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The Antibacterial Efficacy of Silver (I) Cyanoximates Against Streptococcus mutans UA159 Biofilm Growth and Infection in Galleria mellonella Larvae

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A Senior Honors Project Submitted for Fulfillment of Requirements for Honors in the Major of Biology

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Abstract

Streptococcus mutans is the known etiological agent of dental caries. *S. mutans*' virulence factors, namely biofilm formation and lactic acid fermentation, contribute to its pathogenic behavior within the oral cavity. Biofilms are thick, antibiotically resistant communities of bacteria that are much more difficult to treat than planktonic or free-floating bacteria. Continued misuse of antibiotics against dynamic communities, like biofilms, has led to increased research on non-antibiotic alternatives. Amongst these alternatives, there are silver and silver-based compounds. Silver is commonly incorporated into medicine due to its inhibitory and bactericidal effects and antibacterial properties. Silver (I) cyanoximates have demonstrated promising antibacterial activity against various microorganisms that colonize the surfaces of subcutaneous medical devices. To study the antimicrobial properties of silver (I) cyanoximates, specifically Silver(I) Nitrosodicyanomethanide (Ag(ACO)) and Silver(I)α-Oximido-(2-benzoyl)acetonitrile (Ag(BCO)), aerobic and anaerobic growth curves, hydroxyapatite (HA) disc assays, and in vivo treatment assays were performed against *S. mutans* UA159. Both compounds inhibited planktonic growth aerobically and anaerobically at concentrations between 32 ug/mL and 128 ug/mL. Both compounds also inhibited biofilm formation on HA discs at concentrations between 64 ug/mL and 128 ug/mL. To test whether the compounds showed antibacterial activity in-vivo, *Galleria mellonella* larvae were used. All concentrations of Ag(BCO) and Ag(ACO) successfully eliminated, limited, or delayed bacterial infection in *G. mellonella* larvae compared to *S. mutans* UA159 control. Continued research on antibiotic alternatives that target and disrupt biofilm-forming microorganisms contributes to the general understanding of how to treat *S. mutans* biofilms. This may lead to the incorporation of silver (I) cyanoximates in the prevention and treatment of dental caries and other biofilm-related infections.

Introduction

Streptococcus mutans is one of the many bacterial species colonizing the oral cavity. Out of over 700 species, *S. mutans* is the primary etiological agent of dental caries (tooth decay) (Yoshida and Kuramitsu, 2002). Most microorganisms prefer to colonize surfaces as robust and dynamic communities called biofilms (Santos et al., 2018). Like many other bacterial human pathogens, *S. mutans* prefers to develop into biofilms, or dental plaque, on the surfaces of teeth. In the presence of fermentable carbohydrates, namely sucrose, *S. mutans* creates an acidic environment that erodes the tooth's enamel and can lead to tooth decay and periodontal disease (Forssten et al., 2010). Short-term use of antibiotics has been shown to decrease *S. mutans* activity; however, several studies have demonstrated that long-term antibiotic treatment can lead to the development of drug-resistance strains (Hamada and Slade, 1980). Due to the advantages biofilm communities provide and the nature of *S. mutans*, it is more tolerant to antibiotics than planktonic or free-floating cells (Yoshida and Kuramitsu, 2002). The adaptive response of *S. mutans* and other antibiotic-resistant microorganisms has led to recent research on antibiotic alternatives for the prevention and treatment of dental caries and periodontal disease.

The Oral Microbiota

The oral microbiota consists of thousands of diverse species. Commensal species in the mouth protect the oral cavity by competing for resources and creating unfavorable conditions for cariogenic bacteria (Forssten et al., 2010). Oral diseases, like dental caries, appear when there is an ecological imbalance in the naturally occurring oral environment. This imbalance is typically a shift towards increasing acidogenic and acid-tolerating bacteria within the oral community (Marsh, 2006). Saliva has a natural buffering capacity that protects against decay; however,

prolonged exposure to acid-producing bacterial species can still affect the pH levels and lead to dental caries formation (Loesche, 1986).

There are several organic and inorganic substances within the oral cavity that, in combination with saliva, contribute to dental plaque development (Alejandra and Daniel, 2019). *S. mutans* is a gram-positive microorganism and a component of dental plaque, along with other bacteria, saliva, and leftover food particles. *S. mutans* does not have a respiratory chain and instead uses glycolysis to produce ATP. In environments of excess sucrose, *S. mutans* produces lactic acid as the end-product of glycolysis (Dashper and Reynolds, 1996). *S. mutans* significantly contributes to dental caries due to its ability to form acidic, multi-species biofilms. Once *S. mutans* has formed a biofilm, metabolically active bacteria acidify the microenvironment, which begins a process of enamel demineralization and can lead to dental caries (Zhang et al., 2019).

