 mg/kg BW. Compared to the control, doses 100 and 1000 mg/kg BW of SCC22 and AgAc increased (P < 0.05) the plasma Ca level. Ca could be an indicator for renal function and subsequently bone development. For the Ca ion, in rodent and broiler studies that have measured serum or plasma Ca^{+2} (Kim et al., 2008, 2010; Park, 2013; Wen et al., 2017; Ognik et al., 2016 a; Sikorska et al., 2010), no difference was seen in the Ca levels after administration of silver nanoparticles. Ji et al. (2007), however, reported higher Ca level with higher silver dose in an inhalation study. While, Lee et al. (2013) reported decrease in serum Ca levels compared to control group after a single intravenous injection of Ag-NPs at 0.5 and 5 mg/kg BW.

SCC1, and SCC22, at any of the doses, did not affect the plasma enzymes that would indicate liver damage: ALT and ALP neither the serum metabolite total bilirubin (TB) after 7 days of administration. The oral administration of ionic silver in the form of silver acetate (9 mg of silver/kg BW/ day) or nanoparticulate silver (9 mg of 14 nm particles/kg BW/ day) increased

the ALP level in plasma (Hadrup et al., 2012). The author also reported no histological effect on liver tissue.

Product and dose-dependent effects 7 days after single exposure were found in plasma GGT ($P < 0.05$), namely AgAc-treated birds had higher plasma GGT compared to those treated with SCC1. All products tended to decrease plasma GGT at high doses; however, no differences compared to control group. AgAc more rapidly releases its silver ions when compared with SCC1. The increased GGT activity seen with AgAc treatment (product effect) may indicate a reduced availability of glutathione (GSH) as a substrate, because silver ions readily bind to sulfur-containing enzymes. Silver nitrate ($AgNO_3$) was reported to have significant effects on GSH contents reduction in plasma and cytosolic fraction of blood (Khan et al., 2011).

It was reported previously that low antioxidant defenses are associated with raised GGT levels, mainly reduced levels of GSH (Koenig and Seneff, 2015). GGT is mainly needed to enable metabolism of glutathione and glutathionylated xenobiotics in the liver and lungs, and this is a simple explanation for its elevation in association with exposure to xenobiotics. Silver is considered as a xenobiotic (Lansdown, 2010 a). GGT, therefore, is an indicator of depleted supply of glutathione, especially in the liver, which leads to a cascade of problems related to amplify the oxidative stress (Koenig and Seneff, 2015). However, dose dependent effect in the present study of all products was found at high doses. At 1000 mg/kg BW serum GGT levels decreased compared to 10 and 100 mg/kg BW. Scarce literatures regarding GGT and silver ion are available.

Table 8 Effect of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc) on some plasma biochemistry of broiler chickens at d 14 of age[1]

Product	Dose	TP (mg/dL)	Crtea. (mg/dL)	BUN (mg/dL)	TB (mg/dL)	Ca (mg/dL)	ALT (U/l)	ALP (U/l)	GGT (U/l)
Control	0	2.45	0.2	5	0.1	12.3	10	993	21.7
SCC1	10	2.28 *	0.2	5	0.1	11.8	<10	>993	20.5
SCC22	10	2.48	0.2	5	0.1	13.1	<10	>993	22.5
AgAc	10	2.45	0.2	5	0.1	13.1	<10	993	25.0
SCC1	100	2.38	0.2	5	0.1	13.1	<10	>993	21.7
SCC22	100	2.50	0.2	5	0.1	13.3 *	10	>993	22.7
AgAc	100	2.40	0.2	5	0.1	13.9 *	10	>993	24.0
SCC1	1000	2.43	0.2	5	0.1	12.9	10	993	19.7
SCC22	1000	2.52	0.2	5	0.1	13.2 *	10	993	20.8
AgAc	1000	2.35	0.2	5	0.1	13.4 *	10	993	21.5
SEM[2]		0.049	0.00	0.00	0.00	0.259	0.00	0.001	0.972
Main Effects									
Product									
SCC1		2.36 [b]	0.2	5	0.1	12.6 [b]	10	993	20.7 [b]
SCC22		2.50 [a]	0.2	5	0.1	13.2 [ab]	10	993	22.0 [ab]
AgAc		2.40 [b]	0.2	5	0.1	13.4 [a]	10	993	23.5 [a]
Dose									
10		2.40	0.2	5	0.1	12.6 [b]	10	993	22.6 [a]
100		2.43	0.2	5	0.1	13.4 [a]	10	993	22.8 [a]
1000		2.43	0.2	5	0.1	13.2 [a]	10	993	20.7 [b]
P-Value									
Product		0.004	0.99	0.99	0.99	0.002	0.99	0.99	0.005
Dose		0.688	0.99	0.99	0.99	0.003	0.99	0.99	0.0188
Product X Dose		0.181	0.99	0.99	0.99	0.2157	0.99	0.99	0.679

[a-b] Means with different superscript letters within each variable are significantly different at $P < 0.05$.

[1] Each value is the average of 3 replicates/trial with 2 chicks per replicate (n=6).

[2]SEM=standard error mean.

*Indicates comparing to control which served as a reference treatment and was compared with the silver treatments by Dunnett's test ($P < 0.05$).

There was a product main effect at day 14 (P = 0.004) in total plasma protein, where SCC1 and AgAc had a lower concentration than SCC22. Compared to the control, SCC1 at dose 10 mg/kg BW reduced (P = 0.010) the total protein concentration, which also showed slightly decrease in serum Ca. this effect was expected as silver bind readily to serum proteins, however, no such effect at higher doses was seen.

Bone Ash. The results for total bone ash determination are presented in Table 9. Bone mineralization was only evaluated for surviving birds at day 21. No interaction was found between products and the three increasing doses in the % ash content. Yet, there was a product main effect (P = 0.002), where SCC22 and AgAc had lower bone ash than SCC1.

Summarizing the effect of AgAc on Ca seen either in serum or in bone mineralization as indictor of Ca deposition would be totally opposite to SCC1. In other words, SCC1 caused increased % ash and decreased serum Ca levels, which might indicate high precipitation Ca from the blood into bone. In contrast, AgAc (compared to SCC1) caused decrease % bone ash and increased serum Ca, which might indicate open the Ca channels in the bone and increase Ca in the serum. Both actions more likely occurred as a result of dissociation rate. However, toxicity of silver in chicks is more complicated than in different lab animals such as rats (Peterson et al. 1973). This could be attributed to the less development kidney structures in birds.

Table 9 Effect of silver carbene complex 1 (SCC1), silver carbene complex 22 (SCC22), and silver acetate (AgAc) on bone ash percentage of 21-day old broiler chicken dosed with 10, 100, 1000 mg/kg BW

		Bone ash %
Product	Dose	
Control	0	49
SCC1	10	49.04
SCC22	10	47.27
AgAc	10	48.89
SCC1	100	49.54
SCC22	100	48.56
AgAc	100	48.25
SCC1	1000	49.73
SCC22	1000	49.01
AgAc	1000	48.09
SEM[2]		0.767
Main effects		
Product		
SCC1		49.44 [a]
SCC22		48.28 [b]
AgAc		48.41 [b]
Dose		
10		48.40
100		48.78
1000		48.94
P-Value		
Product		0.021
Dose		0.563
Product X Dose		0.419

[a–b] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1] Each value is the average of 3 replicates/trial with 5 chicks per replicate (n=30).
[2]SEM=standard error mean.

Histology. List of each treatment group and a brief summary of histological changes observed in each organ is presented in Table 10. No significant histologic lesions were noted in the duodenum, ileum, and spleen when broiler chicken received SCC1, SCC22, and AgAc up to 1000 mg/kg BW. This result is in line with previously reported studies which indicated no adverse effect on mucosa of small intestine. Hadrup et al. (2012) reported in subacute oral toxicity study for 28 days, no adverse effects occurred on mucosa of the gastrointestinal tract for both particulate silver and silver acetate when delivered at 9 mg silver/kg BW/day. Wen et al., (2017) reported with a single i.v acute toxicity study for 24 h Ag-NP accumulated in main immune system organs including the thymus and spleen. Histopathology results also showed that mild irritations were observed in the thymus and spleen only in the Ag-NP-treated group rather than the ionic silver (Ag^{+}) treated group. Similarly, with 28-day subacute study, no effect on spleen weight or histology, but Ag-NP reduced the spleen weight of the males compared to the control (Hadrup et al., 2012), and the author reported that silver acetate exhibited lower absolute weights of the female's liver and the thymus.

Some mild histopathological changes noted in liver and kidney. Within treatment groups of SCC1 (at 10, 100, 1000 mg/kg BW), and SCC22 (at 10 and 100 mg/kg BW) 66-100% of the birds exhibited mild hepatocellular vacuolation consistent with glycogen and microvesicular lipid (Table 10, Figure 7, B). However, these changes were not dose-dependent and therefore not suspected to be related to silver administration. In liver of poultry, deposition of sliver was studied to be the main site of silver retaining an accumulation (Chauke and Siebrits, 2015; Ahmadi and Rahimi, 2011; Gallocchio et al., 2017, Kulak et al., 2018 a,b). Pathological effect also studied by Ahmadi and Mehrdad (2009) who observed slight necrotic changes in liver of high dose Ag-NP-treated chickens. However, Ognik et al. (2016 b) has not noticed deviations

from the normal structure of liver when using Ag-NPs with larger size with lower dose. Ahmadi et al. (2009) showed that 300, 600, 900 in drinking water did not affect the liver structure. Loghman et al. (2012) evaluated the toxicity of Nano silver and observed pathological and morphological changes in the liver of broiler chickens administered at 4, 8, 12 ppm in drinking water at 4 ppm, the author reported infrequent accumulations in the hepatocytes (cell swelling) and hyperemia. While at 8 and 12 ppm showed dilated central vein, hyperemia with severe vacuolation fatty change. Similarly, in subchronic toxicity (Amin et al., 2016) and acute toxicity (Cho et al., 2018) reported moderate to severe fatty changes of hepatocytes and cytoplasmic vacuolization when silver particulate administered to rats and mice.

Three of the birds of SCC1 at 100 mg/kg BW showed mild, multifocal renal tubular degeneration and necrosis with minimal accumulation of pale basophilic material within the tubules. These changes were not noted in any other group and were considered incidental. The same group showed the lowest kidney relative weight. Two control birds and two birds in the highest treatment groups contained uroliths within the ureter surrounded by a mild to moderate amount of fibrosis (Figure 8). Again, these changes were not dose-related and were considered incidental, most likely associated with dehydration. In the kidney, it was reported that orally administered silver was found to be deposited in the glomerular basement membrane (Walker, 1972). The cause of uroliths in poultry is not fully known, but proposed mechanisms include reduced water intake and increased dietary Ca (Sakhaee et al., 2012). Plasma analysis from this study also found increased Ca levels in the AgAc-treated group (100 and 1000 mg/kg BW), which may explain the pathological changes in the kidney. However, our dietary Ca was 0.95% (lower than NRC recommendation 1%) and control urolitha was not expected to be seen which additionally had higher percentage than in AgAc (seen in 2/6).

Table 10 Brief summary of histological changes observed in liver and kidney after acute oral toxicity

Group	Liver	Kidney
Control	NSF	Large focal urolith in major ureter branch with mild surrounding fibrosis (2/6)33
SCC1-10	mild (2)33% , moderate (4)66% hepatocellular vacuolation (glycogen and microvesicular lipid)	NSF
SCC1-100	mild (2/6)33%, moderate (2/6) 33% hepatocellular vacuolation (glycogen and microvesicular lipid)	Mild, multifocal tubular degeneration, necrosis, and dilation/simplification (3/6) 50%
SCC1-1000	mild (3/6) 50% hepatocellular vacuolation (lipid and glycogen)	NSF
SCC22-10	mild (3/6) 50% hepatocellular vacuolation (lipid and glycogen)	NSF
SCC22-100	mild (3/6) 50%, to moderate (1/6) 16% hepatocellular vacuolation (glycogen and microvesicular lipid)	NSF
SCC22-1000	NSF (6/6) 100%	mild multifocal heterophilic granulomas (1/6) 16%
AgAc-10	NSF (6/6) 100%	NSF
AgAc-100	NSF (6/6) 100%	multifocal uroliths in major ureter branches with mild surrounding fibrosis (1/6) 16%
AgAc-1000	NSF (6/6) 100%	Large focal urolith in major ureter branch with moderate surrounding fibrosis (1/6) 16%

NSF= Non-significant findings

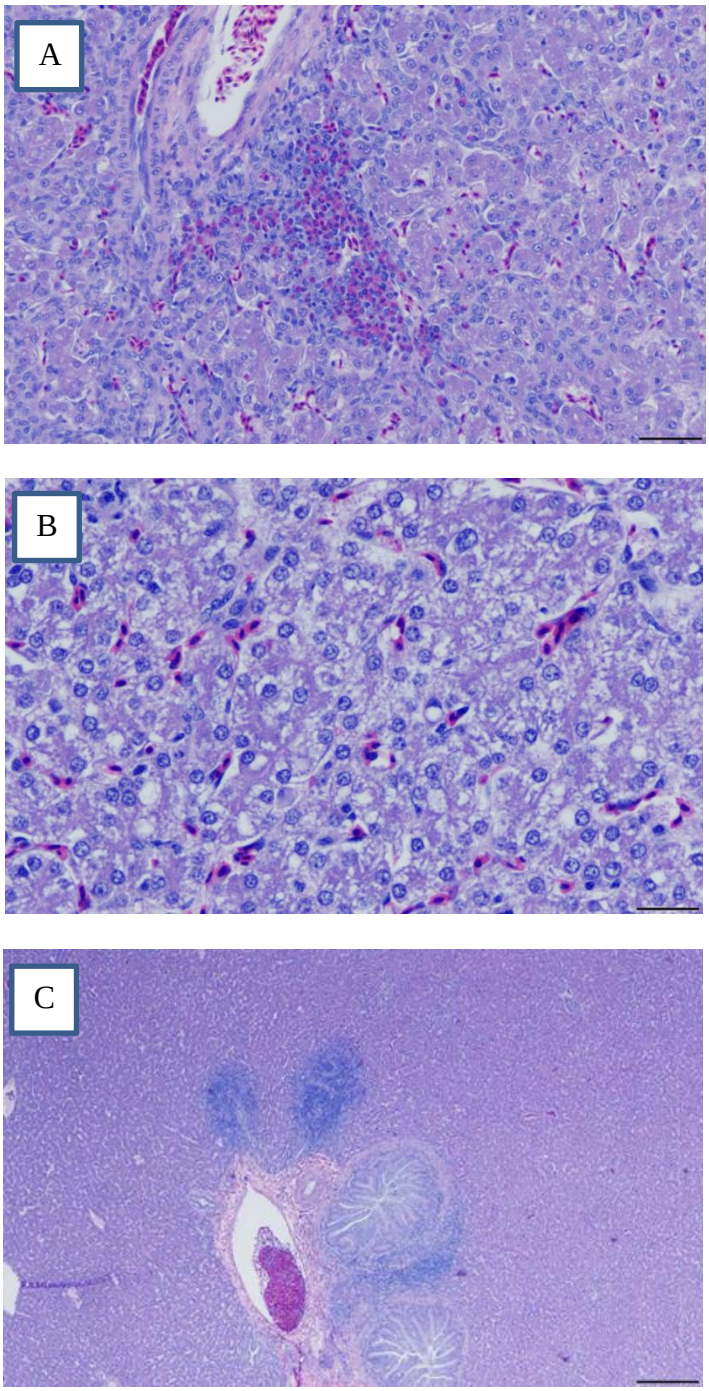


Figure 7 Histology of the liver tissue (H&E stains) in broiler chickens two weeks after single oral exposure to (A) deionized water - Control, (B) silver carbene complex 1 (SCC1) at 10 mg/kg BW - glycogen and lipid type vacuolation of hepatocytes 40x, (C) silver carbene complex 1 (SCC1) at 1000 mg/kg BW