High-sugar diets create the ideal environment for *S. mutans* to increase acid production and acidify plaque. Any carbohydrate within the oral cavity can be used as an energy source for oral bacteria and has cariogenic potential. In the presence of *S. mutans*, an increase in carbohydrates, mainly sucrose, leads to additional acid production that may outcompete the natural salivary buffer system (Forssten et al., 2010). High carbohydrate diets may lead to more frequent plaque acidification and caries development. Sucrose, however, is the most cariogenic carbohydrate because it can form extracellular polysaccharides in the plaque that act as adhesive scaffolding, increasing biofilms' formation (Forssten et al., 2010). Sucrose is most readily utilized because many cariogenic oral bacteria contain specific transport systems and enzymes for the metabolism of sucrose (Zeng and Burne, 2013). Eliminating cariogenic bacteria within

the oral microbiota and modifying high carbohydrate diets can decrease the prevalence of dental caries.

Biofilm formation

Biofilms are aggregates of microorganisms attached to a surface that are covered in a free-floating or attached extracellular polymer matrix (Townsley and Shank, 2017). Biofilms are ubiquitous on many surfaces, as they are the preferred growth method for many bacterial species. Biofilms can grow anywhere from the surfaces of teeth to implanted medical devices. Despite their location, all biofilms self-produce an extracellular polymer matrix and increase individual resistance to host defense and antimicrobial agents when compared to non-attached cells of the same species (Kaplan, 2010). This increase in resistance is due to the inability of antimicrobials to penetrate a mature biofilm, in which each layer is experiencing various stages of metabolic activity. A biofilm can either be a single microbial species or an aggregate of multiple species. Aggregates of multiple species are more common and make biofilm-related diseases very difficult to treat (O'Toole et al., 2000). This is because a treatment that eliminates one microorganism may have a different or no effect on another microorganism within the multi-species biofilm. These advances in protection and antimicrobial activity significantly contribute to the virulence of biofilm-forming microorganisms. An example of a biofilm-forming microorganism is *S. mutans*. *S. mutans* prefers to form a multi-species biofilm, which contributes to its increased resistance to environmental stressors, detergents, host immune responses, and conventional antibiotic treatment (Townsley and Shank, 2017).

Biofilm development occurs in four stages: reversible attachment, accumulation, maturation, and dispersal (Vasudevan, 2014) (Figure 1). Some researchers believe biofilm formation and attachment are initiated as a response to environmental cues, like nutrient

availability (Kaplan, 2010). The first stage is the reversible attachment of free-floating, planktonic cells to a surface. The attachment is typically mediated by nonspecific interactions, acid-base interactions, and electrostatic forces (Kaplan, 2010). In the context of *S. mutans*, there are weak interactions between the microbial cell surface and pellicle-coated tooth. Once the bacteria have been recruited, stronger, short-range interactions (adhesion) result in the irreversible attachment (Marsh, 2004). As more bacteria accumulate, surface proteins on the colonized surface mediate irreversible adhesion, and a monolayer of bacteria and an extracellular matrix, or slime, begin to form (Vasudevan, 2014). The slime typically contains components of the host; for example, *S. mutans*' extracellular matrix contains glycoproteins and leftover food particles found within the saliva.

Accumulation and maturation involve specific interbacterial interactions. Irreversibly attached bacteria can recruit and attach to any biofilm-forming bacteria. The formation and the components of the extracellular matrix help maintain and stabilize the biofilm structure. As bacteria continue to accumulate, the biofilm begins to mature by forming a microcolony. At this point, the biofilm is a heterogeneous community in terms of bacterial metabolism. The deepest layer contains metabolically inactive, dormant, or dead cells. The deepest layer of a mature biofilm is key to resistance to antimicrobials as it contains persister cells, which are highly tolerant to antimicrobials (Lewis et al., 2010).

Once mature, biofilms form a mushroom-like complex structure with channels that allow the distribution of nutrients required for growth (Vasudevan, 2014). Along with several species of microorganisms, macromolecules such as DNA, RNA, lipids, enzymes, etc., comprise a mature biofilm (Vasudevan, 2014). These components contribute towards metabolic processes essential for the survival of the biofilm, and the structural channels allow for the exchange of

oxygen and removal of metabolic waste products (Kaplan, 2010). After a mature biofilm has accumulated a multilayered community that is heterogeneous in metabolic activity, detachment and dispersion of the biofilm can occur. Detachment is initiated by a physical stressor or a depletion of nutrients, allowing cells to colonize elsewhere (Marsh, 2004). Dental biofilms are broken down mechanically by brushing teeth or during a dental procedure. The released bacteria—which may contain genetic variants due to horizontal gene transfer—can colonize new surfaces (McDougald et al., 2012). This stage is called dispersal, which aids in bacterial survival and disease transmission.

The virulence of a biofilm is heavily dependent on host conditions. Virulence is the ability of a microorganism to infect a host and cause disease. *S. mutans*' virulence factors contribute to its ability to form a biofilm, survive in acidic environments, and interact with other microorganisms to develop a multi-species biofilm (Krzyściak et al., 2013). As previously mentioned, biofilms can be single- or multi-species. Multi-species biofilms incorporate complex chemical and electrochemical microbial interactions within various microenvironments (Santos et al., 2018). These complex and physically close interactions enable horizontal gene transfer, which contributes to the antimicrobial resistance of multispecies biofilms.