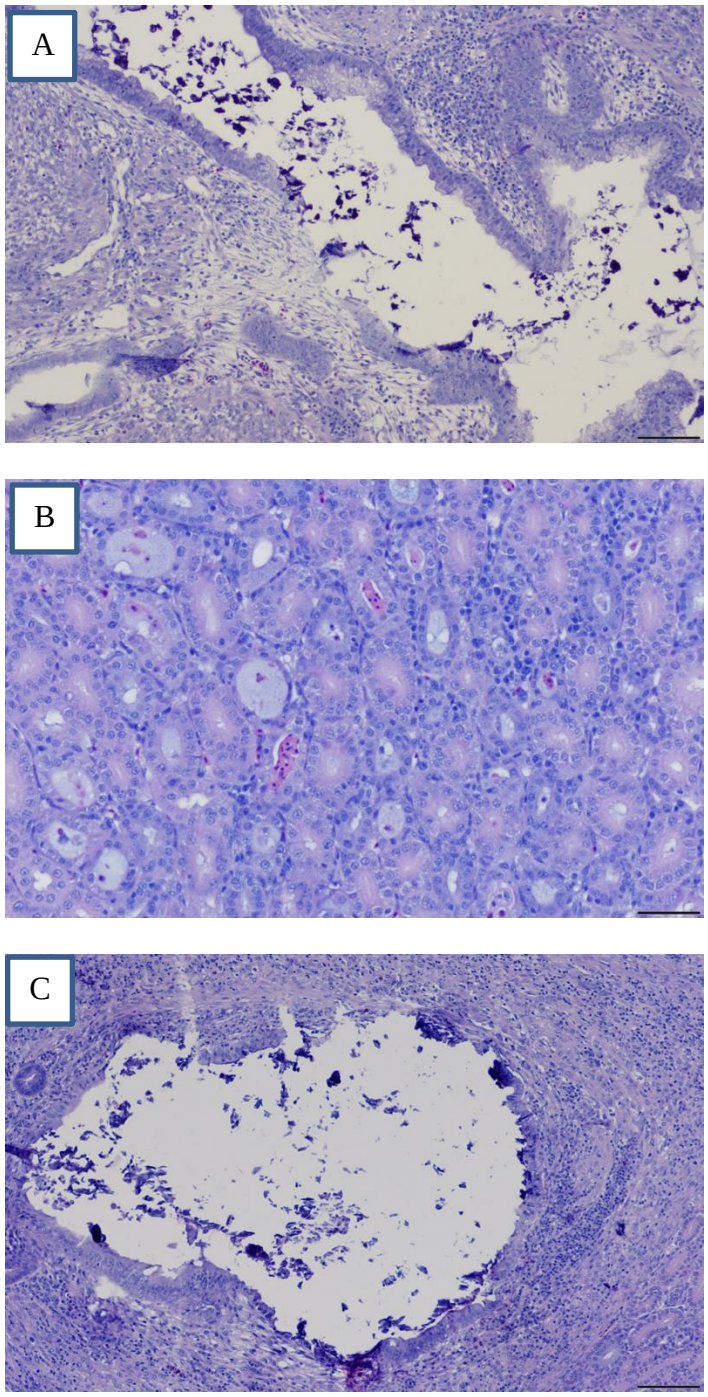


Figure 8 Histology of kidney (H&E stain) in broiler chickens two weeks after single oral exposure to deionized water (A)Control - urolith in major ureter branch 10X , (B) silver carbene complex 1 (SCC1) at 100 mg/kg BW - Renal tubular dilation and mild necrosis 20x, (D) silver acetate (AgAc) at 100 mg/kg BW - Urolith in major ureter branch

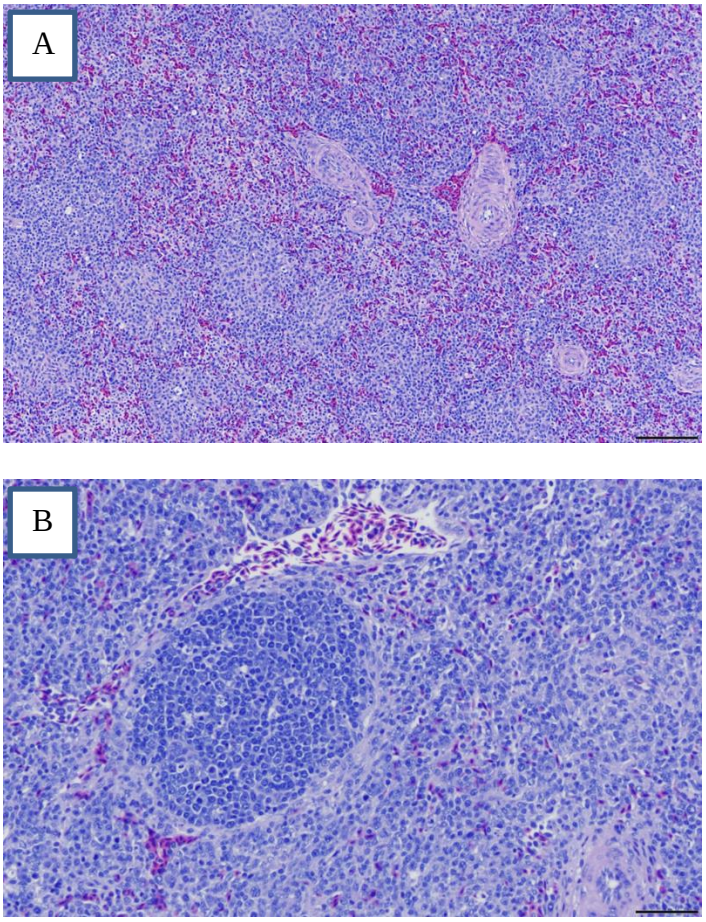


Figure 9 Histology of spleen (H&E stain) in broiler chickens two weeks after single oral exposure to (A) deionized water - Control 4X, (B) silver acetate (AgAc) at 1000 mg/kg BW - Lymphoid nodule in spleen 20x

CHAPTER IV

EVALUATION EFFECT OF SILVER ACETATE ON PERFORMANCE AND CLOSTRIDIUM PERFRINGENS-INDUCED NECROTIC ENTERITIS IN BROILER CHICKEN

Introduction

Necrotic enteritis (NE) in broiler chickens is a bacterial infection caused by the Gram-positive, spore forming; anaerobic *Clostridium perfringens* type A within 2-6 weeks of age (Cooper and Songer, 2010). CP is frequently found in the intestinal tract of healthy poultry, usually at levels lower than 10^2-10^4 CFU/g of intestine content verses 10^7 -10^9 CFU/g in diseased birds (Barnes et al., 1972; Craven et al., 2001). Both clinical and sub-clinical forms of NE are known to cause industry losses of approximately US$6 billion annually (Wade and Keyburn, 2015). One strategy followed by animal producers to reduce or control the enteric bacterial burden of CP and subsequently promotes the growth performance; diets have been fortified with antibiotics as a growth promotor (AGP) (Yegani and Korver, 2008). Banning the use of AGP in European Union and increased restrictions and discouraging the use of AGP in the United States has a major impact on gut health in intensively reared broiler chickens (Castanon, 2007; Timbermont et al., 2011).

Silver has been used as an antimicrobial agent in a variety of ways to control infections since ancient times (Russell and Hugo, 1994). Usually, silver has been used as ionic form (salts), mainly nitrate, sulphate or chloride. The antimicrobial activity of silver ions is characterized by a high effectiveness, low toxicity to host cells, and rare development of resistance (Silver, 2003). Formulations of silver nitrate have been therapeutically well-known since the 18th century (Klasen, 2000). However, many of these inorganic forms rapidly lose effectiveness since the

silver cations are released rapidly at the infection site (Napoli et. al., 2013). The extensive use of silver-based compounds attracted animal-antibiotic alternative researchers to evaluate silver in particulate form as a feed additive in animal production (poultry and swine) relying mainly on the broad antimicrobial activity of silver ion (Sawosz et al., 2007, 2009; Fondevila et al., 2009; Pineda et al., 2012; Vadalasetty et al., 2018). However, scarce literatures evaluated the potential effect of less adverse effect of silver salts on broiler performance, or potential *in vivo* efficacy through a course of a disease using chicken as a model animal.

As was shown in previous chapters, silver acetate (AgAc) under anaerobic conditions exhibited bacteriostatic effect against CP with minimal inhibitory concentration (MIC) being 8 μg/mL. In addition, subjecting *Clostridium perfringens* to 40 μg/mL of AgAc down regulated the expression of virulence genes of CP (plc and netB) by 47- and 36-fold respectively. In terms of its toxicity, using a single oral gavage, showed doses 10 and 100 mg/kg BW with less adverse effect compared to 1000 mg/kg BW. In poultry, maximum tolerable level (MTL) of silver determined by NRC (1980) is 100 mg/kg feed. The objective of this study was to evaluate the potential effect of AgAc (Sigma-Aldrich, 99.9% purity; molecular weight 166.8 g/mol) on broiler chicken performance, *Clostridium perfringens* colonization, and lesion score associated with NE, when delivered at levels up to the MTL.

Materials and Methods

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University (IACUC 2017–0072).

Birds and General Management

Two experiments were conducted at the same time in 2 separated rearing rooms at the USDA-ARS facility (College Station, TX). In Experiment 1, (Feed-added AgAc [F-AgAc]), a

total of 240 one-d-old straight run Cobb-500 broiler chickens was purchased from a commercial hatchery and placed in tow stainless steel battery brooder units (Alternative Design Manufacturing and Supply Inc.) Five birds by pen (2 sq. ft. per cage) were allocated inside environmentally controlled rearing rooms. The room was thermostatically controlled with a 24-hour lighting provided. Birds were fed a corn-soy broiler starter diet (Table 11) either as a control (unsupplemented) or supplemented with graded concentrations of AgAc from the day of hatch until termination of the experiment (d-21).

In Experiment 2, (Water-added AgAc [W-AgAc]), a total of 240 one-d-old straight Ross-300 broiler chickens were purchased from a commercial hatchery and placed into two Petersime battery brooders (5 birds per pen) inside an environmentally controlled rearing room. The room was thermostatically controlled with a 24-hour lighting provided. Each brooder pen contained supplemental heat as required. Birds were fed the control basal diet as in Experiment 1 from the day of hatch until termination of the experiment (d-21). In both experiments, feed and water were provided ad libitum. Birds were monitored daily with regard to general flock condition, temperature, lighting, water, feed, and any unanticipated events for the rearing facility.

Table 11 Feed composition of basal broiler starter diet used in both experiments

Ingredients	%
Yellow corn, grain	62.0
Soybean meal (CP, 48%)	32.0
Soybean oil	2.2
Limestone	1.4
Sodium chloride (salt)	0.50
Monocalcium phosphate	1.6
DL-methionine	0.23
L-Lysine·HCl	0.18
Vitamin premix*	0.25
Mineral Premix**	0.05
Calculated composition	
ME (kcal/kg)	3050
Crude protein	22
Crude fat	3.92
Calcium	0.95
Available phosphorus	0.45
Lysine	1.18
Methionine	0.53

* Provided the following per Kg of diet: vitamin A, 11 IU; vitamin D3, 3,850 IU; vitamin E, 45.8 IU; menadione, 1.5 mg; B12, 0.017 mg; biotin, 0.55 mg; thiamine, 2.93 mg; riboflavin, 5.96 mg; d-pantothenic acid, 20.17 mg; B6, 7.15 mg; niacin,45.8 mg; folic acid, 1.74 mg; choline, 130.3 mg. **Trace minerals premix added at this rate yields (mg/kg): zinc, 60.0; manganese, 60.0; iron, 60.0; copper,7.0;iodine,0.4.

Experimental Design

The *in vivo* efficacy of AgAc delivered either by adding into the feed (as a prophylactic model) or via drinking water (as treatment model) during necrotic enteritis challenge was evaluated. Birds in F-AgAc group were blocked based on initial body weight in 7 dietary treatment groups (6 replicates/5 birds each) as follows: Positive Control (PC) (no AgAc and no challenge and no IBD, n=30), Negative Control (NC) (no AgAc, challenged, and IBD immunized n=30), and 5 dietary AgAc treatments (n=30/treatment) fed 20, 40, 60, 80, 100

mg/kg feed AgAc for 21-d rearing period and challenged. These concentrations were chosen based on our *in vivo* toxicity preliminary study.

For the W-AgAc group, the same protocol was followed except the birds (Ross-308) received AgAc concentrations via drinking water for the last 4 days of the 21-d rearing period. Treated groups received AgAc fresh daily or as needed using a jack-waterer. Water consumption for the last 4 consecutive days was recorded to calculate amount of AgAc delivered. In both experiments, feed consumption and body weight **(BW**) per pen were recorded on d 10, 16 and 21 to calculate weight gain (**WG**) and feed to weight ratio (**FCR**). Mortality and body weight of dead birds was recorded daily and used to adjust FCR. On d-21, all birds were euthanized using CO_2, and necropsied to measure intestinal lesions associated with NE, and intestinal content from 12 birds per treatment were collected for Clostridium perfringens enumeration.

Clostridium Perfringens Challenge

Birds from both trials were administered a commercial infectious bursal disease vaccine (IBD, Bursa-Vac, Merck Animal Health, Summit, NJ) at 10X the recommended dose via the ocular route on day 10 in order to help induce NE by acting as immunosuppressive (McReynolds et al., 2004). Four field isolates of *Clostridium perfringens* type A from different geographical locations confirmed with NE infection (2 isolates from Texas and Virginia and 2 isolates from Georgia) were cultured separately on thioglycollate medium (Becton Dickinson) for 12 h then combined and provided to the appropriate treatment groups (McReynolds et al., 2004). Chicks were challenged via oral gavage (3 mL) with 10^7 CFU of CP/mL on days 16 and 17.

Clostridium Perfringens Enumeration

A section (~15 cm) of the small intestine cranial to Meckel's diverticulum was removed and placed in 10 mL of anaerobic thioglycollate (Becton Dickinson). Then, the sample was

stomached for 30 sec, and 0.5 mL of small intestine contents transferred into 4.5 mL of thioglycollate medium. Ten-fold serial dilutions were performed and plated on Shahidi Ferguson Perfringens (SFP) agar supplemented with 50% egg yolk enrichment, Antimicrobic Vial K (12 mg), and Antimicrobic Vial P (30,000 U) (Becton Dickinson). After the sample was plated, 10 to 12 mL SFP agar base without egg yolk enrichment was overlaid and the plates incubated anaerobically for 24 h at 37°C. Colonies exhibiting typical colony morphology were counted and recorded (McReynolds et al., 2007).

Lesion Score Associated with NE

To evaluate the development of intestinal gross lesions associated with NE, scoring was performed as described by Prescott et al. (1978). The intestine (duodenum, jejunum, and ileum) were examined and scored to evaluate gross lesions associated with NE. The scoring was performed by a licensed poultry veterinarian who was blinded to the treatment group. Lesions were scored on a scale of 0-4. A score of 0 = normal healthy tissue with no gross lesions, a score of 1= thin-walled or friable tissue with a grey appearance, a score of 2 = thin-walled, with focal necrosis, and grey in appearance with minor amounts of gas production, a score of 3 = thin walls with sizable patches of necrosis, gas filled intestine, and small flecks of blood, and a score of 4 = defined by severe extensive necrosis, marked hemorrhage, and large amounts of gas in the intestine.

Statistical Analysis

For performance and Log_{10} CFU *Clostridium perfringens*/gram bacterial recovery data were analyzed as One–Way ANOVA. Lesion scores were analyzed as nonparametric by Kruskal-Wallis test. Additionally, Feed-added AgAc and Water-added AgAc were compared to investigate any possible delivery effect on lesion scores and Log_{10} CFU/g bacterial recovery

associated with NE by Student t test ($P < 0.05$). Pairwise correlations were calculated for each delivery methods to investigate the relationship between the daily silver intake based on body weight and variables studied. Interpretation of the size and strength of the correlation was according to Hinkle et al. (2003). All analyses were conducted using the JMP Pro 12 (SAS, Inc., Cary, NC) and when appropriate means were compared using Tukey's HSD test at $P < 0.05$. Pen average was the experimental unit for all parameters studied.

Results and Discussion

Bird Performance

Performance results for F-AgAc group at days 1-10, 10-16 are presented in Table 12. Compared to the control, there were no differences ($P > 0.05$) in BW, WG, FCR, and mortality on days 10 and 16 when AgAc was fed at different dietary concentrations. On d 10, results showed low negative correlation (Table 16) between average daily silver intake (mg/kg BW) and BW ($r = -0.33$; $P = 0.07$), WG ($r = -0.33$; $P = 0.07$), and negligible negative correlations to FCR (r = -0.12; $P = 0.59$), and mortalities ($r = -0.05$; $P = 0.79$). Similarly, on d 16, there were low negative correlations between daily silver intake and BW ($r = -0.36$; $P = 0.05$), WG ($r = -0.30$; $P = 0.10$), and negligible negative correlations to FCR ($r = -0.01$; $P = 0.96$), and mortalities ($r = -0.08$; $P = 0.67$).

Table 12 Performance of broiler chicken at d 0-10 and d 10-16 fed different concentration of silver acetate (AgAc)[1]

	D 0-10				D 10-16				
Treatment	BW	WG	FCR	Mort	BW	WG	FCR	Mort	Mort *
PC	244	206	1.04	0	530	286	1.19	0	0
NC	253	215	1.02	6.6	547	295	1.19	4.2	10
AgAc20	262	225	1.05	10	562	300	1.21	5.5	13.3
AgAc40	255	218	1.02	0	550	295	1.20	3.3	3.3
AgAc60	259	221	1.05	10	553	294	1.21	0	10
AgAc80	251	213	1.04	3.3	538	286	1.20	0	3.3
AgAc100	244	207	1.03	6.6	528	284	1.20	3.3	10
SEM	6.5	6.5	0.01	4	12.1	7.8	0.01	3.2	5.4

[1]Each value reported is the mean of 6 replicates with 5 chicks per replicate (n=6)
[2]Body weight (BW), weight gain (WG), feed conversion ratio (FCR)
[*]Mortality from d 0-16

Performance results for F-AgAc group at days 16-21 are presented in Table 13. On d 16-21, the highest dietary silver concentration in F-AgAc group (100 mg/kg feed (9.1 mg AgAc/kg BW/day) did not affect the BW, WG, FCR, and mortalities when compared to control groups. Similar negative correlations were found on d 21 when compared to previous days except in WG which showed negligible positive correlation.

Table 13 Broiler chicken performance at d 16-21 after challenge with *Clostridium perfringens* and delivered different concentrations of silver acetate (AgAc) from d 0 in the feed[1]

Treatment*	n	Daily AgAc intake (mg) [3]	Daily AgAc mg/kg BW[4]	BW (g)[2]	WG (g)	FCR (g:g)	Mortality	Mortality*
PC	6	0	0	819	289	1.29	0	0
NC	6	0	0	823	275	1.31	9.7	16.6
AgAc 20	6	1.5	1.9	805	244	1.34	8.3	16.6
AgAc 40	6	3.1	3.7	844	294	1.30	6.6	10.0
AgAc 60	6	4.6	5.4	842	289	1.32	4.2	13.3
AgAc 80	6	6.0	7.2	842	304	1.31	10	13.3
AgAc 100	6	7.2	9.1	791	262	1.31	0	10.0
PSEM				18.8	14.8	0.02	5.2	7.7

[1]Each value reported is the mean of 6 replicates with 5 chicks per replicate (n=6)
[2]Body weight (BW), weight gain (WG), feed conversion ratio (FCR)
[3] Based on total feed consumption for 21 days.
[4] Average daily AgAc intake based on feed consumption for 21 d/average kg body weight, adjusted to mortalities.
* Positive control (No challenge and no AgAc), Negative control (Challenge and no AgAc), and 6 dietary treatments challenged and fed 20, 40, 60, 80, 100 mg AgAc/kg feed for 21 d rearing period.

AgAc delivered for 4 days (18-21) in drinking water up to 100 mg/L water (20.9 mg AgAc/kg BW/day) did not affect ($P > 0.05$) the productive parameters BW, WG, FCR, and mortalities compared to control groups (Table 14). Results showed negligible positive correlations between daily silver intake and BW ($r = 0.12$; $P = 0.51$), WG ($r = 0.14$; $P = 0.46$), and negligible negative correlations to FCR ($r = -0.20$; $P = 0.28$), and mortalities ($r = -0.15$; $P = 0.41$).

Table 14 Broiler chicken performance at day 16-21 after challenge with *Clostridium perfringens* and delivered different concentrations of silver acetate (AgAc) via drinking water for the last 4 days of a 21-day rearing period[1]

Treatment[*]	n	DailyAgAc intake (mg)[3]	Daily AgAc mg/kg BW[4]	BW (g)[2]	WG (g)	FCR(g:g)	Mortality
PC	6	0	0	762	273	1.34	0
NC	6	0	0	750	251	1.31	6.6
AgAc 20	6	3.5	4.8	722	214	1.39	16.6
AgAc 40	6	7.3	9.2	797	284	1.36	10.
AgAc 60	6	11.3	13.8	819	308	1.29	10
AgAc 80	6	13.3	18.0	743	249	1.29	13
AgAc 100	6	16.0	20.9	766	252	1.36	6.6
PSEM				24	24	0.04	5

[1]Each value reported is the mean of 6 replicates with 5 chicks per replicate (n=6)
[2]Body weight (BW), weight gain (WG), feed conversion ratio (FCR)
[3] Based on total water consumption for the last 4 consecutive days.
[4]Average daily AgAc intake for 4 days/average kg body weight, adjusted to mortalities.
[*]PC=Positive control (No challenge no AgAc, and no IBD immunization), NC=Negative control (Challenge no AgAc, and IBD immunization), and 5 treatment groups challenged and delivered 20, 40, 60, 80, 100 mg AgAc/L drinking water for the last 4 days of a 21-day rearing period

The very well toxicity profile for ionic silver has studied decades ago in broiler chickens in many studies in which ionic silver supplemented by either practical or purified diets and drinking water. These studies collectively showed that silver from silver acetate or silver nitrate is toxic at high concentrations (900 mg/kg practical diet in poult and chickens [Jensen et al., 1974; Peterson and Jensen 1975], 1500 mg/kg drinking water fed purified diet [Bunyan et al., 1968]). However, scarce literatures documented the potential effect of ionic silver on broiler

chicken's performance, and on controlling an experimental infection using less adverse concentrations. This makes comparing current result relatively hard.

The performance results of current study collectively indicate whether AgAc included in the feed (from d 1 to 21) or drinking water (4 d) at the concentrations evaluated in this experiment had no negative effect on broiler chicken performance. A practical diet containing graded concentrations 10, 25, 50 and 100 mg/kg silver sulfate for 3 weeks did not affect growth performance and mortality of broiler chickens (Hill et al., 1964). Early silver studies in growing turkeys showed that adding 100 ppm (about 110 mg/kg BW/day) of silver nitrate or silver acetate (4 weeks) to the practical diet did not affect bird performance (Jensen et al., 1974). The results are also consistent with other studies using different forms of silver. Felehgari et al. (2013) showed that silver nanoparticles fed to broiler chickens at 25 and 50 ppm for 21 d did not affect the bird performance parameters BW, WG, and FCR. Similarly, Pineda et al. (2012) reported that providing silver nanoparticles to broiler chickens at 10 and 20 mg/kg in drinking water from d 7 to 36 did not affect bird performance BW, FCR and mortalities.

It was reported the mechanism by which silver can induce adverse (toxic) effect on poultry (mainly reduce BW) is through interfering with metabolism and function of trace minerals such as copper (Cu) and selenium (Se) (NRC, 1980). Hill et al. (1964) reported that 50 and 100 mg/kg of silver increased mortalities and reduced growth rate of chicks fed for 3 weeks a copper purified diet, and supplementation 10 and 25 mg/kg copper prevented the adverse effects of silver. The premix of our diet provided 7 mg/kg copper which could marginally prevented the potential adverse effect of silver as showed with low negative correlation between average daily silver intake and BW and WG. Following oral exposure to both ionic and nanoparticulate silver suspensions, silver has been reported to be deposited as particles in tissues

such as the epidermis, the glomeruli and the intestines. These particles, in the rat intestines, were found to contain sulphur and selenium in addition to silver (Loeschner et al., 2011). This led to the conclusion that Se and other mineral such as Cu are antagonistic to silver ion preventing the bioavailability. Similarly, the calculated Se in our corn-soy diet provided 0.15 mg/kg Se which could play a role to reduce toxic effects of silver ions by reducing their biological availability. In addition, it has been shown that the efficacy of silver can be affected by the interaction of the ions with chloride, which results in the formation silver chloride (AgCl) precipitate (Silver, 2003). It is possible readily released silver ions from AgAc interacted with biologically-relevant compounds (such as organic material or chloride) in the drinking water, preventing their bioavailability.

Beside no adverse effect, no positive effect of adding silver into the feed or water was notice. The metabolic interactions of silver with different essential metals which eventually lead to toxic effect and diminish silver bioactivity is contradicted (at least for this form of silver) to the hypothesis says: as silver ion possesses antimicrobial activity, silver could potentially modify the microbiota profile of gastrointestinal tract (Hadrup et al., 2012), and subsequently could promote the bird performance resembling the antibiotic growth promoter action (Sawosz et al., 2007; Fondevila et al., 2009). Based on this hypothesis, silver availability in the GIT is a key factor to achieve this goal. Sawosz et al. (2007) reported no major effect of colloidal silver on bacterial population in the digestive tract of quails, however, only a significant increase in lactic acid bacteria was observed with 25 mg/kg in drinking water. No effect of silver in particulate form on microbial population including *Clostridium perfringens* when broiler chicken supplemented 10 and 20 mg/kg drinking water for 22 days with no positive effect on bird performance BW and FCR (Pineda et al., 2012). Vadalasetty et al. (2018) reported that the

application of particulate silver via drinking water in the concentration of 50 mg/kg had no antibacterial effect on different intestinal bacterial groups including *Clostridium perfringens*, but this concentration led to decrease the BW. Hadrup et al. (2012) showed neither silver nanoparticle (9 mg /kg BW/day) nor silver acetate (14 mg/kg BW/day) affected the balance between the two main phyla of gastrointestinal tract bacteria in GIT of rates, *Firmicutes* and *Bacteriodetes*. The author suggested that silver nanoparticles and silver acetate in the applied doses did not disturb the microbiological balance of the gastrointestinal environment at the phyla level.

Clostridium perfringens Enumeration and Lesion Score

Following CP challenge at d 16 and 17, CP enumeration and lesion score were performed on d 21. In both F-AgAc and W-AgAc groups, all challenged treatments showed higher ($P < 0.05$) CP enumeration than in unchallenged group which confirmed that the chickens were successfully colonized by the bacteria (Figures 10 and 11). Mortalities were only observed among the challenged treatments, except for highest dietary AgAc concentration (100 mg/kg feed). However, BW, WG, and FCR were not affected ($P > 0.05$). Although we used 4 different isolates of CP type A from confirmed NE outbreaks which screened previously for *plc* gene and were positive (Swaggerty et al., 2016), and further screened in this study for presence of *netB* gene the model did not sustain the expected negative effects on production parameters. Similar results found by (Fasina and Lillehoj, 2018; Fasina et al., 2016) who reported no effect of the challenge on BW, WG, and FCR, however, the challenge groups had 1.5-2-fold higher CP enumeration compared to control group. The author suggested that the mortalities with gross high lesion scores could be an indicator for establishment the infection.

Whether AgAc was added into the feed (for 21 d) or in the water (4 d), there were no differences ($P > 0.05$) seen in *Clostridium perfringens* enumeration compared to challenged treatment (Figures 11 and 12). Pairwise correlation results showed negligible negative correlation between daily silver intake (mg/kg BW) and CP enumeration in both groups, F-AgAc ($r = -0.20$; $P = 0.13$) and W-AgAc ($r = -0.23$; $P = 0.10$).