All biofilms produce an extracellular matrix. Sugar polymers called exopolysaccharides are a major component of extracellular matrices. Exopolysaccharides contribute to microbial adhesion to surfaces and act as a protective coating over the biofilm structure against various antimicrobial agents (Vasudevan, 2014). Different microorganisms produce different kinds of extracellular sugars. *S. mutans*' exopolysaccharides are mainly composed of glucans that act as extracellular 'glue' between cell-cell and cell-surface interactions on the tooth's surface (Forssten et al., 2010). *S. mutans*' extracellular glycans have the ability to adhere to the tooth's

glycoprotein layer, called the enamel salivary pellicle (Koo et al., 2013). In the context of *S. mutans*, interactions between specific exopolysaccharide environments and the host environment can lead to metabolic acidification and acid-dissolution of enamel (Koo et al., 2013). These adhesive, acidification, and multispecies biofilm-forming properties contribute to the pathogenicity of *S. mutans* and contribute to its microbial resistance to conventional antibiotics, host immune response, and environmental stressors (Santos et al., 2018).

The Antimicrobial Response of S. mutans

Antibiotics have been used to fight infectious diseases for decades, yet, antimicrobial-resistant bacteria present many challenges worldwide. Antibiotics work by targeting vital processes in bacteria. Antimicrobials either kill the bacteria or stop it from growing by attacking bacteria-specific attributes in bacterial cell walls, DNA replication, and protein synthesis. However, multidrug-resistant pathogens, including *S. mutans*, have increased in prevalence in the past decades due to the unnecessary and improper use of antibiotics. The unsuccessful use of antimicrobials creates a selective pressure that favors resistance mechanisms in bacteria, increasing their ability to survive and pass on their resistance genes (MacGowan and Macnaughton, 2017). Once bacteria are multidrug-resistant, they are very difficult and sometimes impossible to treat with conventional antimicrobial methods. It is very easy for antibiotic-resistance genes to be passed between pathogenic bacteria through horizontal gene transfer or mutation; therefore, scientists are racing to find antimicrobial alternatives (Frieri et al., 2017).

Biofilms are particularly drug-resistant due to three significant attributes. First, their extracellular matrix comprises a thick outer layer that provides a dense layer of protection from host-immune response and antimicrobials. Second, bacteria that colonize as a community

experience higher rates of horizontal gene transfer and more frequent mutation than planktonic cells due to their physical proximity and social interactions (Gebreyohannes et al., 2019). Finally, the presence of persister cells. As previously mentioned, persister cells are highly tolerant to antibiotics and remain dormant until a stressor, like antimicrobials, is present (Lewis, 2010). Once antimicrobials are distributed, persister cells are the only metabolically active cells remaining, at which point they begin to repopulate the biofilm (Lewis, 2010). Therefore, to stop repopulation, an effective antimicrobial must successfully transverse the extracellular matrix and remain within the biofilm community long enough to treat persister cells. Thus higher, more frequent doses of drugs are needed to treat biofilms that contain persister cells.

Some antibiotic agents have been successful in treating biofilms within the oral cavity. For short-term use, researchers have found that children exposed to sporadic antibiotic treatments, namely penicillin and ampicillin, experience a reduction in dental caries (Staves and Tinanoff, 1991). However, *S. mutans* is a very dynamic human pathogen and will mutate if treated with antimicrobials long-term, rendering traditional antibiotic agents useless (Hamada and Slade, 1980). The necessity for long-term treatment of dental caries continues worldwide.

Experimental Antibiotic Alternatives

The development of long-term antibiotic alternatives that can be implemented in a clinical setting is becoming increasingly important due to the fast-growing population of highly virulent, antibiotic-resistant pathogenic bacteria. Antibiotic resistance has increased the need for natural product antibiotics. Natural product antibiotics are drugs developed from naturally occurring compounds that provide structural diversity and complexity not seen in synthetic antibiotics (Rossiter et al., 2017). Several natural product antibiotics have been discovered that inhibit initial biofilm formation by chemically disrupting the environmental cues responsible for

initiating biofilm formation (Townsley and Shank, 2017). The mechanism of these natural product antibiotics is essentially unknown, and producing natural products is very difficult to implement on a large scale, which makes this alternative challenging to implement in a clinical setting (Ghosh et al., 2019). Antibiotic alternatives must meet specific criteria—for example, water insolubility—that natural products cannot satisfy (Gerasimchuk et al., 2010). Fortunately, some metal-based compounds, like silver, meet these specific needs.

Silver-based compounds are effective antibacterial agents prevalent in the medical community. Specifically, silver nanoparticles (nano-sized molecules less than 100 nm in diameter) have an expansive range of applications, from diagnostic and imaging uses to therapeutics and biomedical implants (Wong and Liu, 2010). Due to its non-specific activity, silver can penetrate, disintegrate, and destroy the cell membrane and intracellular organs of many kinds of bacteria, viruses, and fungi (Sim et al., 2018). The use of silver nanoparticles and silver-baed compounds as antibiotics demonstrates much lower microbial resistance when compared to traditional antibiotics, leading to a worldwide interest in silver-based antibiotic research (Guunathan, 2014). Silver nanoparticles' attractive antibacterial properties have led to the expanded use of silver in the clinical setting.

Silver nanoparticles and silver-based compounds have been found to disrupt essential bacterial processes, which leads to the inhibition of microbial growth and biofilm formation (Gerasimchuk et al., 2010). Silver nanoparticles provide broad-spectrum bactericidal properties and work synergistically with antibiotics, enhancing their antibacterial effect (Gurunathan et al., 2019). Silver nanoparticles promote cell death through protein leakage via increased membrane permeability and elevated formation of detrimental reactive oxygen species (ROS). Though the specific mechanisms of silver compound’s bactericidal activity are unknown, it is hypothesized

that silver nanoparticles bind to the cell membrane and alter its permeability, leading to interference with the cell respiration, replication, and metabolic pathways, eventually inducing cell death (Gurunathan et al., 2019). This silver nanoparticle interference is thought to be mechanistically similar to silver ions.

Though silver presents low toxicity to human cells, silver is highly toxic to microbial species. Particle size, surface charge, and solubility are key factors that contribute to silver's microbial toxicity (Gurunathan, 2019). Silver is biologically active in its soluble monoatomic ionic state (Ag^{+}) (Sim et al., 2018). In this state, silver ions can access and inhibit vital bacterial processes by adhering to and penetrating microbial cell walls (Sim et al., 2018). Positive silver ions also interact with negatively charged DNA and thiol-containing proteins to inhibit DNA replication and protein function (Gurunathan et al., 2019). Once the peptidoglycan wall of bacterial cells is perforated, silver nanoparticles can disrupt the bacterial respiration cycle, metabolic pathways, and aspects of the DNA replication cycle by interacting with bacterial DNA and vital enzymes (Sim et al., 2018). These aspects are what make silver an ideal antibiotic alternative for multidrug-resistant microorganisms.

Prevention of biofilm formation is also essential, as biofilms provide avenues to antibiotic resistance. Silver-based compounds have demonstrated antibacterial activity against various biofilm-forming bacteria. Silver particles also have anti-biofilm properties that prevent biofilm attachment at the initial step of biofilm formation: adhesion to the host surface (Markowska et al., 2013). Silver particles can inhibit oral biofilms by inhibiting *S. mutans*' metabolic activity and lactic acid production, essentially stopping the acidification of the tooth's enamel (Markowska et al., 2013). These anti-attachment and anti-acidification properties make silver-based compounds promising agents to treat dental plaque.

Silver (I) Cyanoximates

Silver is known as an effective anti-caries agent and is, therefore, very common within clinical dentistry. Silver is incorporated into many dental materials due to its antimicrobial and anti-adherence activity on *S. mutans* biofilm formation. For example, silver nitrate was one of the first silver compounds used to arrest and prevent cavities (Peng et al., 2012). Silver diamine fluoride (SDF) is a restorative option that has since replaced silver nitrate, especially in the pediatric population (Hu et al., 2018). Despite concerns associated with silver, including tissue ulceration, these potentially problematic effects are mild and short-lived. For example, both silver nitrate and silver diamine fluoride appear as black staining on cavities caused by an oxidation reaction (Peng et al., 2012). However, these minor adversities do not outweigh the significant anticaries properties of silver compounds.

Several studies have attempted to find additional non-antibiotic alternatives involving silver due to its established effectiveness as an antimicrobial additive. Silver (I) compounds are promising; however, a major challenge for most silver (I) complexes is their sensitivity to light and heat, as well as unwanted interactions with unsaturated organic compounds (Lotlikar et al., 2019). Silver (I) cyanoximates—NC-C(NO)-R, where R is an electron-withdrawing group—exhibits light insensitivity, poor water solubility, high thermal stability, lack of toxicity to organic ligands, and in vitro antimicrobial activity (Gerasimchuk et al., 2010). Along with these antimicrobial attributes, Lotlikar et al. (2019) found that silver (I) cyanoximates effectively inhibit the biofilm growth of gram-positive and gram-negative bacteria and are just as effective against biofilm as planktonic cells. Though the specific mechanism of silver (I) cyanoximates is relatively unknown, silver and cyanoximates demonstrate heightened antimicrobial effects when combined. Due to these properties, silver (I) cyanoximates indicate promising properties as

antimicrobial additives, especially for light-curable fillers and adhesives for indwelling medical devices (Gerasimchuk, 2014).