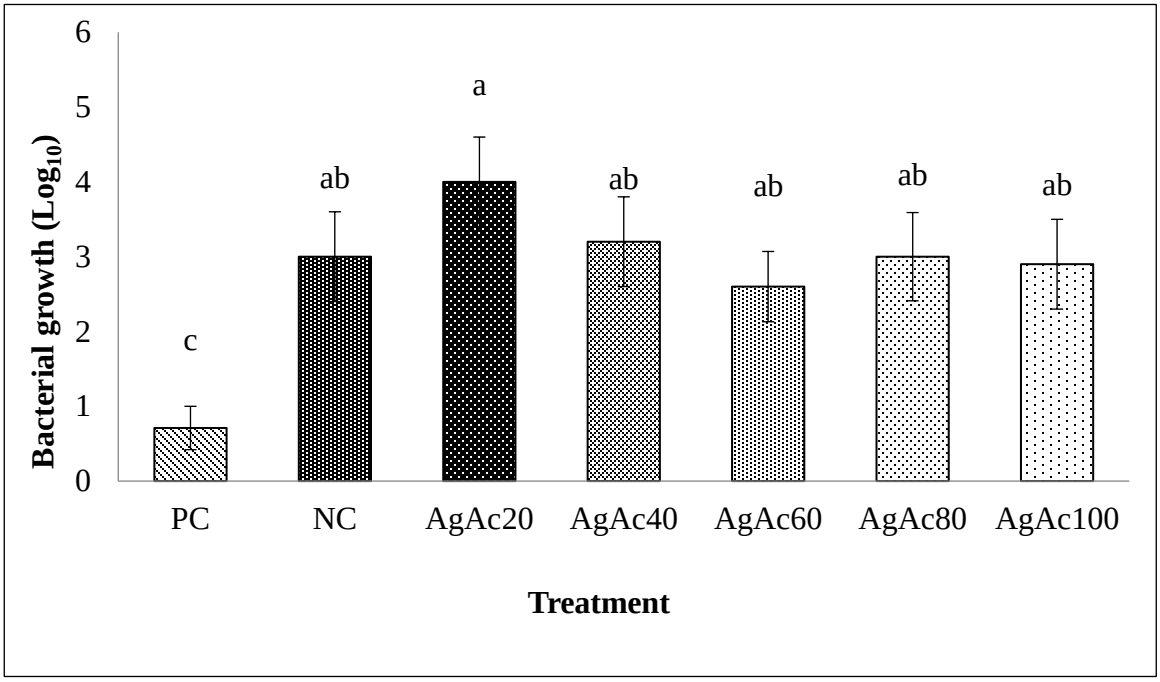


Figure 10 Enumeration of *Clostridium perfringens* of necrotic enteritis experimentally induced in Cobb-500 broiler chickens fed different concentrations of silver acetate (AgAc) for a 21-day rearing period

Birds were blocked in 7 dietary treatment groups (6 replicates/5 birds each) as follows: PC=Positive Control (no challenge no AgAc, and no IBD immunization), NC=Negative Control (challenged only and IBD immunization), and 5 dietary treatments challenged, IBD immunized, and fed 20, 40, 60, 80, and 100 mg/kg feed AgAc for 21 d rearing period. Mean±SEM, mean values of bacterial enumeration= the average $\log_{10}$ CFU *Clostridium perfringens*/gram medileum content recovered from average 2 birds/pen (n=6)

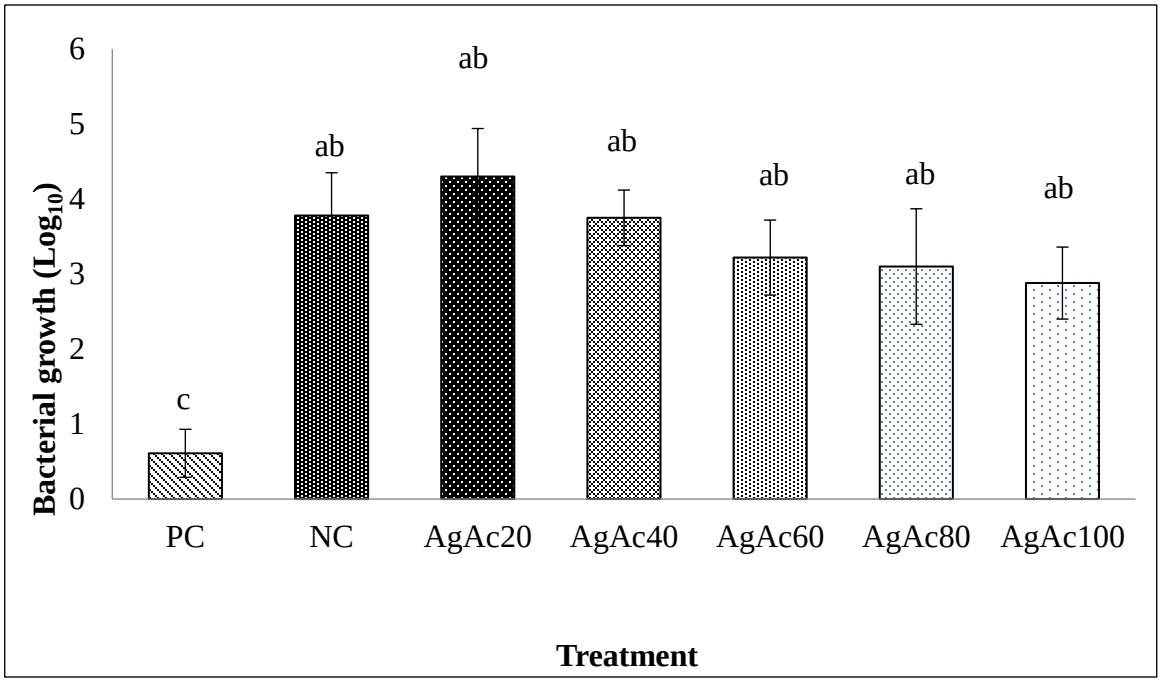


Figure 11 Enumeration of *Clostridium perfringens* of necrotic enteritis experimentally induced in Ross 308 broiler chickens delivered silver acetate (AgAc) via drinking water for the last 4 days of a 21-day rearing period

Birds were blocked in 7 treatment groups (6 replicates/5 birds each) as follows: PC=Positive Control (no challenge no AgAc, and no IBD immunization), NC=Negative Control (challenged only and IBD immunization), and 5 dietary treatments challenged, IBD immunized, and delivered 20, 40, 60, 80, 100 mg/kg water AgAc for the last 4 days of a 21-day rearing period. Mean±SEM, mean values of bacterial enumeration= the average log_{10} CFU *Clostridium perfringens*/gram med-ileum content recovered from average 2 birds/pen (n=6)

Similarly, whether AgAc was added into the feed or in the water, there were no differences ($P > 0.05$) seen in lesion scores compared to challenged treatment (Table 15). In F-AgAc group, pairwise correlation results showed negligible positive correlation between daily silver intake (mg/kg BW) and lesion score ($r = 0.14$; $P = 0.14$), and negligible negative correlation ($r = 0.04$; $P = 0.65$) in W-AgAc group.

Table 15 Lesion scores for broiler chickens challenged with *Clostridium perfringens* and delivered silver acetate (AgAc) in feed and water

	AgAc mg/kg***							
Method[2]	PC*	NC**	20	40	60	80	100	PSEM
F-AgAc	0.63	0.79	0.91	0.83	0.86	0.68	1.1	0.10
W-AgAc	0.30	0.40	0.24	0.49	0.18	0.15	0.39	0.09

*Positive control (No challenge and no AgAc, and no IBD immunization), **Negative control (Challenge no AgAc, and IBD immunization), *** 5 dietary treatments of silver acetate at 20, 40, 60, 80, and 100 mg/kg feed

[1]The intestine (duodenum, jejunum, and ileum) were examined and scored to evaluate gross lesions associated with NE. To eliminate bias, a licensed poultry veterinarian blindly scored all tissues for lesions. Lesions were scored on a scale of 0-4. A score of 0 = normal healthy tissue with no gross lesions, a score of 1= thin-walled or friable tissue with a grey appearance, a score of 2 = thin-walled, with focal necrosis, and grey in appearance with minor amounts of gas production, a score of 3 = thin walls with sizable patches of necrosis, gas filled intestine, and small flecks of blood, and a score of 4 = defined by severe extensive necrosis, marked hemorrhage, and large amounts of gas in the intestine (Prescott et al., 1978).

[2] lesion score in Feed-added AgAc (F-AgAc) and Water-added AgAc (W-AgAc)

In F-AgAc group, lesion score of 2 (thin-walled, with focal necrosis, and grey in appearance with minor amounts of gas production), was observed in all dietary silver treatments with minor incidence percentages (2/21 (9.5%) in concentration 20, 1/22 (4.5%) in concentration 40, 2/23 (8.7%) in concentration 60, 1/21 (4.8%) in concentration 80, 2/24 (8.3%) in concentration 100. In addition, 2/24 (8.3%) in concertation 100 showed lesion score of 3. No

such score lesion was observed in W-AgAc group. The intestinal lesions of the W-AgAc group had lower ($P < 0.05$) lesion scores compared with the F-AgAc group at all concentrations tested including positive and negative controls when subjected to Student *t* test (data not showed). Positive controls (unchallenged) in F-AgAc and W-AgAc showed lesion scoring of 0.64, and 0.30, respectively. However, these lesion scores in both positive controls were not different from their corresponding challenged groups ($P > 0.05$).

Table 16 Pairwise correlations between daily silver intake (mg/kg BW) and studied responses

	r	***P* value**
F-AgAc		
Day 0-10		
Body weight	-0.33	0.07
Weight gain	-0.33	0.07
FCR	-0.10	0.59
Mortalities	-0.05	0.79
Day 10-16		
Body weight	-0.36	0.05
Weight gain	-0.30	0.10
FCR	-0.01	0.96
Mortalities	-0.08	0.67
Day 16-21		
Body weight	-0.06	0.74
Weight gain	0.21	0.26
FCR	-0.21	0.25
Mortalities	-0.08	0.66
CP enumeration	-0.20	0.13
Lesion score	0.14	0.14
W-AgAc		
Day 16-21		
Body weight	0.12	0.51
Weight gain	0.14	0.46
Feed conversion ratio	-0.20	0.28
Mortalities	-0.15	0.41
CP enumeration	-0.23	0.10
Lesion score	-0.04	0.65

Controlling pathogen infections in the gastrointestinal tract of animals using silver-based compounds, in particular silver salt forms, are scarcely reported. Although, *in vitro* antibacterial efficacy of AgAc against *Clostridium perfringens* type A was a bacteriostatic effect with MIC being 8 µg/mL, it seems that *in vivo* efficacy of AgAc, to reduce bacterial colonization in the intestine, was limited. Similar results found by Vadalasetty et al. (2018) who reported that the application of particulate silver via drinking water in the concentration of 50 mg/kg had no antibacterial effect on different intestinal bacterial groups including *Clostridium perfringens* and had no effect on colonization of *Campylobacter jejuni* experimentally infected in broiler chickens although the *in vitro* results showed bactericidal at the same concentration used *in vivo*.

Regardless the lesion scores observed in the PC of both experiments, which could explain the ubiquitous of *Clostridium perfringens* in the environment, AgAc in both delivery methods did not decrease or increase the lesion scores compared to lesion scores of challenged groups. Although there were no significant differences in *Clostridium perfringens* enumeration in both delivery methods groups, the intestinal lesions of the W-AgAc group had lower ($P < 0.05$) lesion scores compared with the F-AgAc group at all concentrations tested including positive and negative controls.

The lesion score of 2 and 3 noticed in small number of birds in F-AgAc group increased the overall average of lesion score compared to W-AgAc. This could indicate (although low number of chickens showed these lesion) that silver could exert its toxicity when supplemented for prolong period at low levels which limiting the use of silver as an antibacterial agent in animal production (Sawosz et al., 2007). However, as the positive and negative controls showed difference in lesion score, the difference seen in lesion score may be due to an effect of strain, rather than an effect of silver administration. It was reported different immunological pathways

are involved in genetic determinants outside the chicken B complex which have a significant impact on birds being either resistant or susceptible to NE (Kim et al., 2014). Therefore, it was strongly suggested that different broiler strains have been shown to have different resistance to pathogens such as *Clostridium perfringens* (Swaggerty et al., 2016).

It was proposed that that the understanding of the potential behaviors of silver ions (whether it dissociated from salts or particulate silver) is not comprehensive in terms of their chemical and biochemical behavior in a biological system (Behra, et al., 2013). The *in vivo* antimicrobial efficacy of silver ion seems to be hindered by its interactions which probably explain the clearer effect on metabolic processes. The results of current study suggest limitation effect of silver acetate on performance; reduce *Clostridium perfringens* colonization and lesion score in broiler chickens.

CHAPTER V

COMPARISON STUDY OF SILVER ACETATE TO PROBIOTIC, PREBIOTIC, AND ANTIBIOTIC IN FULL TERM BROILER PERFORMANCE

Introduction

The use of antibiotics in livestock production is one of the potential contributors to the emergence of antibiotic resistant bacteria. In an effort to improve production efficiency, diets have been fortified with antibiotics, often at sub-therapeutic doses. Long periods of exposure to these sub-therapeutic doses have induced bacterial populations to acquire genetic mutations or plasmids that allow them to survive in the presence of the antibiotics. Recognition of the contribution of antibiotics in feed to the emergence of antimicrobial resistance has led to a 2006 ban on the use of antibiotic growth promoters (AGPs) in the European Union and increased restrictions on the use of AGPs in the United States. As of January 1, 2017, a new regulation of the Food and Drug Administration (FDA) restricts an animal producer from using antibiotics as growth promoters if they are deemed a medically-important antimicrobial with respect to human medicine. Banning AGPs in European was almost immediately followed by health problems in broiler chickens, and an outbreak of *Clostridium perfringens* (CP) infections was seen (Castanon, 2007). Banning antibiotics from animal feed in the United States will likely cause the same challenge, which has promoted research into alternatives to AGP (Niewold, 2006) such as probiotics (Gardiner et al., 2004), organic acids (Partanen and Mroz, 1999), herbs and their extracts (Burt, 2004).

Recently, it was hypothesized that particulate silver could be employed as an alternative to antibiotics in animal production (Sawosz et al., 2007, 2009; Fondevila et al., 2009; Pineda et

al., 2012; Ahmadi and Kurdestani, 2010, Ahmadi, 2012). It was hypothesized that as silver ion possesses antimicrobial activity, silver could potentially modify the microbiota profile of gastrointestinal tract, increase nutrient utilization, and subsequently could promote bird performance, resembling an antibiotic growth promoter action (Saleh and El-Magd, 2018; Hadrup et al., 2012; Sawosz et al., 2007; Fondevila et al., 2009; Pienda et al. 2012). In most of these studies, and in the results from chapter 4, *in vivo* efficacy of adding silver did not show significant alteration in the GIT population (CP in our study). The effect of silver on nutrient utilization is scarcely reported on energy and amino acids digestibility. Similarly, scarce reports exist regarding potential effects of dietary silver on bone mineralization in broiler chickens (Sikorska et al., 2010), although there is evidence that silver can distributed to both soft tissues and bone (Lansdown, 2010 a).

On the other hand, silver could exert toxicity, even at small doses, limiting the use of silver as an antibacterial agent in animal production (Sawosz et al., 2007). The mechanism by which silver can induce adverse (toxic) effects on poultry (mainly reduced BW) is through interfering with metabolism and function of trace minerals such as copper (Cu) and selenium (Se) (NRC, 2005), which are components in the antioxidant system. From *in vivo* and *in vitro* studies, there is a general agreement that the mitochondria are the main sensitive target of silver ions after absorption from the cell membrane (McShan et al., 2014; Stensberg, et al, 2011). After uptake of silver ion in the mitochondria, it can stimulate the production of reactive oxygen species (ROS), because of disruption of the influx of ions and electrons across the mitochondrial membrane. In addition, silver can bind and modulate the glutathione (GSH) function as a non-enzymatic antioxidant by oligodynamic effects which lead to increase ROS production and increased susceptibility to cellular damage.

Silver-induced oxidative stress damage in broiler intestines (jejunum), breast muscles, liver, and plasma has been reported by some researchers using particulate silver (Song et al., 2017; Ognik et al., 2016 a, 2017; Kulak et al., 2018 ab; Ahmadi, 2012). Song et al. (2017) reported that the intestinal and plasma oxidative stress resulted in reduction in body weight and feed intake with no effect on FCR, and dietary treatment with Zn, vitamin E as an antioxidant, or their combination at different inclusion rates failed to alleviate the negative effect of dietary particulate silver (at 1000 mg/L drinking water for 42 days) on the body weight and feed intake of broiler chickens. This results in agreement (in terms of reduction BW) with Paterson et al. (1974) when fed broiler chickens silver acetate or nitrate for 4 weeks at 900 mg/kg feed.

While Kulak et al. (2018) and Ognik et al (2016 ab) demonstrated that oral administration of particulate silver to chickens effects the morphology and functioning of the gastrointestinal tract, as well as parameters of immune and redox status accompanied with intestinal wall accumulation of silver, regardless the size and doses used which were below the MTL. In rodent toxicity studies, it has been reported that oral ingestion of particulate silver at 10 mg/kg for 28 days could lead to destruction of intestinal microvilli, reduce absorption capacity of nutrients and subsequently reduce growth performance (Shahera and Young, 2013). Other pathological changes reported in a rodent study by Jeong et al. (2010) were increased numbers of goblet cells in the intestine that had released an abnormal composition of mucus granules following the oral administration of 30 mg/kg of BW/day of nanoparticles for 28 days. Both, oxidative stress and mechanical injury events could lead to impairment of the intestinal epithelium nutrient absorption function.

Silver is absorbed into the body in its ionized form (Ag^{+}) and it readily binds to intracellular proteins, especially serum albumins and macro-globulins for metabolism and

distribution to different soft tissues and bone (Lansdown, 2010 a). Nano-Ag (50 ppm) was deposited in embryo thigh bones, but did not affect the structure, bone mineral content, or mechanical properties of the bone (Sikorska et al., 2010). Considering silvers role in bone mineralization and the relatively high metabolic rate of modern broiler chickens, bone toxicity of administration silver-based products as an antibiotic alternative is important to study.

Silver acetate has been shown *in vitro* to have positive antimicrobial effects against a wide range bacterial species *Clostridium perfringens* type A, *Salmonella* spp., and *E. coli* isolated from both poultry and swine (chapter 2). However, results from chapter 4 did not show positive or negative effects on performance, and did not reduce necrotic enteritis which agreed with many previous studies.

As a part of evaluating the ability of silver to serve as a replacement of antibiotics, this study was designed to investigate the effects of dietary silver acetate and compare it to a well-known antibiotic growth promoter, and alternative probiotic and prebiotic products using growth performance, ileal digestibility of energy and amino acids, histological changes, bone mineralization, and hepatic glutathione characteristics as variables in a six week grow-out trial.

Materials and Methods

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Birds and General Management

A total of 952 male day-old broiler chicks (Cobb 500) were procured from a commercial hatchery and transported to the Texas A&M Poultry Research Center. Birds were weighed on d 0 and randomly distributed among 56 floor pens (17 birds per pen with 8 replicates per treatment) with recycled litter of pine shavings in a complete randomized block design. Each pen measured

approximately 6’ x 6’ and was equipped with hanging feeders and nipple drinkers. Feed and water were provided *ad libitum*. Birds were observed daily with regard to general flock condition, temperature, water, feed, and unanticipated events for the house, and mortality for each pen. A continuous lighting program was provided for the entire experimental period. Temperature was thermostatically controlled at 33°C during the first 3 d, and then weekly reduced from 2°C to 3°C until reaching 24 - 26°C. All chickens were received the standard vaccinations by the hatchery.

Dietary Treatments

Bird were fed a commercial corn-soybean basal diet in 3-phase feeding program starter consisting of starter (d 0-d 21), grower (d 21-d 35), and finisher (d 35-d 42) phases (Table 17). In each phase, dietary treatments consisted of a control basal diet (Control) without antibiotic or alternative supplementation, basal diet supplemented with 50 ppm antibiotic (bacitracin methylene disalicylate, BMD50), basal diet supplemented with 250 ppm *Saccharomyces cerevisiae* yeast cell wall (YCW) prebiotic (Safmannan, SAF250), basal diet supplemented with 500 g/ton $2x10^6$ of a single bacterial strain of *Bacillus* spp. probiotic (Envera Go plus, Go+), and basal diets supplemented with 10, 50, and 250 ppm silver acetate (AgAc10, AgAc50, and AgAc250, respectively).

Table 17 Feed Composition and calculated nutrients of basal diet

Ingredient (%)	Starter (d 0-d 21)	Grower (d 21-d 35)	Finisher (d 35- d 42)
Yellow corn	60.7	62.3	73.0
Soybean meal (48% CP)	31.9	31.2	21.5
DL-Methionine	0.23	0.23	0.13
Lysine-HCL	0.18	0.06	0.18
Soybean oil	2.06	2.75	1.97
Limestone	1.44	1.41	1.46
Monocalcium diphosphate	1.55	1.32	1.15
Sodium chloride (salt)	0.51	0.36	0.30
Vitamin Premix [1]	0.25	0.25	0.25
Mineral Premix [2]	0.05	0.05	0.05
Calculated nutrient content			
Crud protein (%)	22.00	20.50	18.3
ME (kcal/kg)	3050	3100	3150
Methionine (%)	0.55	0.55	0.55
Methionine+Cystine (%)	0.92	0.91	0.90
Lysine (%)	1.30	1.19	1.19
Crude fiber (%)	2.14	2.13	2.13
Calcium (%)	0.95	0.9	0.85
Available Phosphorus (%)	0.45	0.40	0.35
Sodium (%)	0.22	0.16	0.16
Chloride (%)	0.36	0.16	0.16
Potassium (%)	0.92	0.91	0.90
Titanium dioxide (%)	0.20	-	0.20

[1]Vitamin premix added at this rate yields per kg of diet: 11,023 IU vitamin A, 46 IU vitamin E, 3,858 IU vitamin D_3, 1.47 mg menadione, 2.94 mg thiamine, 5.85 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 7.17 mg pyridoxine, 0.55 mg biotin, 1.75 mg folic acid, 0.017 mg vitamin B_{12}, 130.6 mg choline. [2]Mineral premix added at this rate yield per kg of feed: 7 mg copper, 0.4 mg iodine, 60 mg iron, 60 mg manganese, 60 mg zinc.

Bird Performance

Feed consumption and body weight (**BW**) per pen were recorded on d 21, 35 and 42 to calculate weight gain (**WG**) and feed conversion ratio (**FCR**). Mortality and body weight of dead birds was recorded daily and used to adjust FCR. Broiler productivity index (**PI**) was calculated with the following equation:

$$PI = \text{Livability } (\%) \times \frac{BW(kg)/Age(days)}{FCR}) * 100$$

Energy Digestibility Determination

Birds were offered *ad libitum* the diets containing the titanium dioxide for 3 consecutive days (19, 20, and 21 and 40, 41 and 42) of the experiment as an inert indigestible marker. On d 21, and 42, 2 birds per pen (total 8 pooled samples) were euthanized using CO_2 and the contents of the ileum were collected and pooled per pen for the determination of apparent ileal digestible energy and further for apparent amino acid digestibility. The ileum was defined as that portion of the small intestine extending 4 cm from the vitelline diverticulum to a point 4 cm proximal to the ileo-caecal junction. The digesta were frozen (-20°C) immediately after collection and subsequently freeze-dried. The dried ileal digesta samples were ground using a coffee grinder (Mr. Coffee, Sunbeam Products Inc., Boca Raton, FL) and stored until chemical analyses. Feed and ileal digesta samples were analyzed following Short et al. (1996) procedure to determine the concentration of titanium dioxide. Briefly, using porcelain crucibles 0.3 g of dried ileal or feed samples were ashed for 13 h and then titrated with 10 mL sulfuric acid (7.4 M). Samples were gently boiled until completely dissolved and poured into clean beakers containing 25 mL distillated water. Beaker contents were subsequently poured in 100 mL volumetric flasks and titrated with 20 mL hydrogen peroxide (30%) and diluted to 100 mL using deionized distillated water. Samples were analyzed using a Spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Waltham, MA) at 410 nm. Gross energy of feed and ileal digesta samples were determined using a bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL). Apparent ileal digestible energy (**AID)** was calculated with the following equation:

$$AID = \text{Gross energy of feed} - (\text{Gross energy of ileal digesta} \times \frac{[TiO_2] \text{ feed}}{[TiO_2] \text{ ileal digesta}})$$

Where TiO_2 feed and TiO_2 ileal digesta = concentration of TiO_2 in the diet and digesta samples (g/kg).

Amino Acids Digestibility Determination

On day 21 and 42, samples of digesta and feed were hydrolyzed for 24 h with 6 N hydrochloric acid (HCl) at 110 °C for determination of amino acids concentration using Alliance HPLC System e2695 model (Waters Corporation, Milford, MA). The chromatography was performed on a Supleco C_{18} column (15 cm × 4.6 mm, 3 μm, Sigma) at room temperature and detected by Waters 2475 fluorescent detector excitation at 340 nm and emission at 450 nm. Amino acids standard (Sigma) was used, and Empower 3 advanced software (Waters) was used to control the system operation and results management. Amino acid digestibility coefficients (**AADC**) were calculated according to the following equation:

$$\text{AADC \%} = 100 - \left(\left(\frac{[TiO_2]\ \text{feed} \times \text{AA ileal digesta}}{[TiO_2]\ \text{ileal digesta} \times \text{AA feed}}\right) \times 100\right)$$

Where, AA ileal digesta and AA feed = concentration of amino acid in the ileal digesta and feed samples (g/kg) as fed, TiO_2 feed and TiO_2 ileal digesta = concentration of TiO_2 in the diet and digesta samples (g/kg).

Histology of Small Intestine

On d 21 and d 42, 8 birds/treatment were randomly selected and small intestine was harvested for histological examination. Portions of small intestines (duodenum distal loop), mid-jejunum from the end of pancreatic loop to Meckel's diverticulum, and mid-ileum from Meckel's diverticulum to the ileo-cecum junction) were fixed with 10% formalin solution and were prepared using standard paraffin embedding procedures by sectioning at 5 μm thickness, and staining with hematoxylin and eosin.

Bone Mineralization Analysis

On d 42 of the experiment, eight birds per treatments were randomly selected and euthanized using CO_2. Total bone mineral content **(BMC)** and bone mineral density **(BMD)** were obtained using a Dual X-ray absorptiometry **(DXA)** scan (GE Lunar Prodigy, Boston, MA) located at the Applied Exercise Science Laboratory of Texas A&M University. Bone mineral content is defined as the total bone mineral found in a specific area measured in grams. Bone mineral density is derived using BMC (g) divided by an area (cm^2) of interest. Chickens were placed in prone position with their wings and legs at the sides of the body throughout the scan. Data were analyzed using the small animal <20 kg software (GE Lunar Prodigy, Boston, MA).

Hepatic Glutathione, Cysteine and Cystine

At d 42 of age, 8 birds/treatment (1 bird pen) were randomly selected and euthanized using CO_2 for measurement total glutathione (reduced (GSH) and oxidized (GSSG) glutathione) and cysteine/cystine concentrations in liver as an indicator for antioxidant status using HPLC (*o*-phthalaldehyde (OPA) method) as described previously (Hou et al., 2018). Liver portions were removed and immediately frozen in liquid nitrogen and stored at −80°C. Extraction of GSH/GSSG, and cysteine/cystine from liver tissues were conducted by homogenizing the tissue (100 mg) with homogenization buffer containing 50 mL of 12 mM iodoacetic acid + 50 mL of 1.5 M perchloric acid ($HClO_4$) in a glass homogenizer. The solution transferred to 15-mL polypropylene tubes, and the homogenizer glass was rinsed with 1.5 mL of homogenization buffer, and all homogenate combined, and then, 0.75 mL of 2 M K_2CO_3 were added, centrifuged at 3000 g for 5 min and the supernatant used for derivatization with iodoacetic acid to S-carboxymethyl. For GSH/cysteine analysis, 50 μL of 100 μM GSH/100μM cysteine standard (or sample) and 100μL of 40 mM sodium borate were added into a 4-mL glass vial. For

GSSG/cystine analysis, 50 μL of 50 μM GSSG/ 50 μM cystine standard (or sample) and 100 μL of 28 mM 2-mercaptoethanol were added into a 4-mL glass vial. The vials vortexed for 10 sec, and after 10 min, 50 μL of 25 mM iodoacetic acid to each vial was added, vortexed for 10 sec. After 5 min, 0.1 mL of 1.2% benzoic acid and 1.4 mL of HPLC H_2O to each vial were added, and then vials placed in the autosampler. The autosampler was programmed to mix 25 μL of sample (or standard) with 25 μL of the *o*-phthalaldehyde (OPA reagent) solution for 1 min and then deliver the derivatized solution into the HPLC column without any delay.

The samples were subjected to chromatography using a Waters HPLC apparatus consisting of a Model 600E Powerline multisolvent delivery system with 100-μL heads, a Model 712 WISP autosampler, a Waters 2475 Multi λ Fluorescence detector, and a Millenium-32 Workstation. A Supelco C_{18} guard column (4.6 mm × 5 cm, 20–40 μm, Sigma) and a Supelco C_{18} column (4.6 mm × 15 cm, 3 μm, Sigma) were used to determine the amount of GSH/cysteine and GSSG/cystine in nanomole per milligram tissue. The amount of GSH/cysteine and GSSG/cystine in an unknown sample was calculated by the Waters Workstation on the basis of known amounts of GSH/cysteine and GSSG/cystine standards, and hepatic total GSH calculated as the sum of GSH and 2 GSSG.

Statistical Analysis

Collected data were analyzed as one way- ANOVA using the GLM Procedure of JMP for a complete randomized block design where treatment diets were used as the fixed factor in the model. When significance was detected, contrast means were compared using Student *t* test ($P < 0.05$).

Results and Discussion

Performance

Dietary supplementation of AgAc at 10 and 50 ppm did not show different effects on BW, WG, mortality, and FCR compared to the Control unsupplemented diet during all production phases (Table 18). In the grower phase, inclusion BMD50 improved the BW ($P < 0.05$) compared to all inclusion rates of AgAc.

Diet supplemented with BMD50 improved FCR by 6.5% in the starter ($P < 0.05$), 2.5% grower, and 2.4% finisher ($P > 0.05$) phases when compared to the Control group. No such improvement was observed in chicks fed AgAc at all concentrations tested. Dietary AgAc at 250 ppm reduced BW ($P < 0.05$) compared to the Control diet, BMD50, and dietary AgAc at 10 and 50 ppm in all phases of production. Dietary AgAc at 250 reduced WG in a dose dependent manner during the starter and grower phases. Dietary AgAc at 250 ppm also reduced ($P < 0.05$) the productivity index in starter, grower, and finisher phases when compared to the control, BMD50, and AgAc at 10 and 50 ppm. No significant effect was noticed on FCR among treatment groups in grower and finisher phases.