Studies by Gerasimchuk (2014) and Lotlikar et al. (2019) show that silver (I) cyanoximates have a concrete potential to be used as additives for the 'glue' that holds indwelling devices in place and incorporated into materials, like composite, which is used to fill cavities. The diverse applications of silver (I) cyanoximates continue to be explored today. Silver (I) cyanoximates' antimicrobial and light-insensitive attributes open the doors to practical uses in the clinical dental setting to prevent dental caries.

Among other silver (I) cyanoximate compounds, silver (I) Nitrosodicyanomethanide (Ag(ACO)) and silver (I)α-Oximido-(2-benzoyl)acetonitrile (Ag(BCO)) were tested against *S. mutans* growth as antimicrobial alternatives. Recent studies by Dr. Santiago's lab have found that these silver (I) cyanoximate compounds successfully inhibit planktonic and biofilm growth in an in vitro setting (data not published). Ag(ACO) and Ag(BCO) have similar minimum biofilm inhibitory concentrations against *S. mutans* at 64 ug/mL (data not published). One promising statistic showed that at 64 ug/mL, Ag(ACO) demonstrated almost 50% disruption of preformed biofilms (data not published).

Although silver (I) cyanoximate activity involving biofilm growth inhibition and disruption are important areas of research, the activity of these compounds in vivo are necessary to determine drug safety and in vivo antibacterial efficacy. *Galleria mellonella* larvae are commonly used as an infection model for many human pathogens (Harding et al., 2013). This is because, similar to human macrophages in their phagocytotic processes, the larvae's immune system involves hemocytes, which are a phagocyte that can take up and destroy bacterial invaders (Cools et al., 2019; Harding et al., 2013). Typically rodents are preferred for in vivo

models; however, *G. mellonella* larvae are less expensive, easy to handle, naturally live and can be maintained at 37℃—the temperature at which human pathogens live— and have an innate immune system mechanistically similar to the mammalian innate immune system (Cools et al., 2019). Because of this, *G. mellonella* larvae have become increasingly popular in the antimicrobial testing of several human pathogens. Previous research has demonstrated that *G. mellonella* is a proficient model system for several human pathogens due to its advanced antimicrobial defenses and innate immune response, which are analogous to the mammalian infection process (Harding et al., 2013; Jander et al., 2000).

Future Directions and Applications

This research is essential to the dental field due to the increasing number of antibiotic-resistant oral bacteria. Antimicrobial implications of silver (I) cyanoximates in clinical dentistry could decrease oral diseases such as dental caries and periodontal disease. Today, the only preventative treatments for dental caries are mechanical oral hygiene processes and maintenance, which is not enough to prevent caries (Forssten et al., 2010). This research has the opportunity to shift dental care from primarily curative treatment to preventative strategies by incorporating alternative antimicrobial agents into everyday dental healthcare products (Forssten et al., 2010).

This research aims to further our understanding of the antimicrobial effects of silver (I) cyanoximate compounds on *S. mutans* biofilm growth to provide a more effective avenue of caries prevention. Several studies have tested the properties of silver compounds, namely research on silver (I) cyanoximates as antibiotic alternatives, their anti-adherence, and light and heat insensitivity. Despite this, very little is known about the specific mechanisms of silver (I) cyanoximates on the bacterial growth of *S. mutans*.

Previous studies in Dr. Santiago's lab showed that among other silver (I) cyanoximate compounds, Ag(ACO) and Ag(BCO) have antimicrobial activity against planktonic and biofilm growth of *S. mutans* UA159 and are able to destroy pre-formed biofilms (data not published). The previous study determined the effectiveness of silver (I) cyanoximates within an in vitro system using MIC, MBIC, and biofilm disruption assays. This project, however, aims to assess silver compounds' effectiveness in inhibiting biofilm growth within an in vitro model system using hydroxyapatite (HA) discs soaked in a salivary pellicle solution. Teeth are made of a bone mineral called hydroxyapatite which, in combination with the synthetic salivary pellicle, creates an in-vitro model system for bacteria to adhere to. A salivary pellicle is a network of salivary glycoproteins that serve as a protective layer around the tooth and act as velcro with EPS, which bacteria excrete. Prepared hydroxyapatite discs reveal a more accurate demonstration of *S. mutans* growth on a tooth-like surface without experimenting on a living organism.

The structures of Ag(ACO) and Ag(BCO) are shown in Figure 2. These compounds were tested under anaerobic environmental conditions to mimic the conditions *S. mutans* may encounter in the oral cavity. Anaerobic growth was observed in the presence of silver (I) cyanoximates because as a biofilm accumulates, it is subjected to fluctuations in oxygen availability (Ahn et al., 2009). Differences in the anaerobic effectiveness of Ag(ACO) and Ag(BCO) compared with previously determined aerobic effectiveness were determined using bacterial growth curves, minimum inhibitory concentration (MIC) assays, minimum biofilm inhibitory concentration (MBIC) assays, and biofilm disruption assays (data not published). Increasing concentrations of Ag(ACO) and Ag(BCO) were tested in vitro using hydroxyapatite (HA) disc assays and injected into *S. mutans*-infected *Galleria mellonella* larvae.

Materials and Methods

Growth Conditions

S. mutans lab strain UA159 was struck onto Brain Heart Infusion (BHI) agar to isolate single colonies. Plates were incubated at 37℃ for 24-48 hours in a 5% (v/v) CO_2/ 95% air atmosphere. Overnight cultures were prepared by suspending an isolated colony into BHI or Typtone (TY) 1% sucrose broth and incubated at 37℃ in a 5% (v/v) CO_2/ 95% air atmosphere for 24-48 hours.

Preparation of Compounds

Ag(ACO) and Ag(BCO) compounds were resuspended in dimethyl sulfoxide (DMSO) to create three respective 1 mg/mL stock solutions. Two-fold serial dilutions were conducted from 128 ug/mL to 1 ug/mL for each compound for growth curves, hydroxyapatite (HA) disc assays, in vivo *G. mellonella* drug assays, and *G. mellonella* treatment assays. Media (BHI or TY 1% sucrose) inoculated with UA159 and media only were used as positive and negative controls, respectively.

Aerobic and Anaerobic Growth Curves

Two 100-well honeycomb plates were inoculated: one under aerobic growth conditions and the other under anaerobic growth conditions. The aerobic 100-well honeycomb plate was inoculated with positive and negative controls and increasing concentrations of Ag(BCO) from 1ug/mL to 128 ug/mL. The anaerobic 100-well honeycomb plate was inoculated with positive and negative controls, increasing concentrations of Ag(BCO) from 1ug/mL to 128 ug/mL, and 25 mL mineral oil in each inoculated well. The optical density (OD_{600}) was read every hour, starting at inoculation using a Bioscreen C Automated Growth Curve Analysis System incubated at 37℃ for 48 hours or until cultures reached the stationary phase. The same protocol was

followed to generate aerobic and anaerobic growth curves of UA159 in the presence of Ag(ACO). OD_{600} was plotted against time for all experimental conditions to create growth curves. (Ahn et al., 2009).

Hydroxyapatite (HA) Disc Assay

The salivary pellicle solution was prepared by combining human saliva with absorption buffer (50mM KCl; 1mM KH2PO4; 1mM CaCl2; 0.1mM MgCl2 – pH 6.5) at a 1:1 ratio. The solution was centrifuged for 10 minutes at 3000 rpm, and the supernatant was collected and filtered through a bottle-top filtration unit. Sterile hydroxyapatite discs were soaked in the prepped salivary pellicle solution for 24 hours at room temperature. The soaked discs were placed into another 24-well plate prepped with sterile orthodontic wire racks and vertically positioned hydroxyapatite discs in the wire. The plate was inoculated with positive and negative controls, increasing concentrations of Ag(BCO) from 4 ug/mL to 128 ug/mL and diluted 1:10 *S. mutans* UA159 culture in TY 1% sucrose. The 24-well plate was incubated at 37℃ for 24 hours in a 5% (v/v) CO2/ 95% air atmosphere. To recover the 24-hour biofilm, hydroxyapatite discs were soaked in fresh media to remove loosely adhered cells, which were then physically scraped with a sterile spatula and placed into another 24-well plate with fresh TY 1% sucrose media. Ten-fold serial dilutions were performed on a sample from each well from 10^{-1} to 10^{-4}. Serial dilutions were plated using the spot plate technique and a 10 uL sample from each well. Dilutions from 10^{-3} to 10^{-6} were used to determine the bacterial load. After inoculation, all plates were incubated for 48 hours at 37°C in a 5% (v/v) CO_2/ 95% air atmosphere. After incubation, colonies were counted using a Quebec colony counter to calculate CFU/mL for each drug concentration. The same protocol was followed to generate hydroxyapatite disc assays in the presence of Ag(ACO). Experiments were performed in triplicate. (Kovacs et al., 2017).

In Vivo Drug Toxicity

Galleria mellonella or wax moth larvae were stored in wood chips at 4℃ until use. Only non-melanized, healthy-looking larvae were injected with 5 uL aliquots of saline control, negative non-injected control, and increasing concentrations of Ag(BCO) from 4 ug/mL to 128 ug/mL through the hindmost right proleg using a sterile 10 uL Hamilton syringe. The worms were incubated at room temperature for five days, and every 24 hours, larvae were scored as dead or alive based on the waxworm's movement and melanization. For each condition, at least 10 larvae were tested. The same protocol was followed using Ag(ACO) injections. (Ignaskiak and Maxwell, 2017).