Table 18 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed on bird performance of male broilers

		Treatment							
D 0-21		PC	BMD50	SAF250	GO+	AgAc10	AgAc50	AgAc250	*P* value
	BW (g)	879±31 [a]	884±32 [a]	881±31 [a]	831±30 [bc]	870±15 [ab]	850±34 [ab]	814±38 [c]	0.001
	WG (g)	835±31 [a]	840±31 [a]	837±31 [a]	786±30 [bc]	825±15 [a]	806±34 [ab]	770±38 [c]	0.001
	FCR (g:g)	1.32±0.04 [b]	1.24±0.04 [a]	1.27±0.03 [ab]	1.33±0.04 [b]	1.32±0.05 [b]	1.32±0.03 [b]	1.32±0.04 [b]	0.049
	PI[†]	301±16 [ab]	307±31 [a]	310±23 [a]	278±12 [cd]	292±18 [abc]	284±10 [bcd]	265±20 [d]	0.002
	Mortality (%)	0.75±2.1	4.5±4.2	0.75±2.1	1.5±2.7	2.3±3.1	2.3±3.1	4.5±5.3	0.220
D 22-35									
	BW (g)	2162±64 [ab]	2213±68 [a]	2139±93 [b]	2137±51 [b]	2138±78 [b]	2123±89 [b]	2010±56 [c]	0.001
	WG (g)	1090±51 [ab]	1129±46 [a]	1065±74 [b]	1116±51 [ab]	1077±70 [ab]	1084±62 [ab]	997±36 [c]	0.001
	FCR (g:g)	1.61±0.03 [ab]	1.57±0.08 [a]	1.61±0.03 [ab]	1.63±0.04 [b]	1.63±0.04[b]	1.62±0.04[ab]	1.62±0.03[ab]	0.206
	PI	369±13 [ab]	372±26 [a]	368±26 [ab]	355±16 [ab]	358±9 [ab]	350±13 [b]	324±20 [c]	0.001
	Mortality (%)	0.88±2.5	1.0±2.8	0.0±0.0	1.88±3.5	0.0±0.0	2.8±3.8	2.0±3.7	0.387
D 36-42									
	BW (g)	2881±99 [a]	2905±117 [a]	2843±134 [a]	2863±69 [a]	2840±108 [a]	2839±123[a]	2679±94 [b]	0.002
	WG (g)	718±61	691±53	735±79	725±30	701±71	717±51	669±58	0.453
	FCR (g:g)	1.71±0.03[ab]	1.67±0.05[a]	1.73±0.06[ab]	1.71±0.03[ab]	1.71±0.04[ab]	1.74±0.04 [ab]	1.71±0.06[ab]	0.115
	PI	382±17 [a]	384±24 [a]	383±33 [a]	379±17 [a]	375±15 [a]	365±10 [a]	340±32 [b]	0.001
	Mortality (%)	1.88±3.4	**0.0±0.0**	**0.0±0.0**	0.0±0.0	1.0±2.8	0.0±0.0	1.0±2.8	0.478
	Total Mortality[*]	3.0±4.5	5.3±5.1	0.86±2.3	3.0±4.5	3.0±4.5	4.5±4.2	6.8±6.8	0.308

[a-c] Values within rows with different superscripts are significantly different at $P<0.05$

[†]Productivity Index (PI)=Livability(%)xBW(kg)/Age(days)/FCRx100

[*] Total mortality from d 0 to d 42

Mean±SD

Apparent Ileal Digestibility of Energy

Results of apparent ileal digestible energy at days 21 and 42 are presented in Figures 12 and 13, respectively. There were no statistical differences ($P > 0.05$) between dietary treatments in apparent ileal digestibility of energy. Dietary supplementation with prebiotic (yeast cell wall) increased ($P < 0.05$) the digestible energy at day 21 compared to BMD50 and all AgAc inclusion rates.

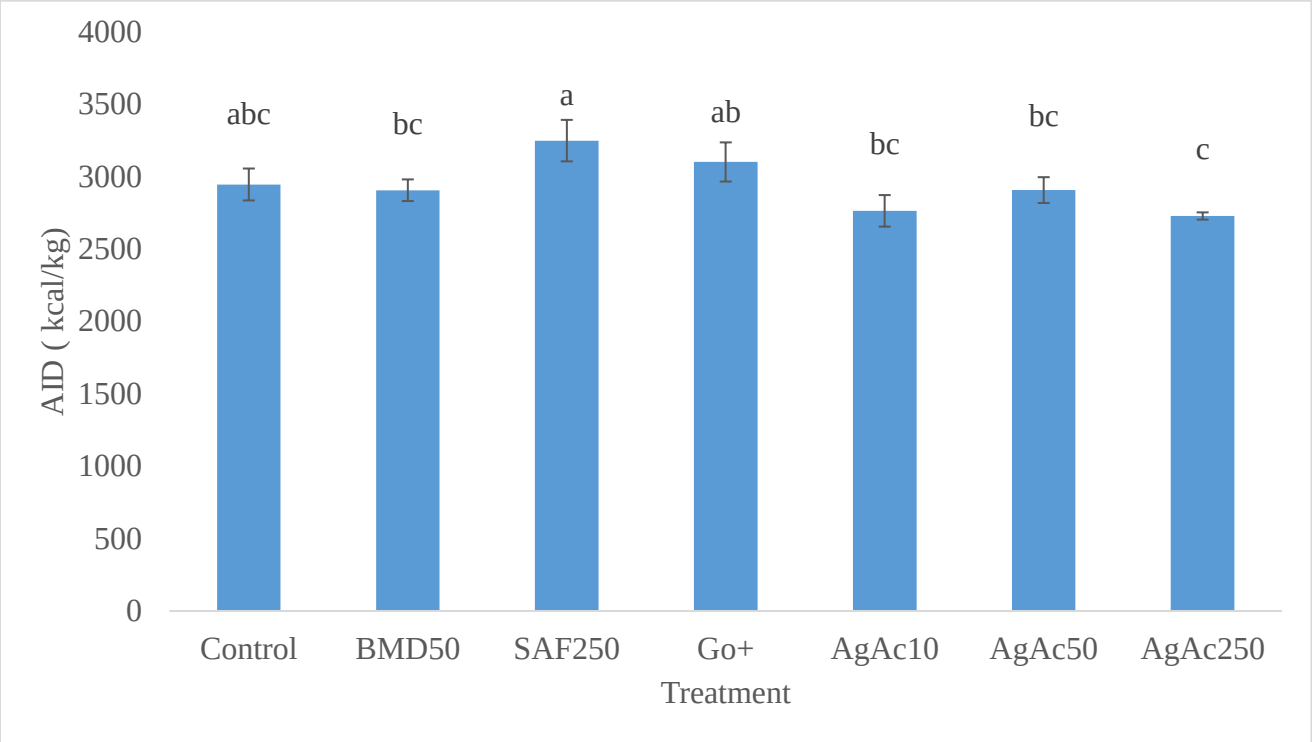


Figure 12 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed) on apparent ileal digestible energy of male broilers at 21 d of age

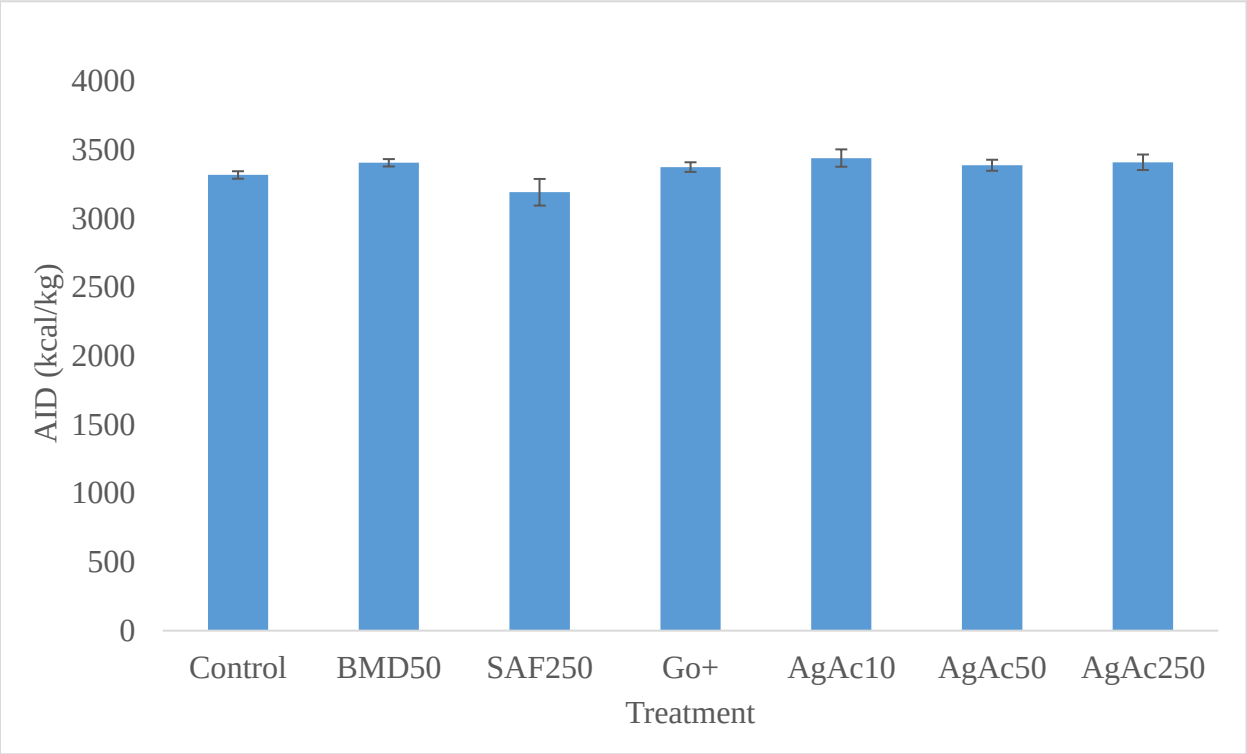


Figure 13 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed) on apparent ileal digestible energy of male broilers at 42 d of age

Apparent Ileal Digestibility of Amino Acids

Apparent ileal amino acid digestibility coefficient percentages at days 21 and 42 are presented in Tables 19 and 20, respectively. On d 21, AgAc supplementation at 10 and 50 ppm improved most amino acid digestibility coefficients relative to the control and coefficients were similar to the BMD50, SAF250, and Go+ treatment group, where dietary BMD50, SAF250, and Go+ improved ($P < 0.05$) all amino acid digestibility coefficients compared to Control group.

However, when silver acetate was supplemented at 250 ppm amino acids coefficients were reduced compared to the BMD50, SAF250, and most amino acids in Go+ treatment groups.

On day 42, the inclusion of BMD50 improved the amino acid digestibility coefficient relative to the Control. The digestibility coefficients for lysine, threonine, isoleucine, leucine, valine, phenylalanine, serine, glycine, alanine, arginine, tyrosine, and glutamic acids were significantly increased ($P = 0.001$). Dietary Go+ at 500 g/ton had no effect on all amino acid digestible coefficients when compared to the Control group. Although dietary SFA250 improved all amino acid digestibility coefficients at day 21 (starter phase), it had the lower digestibility at day 42 (finisher) for all amino acids comparing to the Control, BMD50, and Go+ treatment groups. These results suggest that the host might respond to these feed additives at the early stage of life and not at later stages. The antibiotic BMD50 improved amino acid digestibility coefficients at both day 21 and 42 compared to the both probiotic and prebiotic.

For the AgAc treatments, values were largely unchanged when compared to the Control. There was a significant reduction in methionine digestibility at the AgAc10 level, however, AgAc50 and AgAc250 was similar to both the Control and BMD50 treatments. There was a reduction in digestibility coefficients for histidine, threonine, and aspartic acid with the AgAc250 level, when compared to the Control.

Table 19 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed on apparent ileal amino acid digestibility coefficient percentages of male broilers at 21 d of age

Amino acid	PC	BMD50	SAF250	GO+	AgAc10	AgAc50	AgAc250	*P* value
Lysine	86±1.2 ^{c}	97±0.3 ^{a}	98±0.2 ^{a}	92±1.9 ^{b}	92±0.7 ^{b}	96±0.5 ^{ab}	87±0.8 ^{c}	<0.001
Methionine	90±1.0 ^{e}	98±0.3 ^{ab}	98±0.3 ^{a}	94±1.9 ^{cd}	95±0.4 ^{bcd}	97±0.3 ^{abc}	92±0.5 ^{de}	<0.001
Threonine	69±2.2 ^{c}	96±0.8 ^{a}	97±0.5 ^{a}	82±5.1 ^{b}	89±1.6 ^{ab}	92±1.6 ^{a}	81±1.6 ^{b}	<0.001
Isoleucine	80±1.7 ^{d}	96±0.4 ^{a}	98±0.2 ^{a}	88±3.1 ^{c}	90±1.2 ^{bc}	95±0.5 ^{ab}	85±1.1 ^{cd}	<0.001
Leucine	82±1.6 ^{d}	97±0.4 ^{ab}	98±0.2 ^{a}	89±2.9 ^{c}	92±0.9 ^{bc}	96±0.6 ^{ab}	88±1.0 ^{c}	<0.001
Valine	79±1.7 ^{d}	96±0.6 ^{ab}	97±0.3 ^{a}	87±3.4 ^{c}	89±1.3 ^{bc}	94±0.8 ^{ab}	84±1.1 ^{cd}	<0.001
Histidine	81±1.2 ^{c}	98±0.4 ^{a}	98±0.4 ^{a}	90±3.2 ^{b}	94±0.9 ^{ab}	96±0.6 ^{a}	90±0.9 ^{b}	<0.001
Phenylalanine	83±1.4 ^{d}	97±0.5 ^{ab}	98±0.2 ^{a}	89±2.8 ^{c}	92±1.1 ^{bc}	95±0.7 ^{ab}	87±1.1 ^{c}	<0.001
Serine	79±1.6 ^{d}	97±0.6 ^{ab}	98±0.3 ^{a}	87±3.4 ^{c}	91±1.3 ^{bc}	95±0.6 ^{ab}	85±1.3 ^{c}	<0.001
Glycine	75±1.77 ^{c}	94±0.5 ^{a}	97±0.4 ^{a}	84±3.6 ^{b}	84±1.3 ^{b}	93±0.8 ^{a}	78±1.1 ^{bc}	<0.001
Alanine	80±1.7 ^{d}	96±0.5 ^{ab}	98±0.2 ^{a}	88±2.9 ^{c}	90±1.1 ^{bc}	95±0.8 ^{ab}	86±1.1 ^{c}	<0.001
Arginine	87±1.1 ^{d}	98±0.4 ^{a}	98±0.2 ^{a}	92±2.2 ^{bc}	95±0.9 ^{abc}	97±0.7 ^{ab}	91±0.8 ^{c}	<0.001
Tyrosine	74± 1.7 ^{d}	95±0.4 ^{a}	96±0.3 ^{a}	87±3.4 ^{bc}	83±1.3 ^{c}	91±0.7 ^{ab}	73±1.6 ^{d}	<0.001
Glutamic acid	86±1.5 ^{d}	98±0.4 ^{a}	98±0.2 ^{a}	90±2.5 ^{bcd}	94±0.9 ^{abc}	96±0.7 ^{ab}	90±0.9 ^{cd}	<0.001
Aspartic acid	78±1.9 ^{c}	96±0.6 ^{a}	98±0.3 ^{a}	86±3.7 ^{b}	91±1.5 ^{ab}	94±1.2 ^{a}	85±1.4 ^{b}	<0.001

Mean±SEM, each value is the mean of 8 samples

$^{a-c}$ Values within rows with different superscripts are significantly different at $P < 0.05$

Table 20 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed on apparent ileal amino acid digestibility coefficient percentages of male broilers at 42 d of age