Treatment Assay

Galleria mellonella or wax moth larvae were stored in wood chips at 4℃ until use. Only non-melanized, healthy-looking larvae were infected through the hindmost right proleg with *S. mutans* UA159 (OD_{600} normalized to 0.6) using a sterile 5 uL Hamilton syringe. Control groups were injected with TY 1% sucrose only, and an uninjected control group was present. After treatment, the larvae were incubated at room temperature for one hour. One hour post-injection, 3 replicates for each condition (bacterial, media, or no injection) were injected with 5 uL of increasing concentrations of Ag(BCO) from 4 ug/mL to 128 ug/mL and a saline control through injection to the hindmost left proleg. The larvae were incubated at room temperature for five days. Every 24 hours, larvae were scored as dead or alive based on the waxworm's movement and melanization. The same protocol was followed using Ag(ACO) injections. (Cools et al., 2019).

Results

Growth Curves

Oxygen has a potent influence on the expression of genes and the activity of physiological and biochemical pathways in bacteria. We have found that oxygen significantly altered virulence-related phenotypic properties of *S. mutans*, the primary etiological agent of human dental caries (Ahn et al., 2009). Aerobic and anaerobic growth curves were generated to determine if growth conditions have an effect on Ag(BCO) and Ag(ACO) compounds' ability to inhibit *S. mutans* UA159 growth. Growth was tracked over a 48-hour time period in the presence of increasing concentrations of each compound from 1 ug/mL to 128 ug/mL. Higher concentrations of both compounds have a greater negative effect on *S. mutans* growth. For Ag(BCO), significant decreases in growth compared to the UA159 control occurred at 64 ug/mL and 128 ug/mL, with a small delay in the log and stationary phases in the presence of 32 ug/mL Ag(BCO) (Figure 3). For Ag(ACO), significant decreases in growth compared to the UA159 control occurred at 64 ug/mL and 128 ug/mL. Unlike Ag(BCO), Ag(ACO) did not experience as much delay in the log phase at 32 ug/mL (Figure 4).

Growth curves were performed anaerobically by overlaying the inoculated media with mineral oil. For both Ag(BCO) and Ag(ACO), there is no significant difference in aerobic and anaerobic *S. mutans* growth (Figures 3 and 4). For both compounds, growth conditions do not affect the effectiveness of the drug on *S. mutans* growth.

Hydroxyapatite Disc Assay

S. mutans colonizes the oral cavity by attaching to the tooth's surface and forming a biofilm. To mimic this scenario and to test the compound's efficacy in vitro, we performed hydroxyapatite (HA) disc assays. 24- well plates were inoculated with the silver (I) cyanoximate

compounds and bacterial culture pre-incubation to observe biofilm formation inhibition in the presence of the compounds. Biofilm growth was measured based on CFU/mL. Ag(BCO) and Ag(ACO) had complete inhibition of biofilm growth at 128 ug/mL and 64 ug/mL (Figure 5). At drug concentrations lower than 32 ug/mL, the calculated CFU/mL varies; however, biofilm growth in the presence of each compound remains lower than the positive UA159 control (Figure 5).

In Vivo Drug Toxicity

As our goal is to test the use of silver(I) cyanoximates in dentistry, we first tested the toxicity of the compounds using an invertebrate animal model. Therefore, to ensure silver (I) cyanoximate compounds were not toxic to *G. mellonella* larvae, an in vivo drug assay was performed. Specific concentrations of each drug were injected into the hindmost right proleg, and larvae were incubated at room temperature for 5 days. Every 24 hours, larvae were scored as alive or dead based on movement and melanization. This was an important assay to ensure specific concentrations of Ag(BCO) and Ag(ACO) alone would not kill or harm *G. mellonella* larvae. All tested concentrations of Ag(BCO) and Ag(ACO) were no more harmful to the larvae than the PBS control and were not toxic to *G. mellonella* larvae (Figure 6).

Treatment Assay

The ability of Ag(BCO) and Ag(ACO) to eliminate or limit a bacterial *S. mutans* infection in vivo is important information to determine the future applications of silver (I) cyanoximates, including their incorporation into dental materials. Model organisms, *Galleria mellonella* larvae, were used to determine the in vivo effect of silver (I) cyanoximates using treatment assays. *G. mellonella* or wax moth larvae were injected with media control, a normalized culture of *S. mutans* UA159 (OD_{600}= 0.6), or no treatment and incubated at room

temperature. One hour post-injection, at least three larvae per condition were injected with saline control or increasing concentrations of Ag(BCO) and Ag(ACO), respectively. All concentrations of Ag(BCO) and Ag(ACO) successfully eliminated, limited, or delayed bacterial infection in *G. mellonella* larvae compared to *S. mutans* UA159 control (Figure 7). Compared to the untreated and PBS-treated larvae (controls) with 0% survival after 2 days, larvae experienced higher percent survival when treated with 16 ug/mL, 64 ug/mL, and 128 ug/mL Ag(ACO) and 128 ug/mL Ag(BCO). Larvae treated with 16 ug/mL Ag(ACO) experienced 100 % survival after 5 days, living at least 3 days longer than the untreated control groups (Figure 7).