Amino acid	Control	BMD50	SAF250	GO+	AgAc10	AgAc50	AgAc250	*P* value
Lysine	83±1.6 ^{bc}	92±1.1 ^{a}	73±1.4 ^{d}	82±1.4 ^{bc}	81±2.2 ^{c}	88±0.5 ^{ab}	86±0.5 ^{bc}	0.001
Methionine	91±0.8 ^{a}	93±0.6 ^{a}	85±1.2 ^{c}	93±0.7 ^{a}	87±1.4 ^{bc}	90±1.1 ^{ab}	90±2.1 ^{ab}	0.001
Threonine	74±2.2 ^{bc}	89±1.5 ^{a}	58±3.5 ^{d}	77±2.0 ^{ab}	66±4.8 ^{bcd}	72±2.7 ^{bc}	61±4.0 ^{cd}	0.001
Isoleucine	81±1.5 ^{b}	91±1.0 ^{a}	70±2.0 ^{c}	82±1.6 ^{b}	78±2.8 ^{b}	82±1.2 ^{b}	78±1.4 ^{b}	0.001
Leucine	82±1.4 ^{b}	93±0.9 ^{a}	72±1.9 ^{c}	83±1.4 ^{b}	80±2.6 ^{b}	86±0.9 ^{b}	81±1.2 ^{b}	0.001
Valine	81±1.2 ^{b}	90±0.9 ^{a}	70±2.1 ^{c}	82±1.5 ^{b}	77±2.7 ^{bc}	76±1.9 ^{bc}	76±1.7 ^{bc}	0.001
Histidine	84±1.5 ^{ab}	93±1.1 ^{a}	71±2.3 ^{c}	85±1.2 ^{ab}	78±2.9 ^{bc}	80±1.7 ^{bc}	71±3.5 ^{c}	0.001
Phenylalanine	81±1.2 ^{b}	92±0.8 ^{a}	69±2.3 ^{c}	83±1.5 ^{b}	80±2.7 ^{b}	84±1.3 ^{b}	79±1.6 ^{b}	0.001
Serine	80±1.7 ^{b}	92±1.1 ^{a}	66±2.6 ^{c}	82±1.7 ^{b}	75±3.3 ^{bc}	82±1.5 ^{b}	75±2.1 ^{bc}	0.001
Glycine	81±1.5 ^{bc}	90±1.2 ^{a}	68±2.1 ^{d}	83±1.4 ^{ab}	72±3.9 ^{cd}	80±1.5 ^{bc}	73±2.2 ^{cd}	0.001
Alanine	80±1.4 ^{b}	92±0.9 ^{a}	70±1.9 ^{c}	83±1.4 ^{b}	80±2.7 ^{b}	84±1.4 ^{b}	79±1.5 ^{b}	0.001
Arginine	86±1.2 ^{b}	95±0.8 ^{a}	75±2.1 ^{c}	88±1.2 ^{b}	83±2.5 ^{b}	87±1.0 ^{b}	84±1.5 ^{b}	0.001
Tyrosine	82±0.8 ^{bc}	89±1.1 ^{a}	74±1.5 ^{d}	85±0.9 ^{ab}	78±1.9 ^{cd}	80±1.4 ^{bcd}	79±1.6 ^{bcd}	0.001
Glutamic acid	86±1.0 ^{b}	94±0.8 ^{a}	79±1.7 ^{c}	88±1.2 ^{b}	85±1.8 ^{b}	87±1.1 ^{b}	82±1.3 ^{bc}	0.001
Aspartic acid	84±1.2 ^{ab}	92±1.2 ^{a}	72±1.9 ^{c}	86±1.3 ^{ab}	75±3.6 ^{c}	80±1.5 ^{bc}	74±1.9 ^{c}	0.001

Mean±SEM, each value is the mean of 8 samples

$^{a-c}$ Means within rows with different superscripts are significantly different at $P < 0.05$

Histology of Small Intestine

Histomorphologic assessment of intestinal samples at day 21 and 42 revealed no significant abnormalities apart from minimal to mild enteritis in the duodenum and jejunum associated with infiltration of the lamina propria by lymphocytes and plasma cells and expansion of villus enterocytes by coccidian microgametes, macrogametes, schizonts, and oocysts. Few animals from all groups exhibited these signs with no significant association with treatment. Villus length and crypt morphology were within normal limits and no pigmentation was observed within enterocytes.

Bone Mineralization

Bone mineral content and bone mineral density results are presented in Figures 14 and 15, respectively. For total bone mineral content, neither the BMD50 treatment nor any of the AgAc treatments were different when compared to the Control. There was a dose-related response among AgAc treatments, in which the mineral content of AgAc250 was significantly lower than AgAc10 and AgAc50. Bone mineral density values were highest in the AgAc50 treatment, significantly higher than the BMD50, SAF250, and Go+ treatment groups. Dietary SAF250 and Go+ did not affect the bone mineral density and content when compared to Control group. Similar to what was seen with total mineral content; AgAc250 had a reduced density when compared to both the Control and AgAc50 treatments.

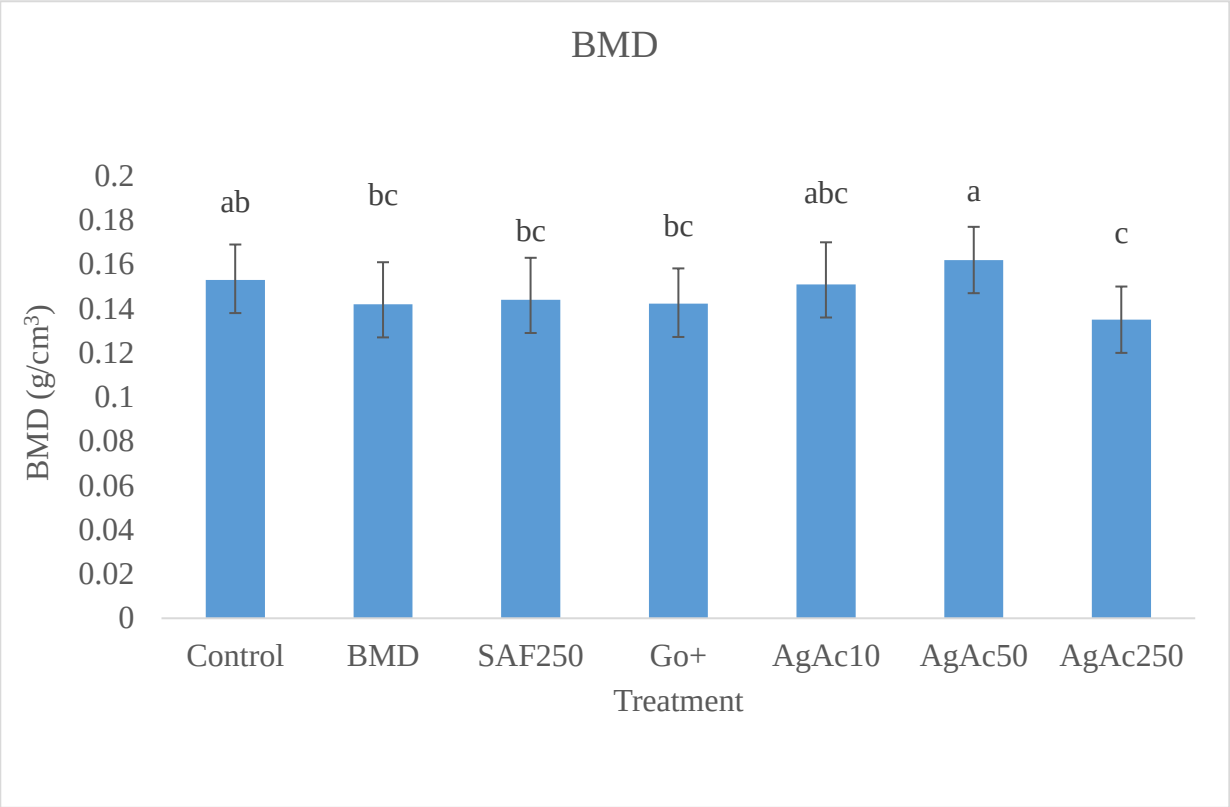


Figure 14 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed) on bone mineral density (BMD) of male broilers at 42 d of age

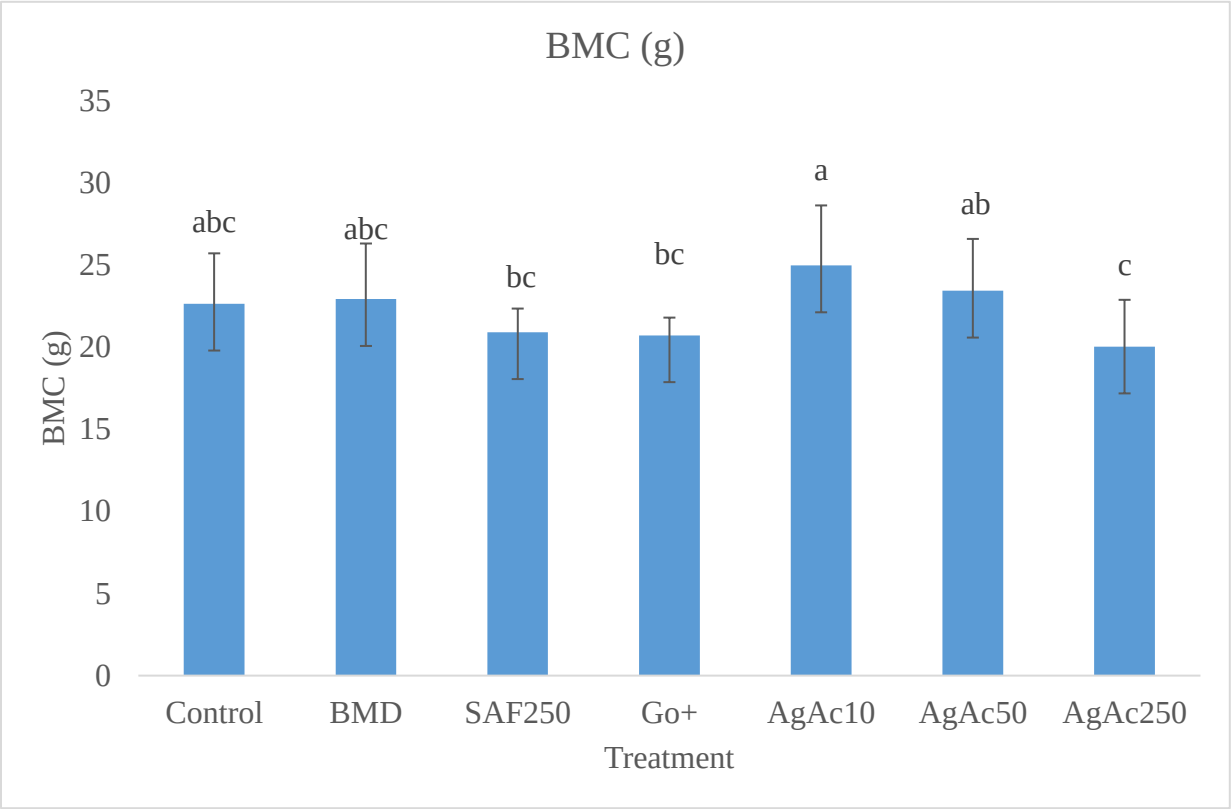


Figure 15 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed) on bone mineral content (BMC) of male broilers at 42 d of age

Hepatic Glutathione, Cysteine and Cystine

Results of total glutathione, reduced (GSH) and oxidized (2 GSSG), and the related amino acids cysteine and cystine are presented in Table 21. The results showed that dietary AgAc at 10 and 50 ppm for 42 days did not affect ($P > 0.05$) hepatic total GSH compared to other treatment groups. Dietary AgAc at 250 ppm reduced ($P < 0.05$) hepatic total GSH compared to Control, BMD50, SAF250, and Go+ treatment groups. There was no adverse effect of dietary AgAc at 10, 50, and 250 ppm ($P > 0.05$) on hepatic cysteine concentrations compared to Control and other treatment groups. Similarly, there was no effect on hepatic cystine concentrations at 10 and 50 ppm when compared to other treatment groups. Dietary AgAc at 250 ppm, however, showed the lowest hepatic cystine concentrations when compared to all other treatment groups, in which the cystine concentration in all 8 samples averaged 0.001 vs 0.06 nmol/mg tissue for the Control.

Table 21 Effect of dietary treatments (bacitracin methylene disalicylate (BMD50), Safmannan (SAF250), Envera Go plus (Go+), and silver acetate (AgAc) at 10, 50, and 250 mg/kg feed) on liver concentrations of total glutathione (reduced (GSH) and oxidized (GSSG), cysteine and cystine of male broilers at 42 d of age

Treatment	Total GSH (nmol/mg tissue)	Cysteine (nmol/mg tissue)	Cystine (nmol/mg tissue)
Control	2.81±0.45 ^{ab}	0.11±0.02	0.06±0.01 ^{abc}
BMD50	2.85±0.41 ^{ab}	0.09±0.03	0.04±0.01 ^{c}
SAF250	2.68±0.39 ^{ab}	0.14±0.03	0.07±0.01 ^{ab}
Go+	3.37±0.45 ^{a}	0.17±0.05	0.08±0.01 ^{a}
AgAc10	2.29±0.32 ^{bc}	0.07±0.02	0.06±0.01 ^{abc}
AgAc50	2.33±0.17 ^{bc}	0.09±0.01	0.05±0.01 ^{bc}
AgAc250	1.58±0.29 ^{c}	0.09±0.02	0.001±0.00 ^{d}

$^{a-c}$ Means within a column with different superscripts are significantly different at $P < 0.05$
Mean±SEM (n=8)

The purpose of this study was to investigate the potential effects of dietary silver acetate supplemented feed on broiler performance, energy and amino acid digestibility, intestinal histology, bone mineralization, and hepatic antioxidant status compared with an antibiotic growth promotor. The NRC (2005) stated that silver is a relatively nontoxic element when consumed with a diet that contains rich amounts of copper, selenium, and vitamin E, as the mechanism by which silver induce adverse (toxic) effect on poultry (mainly reduce BW) is through interfering with metabolism and function of such trace minerals (components of antioxidant system). The maximum tolerable level (MTL) of silver with no adverse effect on poultry was set at 100 mg/kg feed. Thus, based on NRC recommendations, the addition of silver as growth promotor should be within this range of inclusion to avoid toxic effects.

The results showed that dietary supplementation of AgAc at 10 and 50 ppm did not show different effects on BW, WG, mortality, and FCR compared to the control unsupplemented diet group during all phases. The result is consistent with previous 21-d-battery study (chapter 4) when broiler chicken fed gradient concentration of AgAc 20, 40, 60, and 100 mg/kg feed. The results are also consistent with other studies using particulate silver (Felehgari et al., 2013; Pineda et al., 2012; Ahmadi, 2012; Kulak et al., 2018). Ahmadi (2012) showed that the addition of Ag-NPs to broiler diets (20, 40, 60 ppm) did not improves performance in comparison with a control treatment with respect to body weight, feed intake, feed conversion ratio and feed efficiency of broilers through a 42-d trial period.

In all these studies, the inclusion rate of particulate silver was below the MTL, and the diets contained adequate mineral and vitamin premixes. Diet composition (adequate mineral and vitamins premix) could be the reason for no significant positive effects of adding silver at these

concentrations. In contrast, Saleh and El-Magd (2018) reported that dietary supplementation with silver nitrate (100 ppm) and particulate silver (50 ppm) for 12 days improved broiler performance body weight gain, feed intake, and feed conversion ratio. Vadalasetty et al. (2018), however, reported that the application of particulate silver via drinking water for 30 days in the concentration of 50 ppm led to decrease the BW and WG (average body weight gain) with no impact on FCR. Ahmadi and Rahimi (2011) also reported negative effect of particulate silver when supplemented in drinking water (4, 8, 12 ppm) for 42 days on BW and FCR of broiler chickens.

Dietary AgAc at 250 ppm reduced BW ($P < 0.05$) compared to the Control diet, BMD50, and dietary AgAc at 10 and 50 ppm in all phases of production. Dietary AgAc at 250 reduced WG in a dose-dependent manner during the starter and grower phases. In turkeys, 300 mg silver acetate/kg diet depressed growth performance (Jensen et al., 1974). Song et al. (2017) reported that the intestinal and plasma oxidative stress resulted in a reduction in body weight and feed intake with no effect on FCR, and dietary treatment with Zn, vitamin E as antioxidants, or their combination at different inclusion rates failed to alleviate the negative effect of dietary particulate silver (at 1000 mg/L drinking water for 42 days) on the body weight and feed intake of broiler chickens. Peterson and Jensen (1974) showed that adding 900 ppm silver nitrate for 4 weeks to a practical diet for chicks significantly depressed growth, reduced the copper content of blood, spleen, brain, liver and 50 ppm Cu supplementation only partially corrected the growth depression. This result indicates that exceeding the recommended dose of MTL in broiler could result in adverse effect and the diet mineral premix added in this trial did not attenuate the adverse effect on BW and WG.

Results in this study indicated that dietary supplementation of AgAc at all concentrations did not affect ($P > 0.05$) the apparent ileal digestible energy on day 21 and 42. Similar results were found by Pienda et al. (2012) who reported that no treatment effects on intake of metabolizable energy (ME) when particulate silver at 10 and 20 ppm was supplemented in drinking water for 4 weeks.

There is no literature available evaluating the effect of dietary silver on apparent amino acid digestibility. A study by Saleh and El-Maged (2018) evaluated dietary silver nitrate (100 mg/kg feed) and particulate silver (50 mg/kg feed) on protein digestibility and the author's reported increased protein digestibility, and nitrogen (N) content in the muscle tissue in the chicks fed particulate silver and silver nitrate compared with that in the control group. Pienda et al. (2012), reported high N intake and more N retention per kg metabolic body size of broiler chickens with supplementation of 10 ppm particulate silver for 4 weeks. The results of amino acid digestibility coefficients on d 21 of the current study showed that AgAc supplementation at 10, 50, and 250 ppm increased most amino acid digestibility coefficients relative to the Control. It is probable that the increased N intake, retention and content in muscle tissues observed by Pienda et al. (2012) was due to increased amino acid digestibility. However, on day 42, most amino acid digestibility coefficients for AgAc treatments were not different from the Control. This inconsistent effect was not seen for BMD50 which showed increases amino acid digestibility coefficients for both days 21 and 42 when compared to the Control group.

Results of amino acid digestibility on d 42 showed a reduction in digestibility coefficients for histidine, threonine, and aspartic acid with the AgAc250 level, when compared to the Control. The reason behind this effect is not clear as no literature is available to compare with. However, histidine is known to have a strong association with silver, readily forming chelates.

The high levels of silver in the AgAc250 diet may have potentially become bound with histidine residues in the GI tract, leading to the reduction in the digestibility coefficient seen at that dose.

Kulak et al. (2018) and Ognik et al. (2016 ab) demonstrated that oral administration of particulate silver to chickens effects the morphology and functioning of the gastrointestinal tract, as well as the parameters of immune and redox status accompanied with intestinal wall accumulation of silver, regardless of the size and doses used. In rodent toxicity studies, it has been reported that oral ingestion of particulate silver at 10 and 20 mg/kg for 28 days could lead to destruction of intestinal microvilli, reduce absorption capacity of nutrients and subsequently reduce growth performance (Shahera and Young, 2013). Another pathological change reported in a rodent study by Jeong et al. (2010), were increased numbers of goblet cells in the intestine with abnormal composition of mucus granules following the oral administration of 30 mg/kg of BW/day of nanoparticles for 28 days. On the other hand, some studies reveled no adverse effect on intestinal histology when applying particulate silver with different doses, exposure duration, and route of administration in quail (Sawosz et al., 2007), broiler chickens (Ahmadi et al. 2009), weaned pigs (Fondevila et al., 2009), and mice (van den Brule et al., 2016). In the present study, histological analysis of the tissue samples from the duodenum, jejunum, and ileum of the chicks receiving AgAc at all concentrations showed no significant histological abnormalities on day 21 and day 42.

Ognik et al. (2016 b) reported the histological analysis of tissue samples from the jejunum of chicks receiving particulate silver and silver acetate (5 mg/kg BW/day) with no deviations from the norm; the villi were slender, finger-shaped and regular, which indicates that the silver nanoparticles had no negative effect on the histological picture of the jejunum. Ag-nanoparticles did not show any damaging properties on enterocytes of duodenal villi of quail

when particulate silver was added to drinking water at concentrations of 0, 5, 15 and 25 mg/kg for 12 days (Sawosz et al., 2007). Ahmadi et al. (2009) reported no adverse effect on intestinal histology when silver NP was fed at 300 ppm.

For the current study the results of bone mineralization indicated that silver from silver acetate could interfere with bone formation. In broiler chickens, Peterson and Jensen (1974) noticed that among mortalities weak bones were a noticeable clinical sign after feeding an industry-type diet supplemented with 900 ppm silver nitrate for 4 weeks. Jensen et al. (1974) did not mention such effects on young turkeys when fed the same concentration of 900 ppm, nor at lower concentrations of 100 and 300 ppm. In our previous work with acute oral toxicity of silver acetate (chapter 3), the mortalities at dose 1000 mg/kg of BW showed very weak and pliable bones, and AgAc (100 and 1000 mg/kg BW) increased plasma Ca levels compared to control. In a recent study done by Ognik et al. (2017), it was found that administration of silver nanoparticles to intestine of chickens did not interfere with Ca absorption but there was a decrease in the absorption of K and Fe.

As was seen in this study, bone mineral content among AgAc treatments did not differ from the Control or BMD50 treatments. However, AgAc250 was reduced bone mineral content relative to the AgAc10 and AgAc50 doses. This suggests that at lower concentrations, AgAc does not have a negative impact on Ca utilization, but there may be an impact on bone mineralization and density as the dose increases. Similar results by Sikorska et al., 2010 showed a tendency ($P > 0.05$) towards increasing mineral content of Ca, Cu, and iron in thigh bone of chicken embryo, indicating that particulate silver may influence bone mineralization, and it could be speculated that particulate silver has the ability to stimulate the hydroxylapatite formation It

was also shown in an *in vitro* study that silver binds to the hydroxyapatite complex and can displace calcium and magnesium ions (Gould et al., 1987; Lansdown, 2009).

In this study, the AgAc250 treatment had the lowest digestibility for histidine (significantly lower than both Control and BMD treatments). Histidine is known for being involved in Zn uptake (Wapnir et al., 1983). Given that Zn is critical in bone formation and affects bone mass (Nielson et al, 1966; Yamaguchi, 1998), there may be a relationship between reduced levels of histidine and the impact seen on the bone parameters at the high levels of dietary AgAc. This hypothesis needs more investigation. Data showed that using dietary silver as silver acetate in broiler diets could result in adverse effects at concentrations as low as 250 ppm, and low concentrations ($\leq$ 50 ppm) did not show improvement in productive parameters over the PC or similar to BMD50; these could be limitations for using this form of silver as an antibiotic alternative.

Glutathione (GSH, L-Ɣ-glutamyl-L-cysteinyl-glycine) is the predominant low-molecular-weight thiol in animal and plant cells (Wu et al., 2004). Glutathione is a significant endogenous antioxidant involved in many vital cellular functions (Meister, 1983) such as protection from the actions of free radicals and reactive oxygen species (Enkvetchakul et al., 1995). Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine with cysteine being the rate limit amino acid for GSH synthesis. Because of the cysteine residue, GSH is readily oxidized nonenzymatically to oxidized glutathione or glutathione disulfide (GSSG) by electrophilic substances (such as free radicals and reactive oxygen/nitrogen species). Cellular GSH concentrations are reduced significantly as a result of many conditions such as protein malnutrition, oxidative stress, and many pathological conditions (Wu et al., 2004).

It was reported previously that silver ion induce oxidative stress damage in broiler chickens tissues such as intestines, breast muscles, liver, and plasma (Song et al., 2017; Ognik et al., 2016 a; Kulak et al., 2018), although studies regarding silver induce oxidative stress are somewhat contradictory (Ebabe et al., 2013; Munger et al., 2014). Kulak et al. (2018) reported that administration of Ag-NPs at dose of 9.47 mg/bird can induce oxidative reactions in the blood, small intestinal wall, liver and breast muscle of chickens and can increase in the content of the oxidized form of glutathione which leads to depletion of the relative amount of the reduced form of glutathione. The author reported no effect on bird performance. Ognik et al., (2016 a) reported that administration of particulate silver (lipid-coated or uncoated) at 5 mg/kg BW did not significantly affect the total glutathione (GSH+GSSG) in blood serum of broiler chickens at 24 and 38 days of age. The results of the current study showed that dietary AgAc at 10 and 50 ppm for 42 days did not affect the hepatic total GSH compared to other treatment groups. Dietary AgAc at 250 ppm reduced hepatic total GSH compared to Control, BMD50, SAF250, and Go+ treatment groups. There was no adverse effect of dietary AgAc at 10, 50, and 250 ppm on hepatic cysteine concentrations compared to Control and other treatment groups. Similarly, there was no effect on hepatic cystine concentrations at 10 and 50 ppm when compared to other treatment groups. Dietary AgAc at 250 ppm, however, showed the lowest hepatic cystine concentrations when compared to all other treatment groups.

CHAPTER VI

CONCLUSION

The poultry industry community and researchers are searching for suitable antibiotic alternative after the adoption of the veterinary feed directive implemented in January 2017. Silver was introduced recently as an antibiotic alternative option, however, its registration as a feed additive is an early requisite before being applied in practice (Fondevila, 2009).

In experiment 1, the *in vitro* antimicrobial efficacy experiment was conducted using two silver ion forms, two separate silver carbene complexes (SCCs) with different carrier molecules (SCC1 with a methylated caffeine backbone and SCC22 with a dichloroimidazolium backbone), and silver acetate were investigated against four 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 poultry field isolates of *Salmonella* Heidelberg, *Salmonella* Enteritidis, and *Salmonella* Montevideo with MICs and MBCs ranges from 16-21 µM (6-8 µg/mL) and 16-32 µM (6-12 µg/mL), respectively. *Clostridium perfringens* type A (CP) 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 CP was >85 µM for SCCs, and >192 µM for AgAc (>32 µg/mL for all compounds). Ten hours incubation of CP 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 CP with higher concentration (100 µg/mL) of each SCC for 10 h inhibited more bacteria compared to the untreated bacterial cells, however, no differences in the ultrastructure of lysed bacteria was seen and this concentration might not induce viable but non-culturable states 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 an inhibitory effect against the Gram-positive anaerobic *Clostridium 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.

The challenge of using silver as an antimicrobial agent is the effective therapy with low toxic effect on host cells, which requires the therapeutic window being safe. The preliminary *in vivo* study (chapter 3) was conducted to investigate the potential toxic effect of SCC1, SCC22, and AgAc on 300 7-day old broiler chickens in 2 independent replicates trials using acute oral toxicity, prior to including them in chicken feed or water to evaluate their *in vivo* antimicrobial efficacy. Determination of the median lethal dose (LD_{50}), bird performance, relative organ weight, bone mineralization, blood biochemistry, and histological changes were evaluated. Over the following 24 h and 14 days, none of the products at any given dose caused 50% mortality. During the two-week observation period after dosing with SCC1 and SCC22, no differences were observed in bird performance for these silver formulations and the control group. Compared to the control and SCCs, single administration of silver acetate at dose 1000 mg/kg BW reduced (P<0.05) BW after 7 and 14 days of administration, although the birds showed normal weight gain after 14 days of administration compared to the control. In addition, no adverse effects of SCC1, SCC22, and AgAc on relative organ weight of vital internal organs, bone mineralization, or plasma enzymes (ALT, ALP, and GGT) and metabolites (blood urea nitrogen, creatinine, and total bilirubin) were noted compared to the control group. Gross and histological examination after 14 days of administration did not show any significant

pathological changes in jejunum, ileum, spleen, liver, and kidney. Gradual release of silver ions, appear to be well tolerated by broilers as judged by indicators of LD_{50}, performance, and blood biochemistry, supporting the safety and efficacy of SCC products for use in broiler feed as an alternative to traditional antibiotics.

The objective of the third experimental study was to evaluate the potential effect of silver acetate (AgAc) on broiler chicken performance and necrotic enteritis development. The *in vivo* antimicrobial efficacy of AgAc delivered either by adding into the feed or via drinking water during necrotic enteritis challenge and Infectious Bursal disease immunization was evaluated in two experiments studies. Five concentrations of AgAc up to the maximum tolerable level set by NRC (20, 40, 60, 80, and 100 mg/kg feed) were fed. Whether or not AgAc was added into the feed (for 21 d) or in the water (4 d), there were no differences ($P > 0.05$) seen in bird performance, *Clostridium perfringens* enumeration, and lesion score compared to the positive and negative Controls. These results suggest limitations on the effect of silver acetate on performance and reduced intestinal *Clostridium perfringens* colonization in broiler chickens, although the *in vitro* efficacy results showed bacteriostatic and bactericidal against different enteric poultry pathogens.

In experiment 4, we further evaluated the potential effect of silver acetate in comparison to an antibiotic and its selected alternatives (probiotic and prebiotic) on performance, energy and amino acid digestibility, intestinal histology, total bone mineral content and density, and hepatic glutathione. Dietary supplementation of AgAc at 10 and 50 ppm did not show different effects on performance compared to the Control group during all production phases. Dietary AgAc supplemented at 250 ppm showed reduced BW ($P<0.05$) compared to the Control, BMD50, and dietary AgAc at 10 and 50 ppm. There were no differences among groups in apparent ileal

digestible energy. On d 42, there was a reduction in amino acid digestibility coefficients for histidine, threonine, and aspartic acid with the AgAc250 ppm treatment, although no histopathological changes were observed in duodenum, jejunum, or ileum. For bone mineralization, the bone mineral content of birds fed AgAc250 ppm was significantly lower than AgAc10 and AgAc50. AgAc250 had a reduced bone mineral density when compared to both the Control and AgAc50 treatments. These results suggest that using silver acetate in broiler diets could result in adverse effects at concentrations of 250 ppm, and lower concentrations did not show improvement in performance over the Control or BMD50, and probiotic and prebiotic alternatives. Results from chapter 4 (adding silver in feed for 21 days) and from chapter 5, (adding silver for 24 days) are in line with other publications using particulate silver which showed no significant effects, suggesting the limitation of using this inorganic form as an antibiotic alternative, and further chronic and subchronic toxicity studies are still required for future studies to fully elucidate the mechanism of action of silver carbene complexes.

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