Discussion

The scope of this project was to test the efficacy of Ag(ACO) and Ag(BCO) on *S. mutans* activity in vitro and in vivo. Biofilm development is a major virulence factor of *S. mutans*, the causative agent of dental caries (Yoshida and Kuramitsu, 2002). Previous data in the Santiago lab has established the in vitro antimicrobial activity of Ag(BCO) and Ag(ACO) on both planktonic and biofilm growth of *S. mutans* (data not published). At high concentrations, these silver (I) cyanoximate compounds decrease both planktonic and biofilm growth of *S. mutans*. *S. mutans* is a known facultative anaerobe and experiences anaerobic pockets within a biofilm community (Wong and Liu, 2010). The effects of the silver compounds on the growth of *S. mutans* in varying conditions (with and without oxygen) were of interest because the oral cavity has fluctuating oxygen levels (Ahn et al., 2009). These oxygen fluctuations are determined by biofilm maturity. Cells that begin colonization are exposed to an oxygen-rich environment, whereas cells within a mature biofilm experience limited oxygen diffusion (Ahn et al., 2009). Therefore, we aimed to test the effectiveness of silver (I) cyanoximates anaerobically.

S. mutans is a facultative anaerobe; therefore, to establish the efficacy of silver (I) cyanoximates in oxygen-rich versus oxygen-poor (within a mature biofilm) environments, anaerobic growth curves were performed. There is very little data on the mechanism of silver (I) cyanoximates in an anaerobic environment; however, common antimicrobials, like penicillin, ampicillin, and amoxicillin, often experience enzymatic inactivation via beta-lactamases in the presence of oral anaerobes (Brook et al., 2013). It is hypothesized that silver compounds' bacteriostatic and bactericidal effects on oral microbes are due to their interactions with microbial DNA or inactivation of bacterial electron transport chains (ETC) by reacting with the sulfhydryl groups in the essential enzymes of the ETC (Gerasimchuk et al., 2010). If no oxygen is present, bacterial metabolism shifts to fermentation and genes encoding for components of the TCA cycle are transcriptionally inactive (Ahn et al., 2009). Our data show no significant difference in the anaerobic efficacy of Ag(ACO) or Ag(BCO) on the planktonic growth of *S. mutans* compared to aerobic conditions (Figures 3 and 4). This data is crucial in the continued research on the mechanism of action of silver (I) cyanoximates.

The similarity of the anaerobic and aerobic growth curves demonstrated that growth conditions do not affect the efficacy of the silver (I) cyanoximate compounds on *S. mutans* growth (Figures 3 and 4). As growth conditions have no effect on the silver (I) cyanoximate effectiveness, we then focused our research on drug in vitro testing. Thus, our research shifted to determining the efficacy of Ag(BCO) and Ag(ACO) on *S. mutans* biofilm growth using hydroxyapatite discs. Hydroxyapatite discs create in vitro surfaces that mimic tooth surfaces in the oral cavity (Forssten et al., 2010). The antimicrobial properties of silver compounds allow us to hypothesize that Ag(BCO) and Ag(ACO) would decrease or have negative effects on biofilm growth of *S. mutans*. Our results support this hypothesis, demonstrating that with higher

concentrations of Ag(BCO) and Ag(ACO), *S. mutans* experienced decreased biofilm growth compared to the UA159 control group.

Once developed, biofilms are extremely difficult to treat. This is due to a lack of antibiotic penetration, quorum sensing abilities, adaptive stress responses, and the formation of persister cells within the dynamic multi-species community (Lotlikar et al., 2019). Therefore, anti-biofilm non-antimicrobial development is crucial. Hydroxyapatite disc assays were performed to gather quantitative data suggesting whether or not Ag(BCO) and Ag(ACO) prevent biofilm growth of *S. mutans*. Natural tooth surfaces in the oral cavity are coated in a protective film called the acquired salivary pellicle, which protects against S. mutans' acidic attacks (Vacca-Smith et al., 1996). To accurately mimic the oral environment, the hydroxyapatite discs were soaked in human saliva to adhere salivary proteins and glycoproteins to the discs before inoculation with *S. mutans* (Vacca-Smith et al., 1996). 24-well plates were inoculated with increasing concentrations of Ag(BCO) and Ag(ACO), respectively, and *S. mutans*. Wells subjected to 128 ug/mL and 64 ug/mL showed no biofilm growth in both compounds (Figure 5). Lower concentrations of the drug experienced reduced CFU/mL compared to the *S. mutans* UA159 control, still demonstrating a reduction in biofilm growth (Figure 5). Slightly higher concentrations of Ag(BCO) and Ag(ACO) were required for the complete inhibition of biofilm growth compared to concentrations required for the inhibition of planktonic growth (Figures 3A, 4A, and 5). We expected to see these differences due to the limited ability of antimicrobials to diffuse through a biofilm. This data illustrates that biofilms are more resistant to environmental stressors than planktonic bacteria and are extremely difficult to treat (Lotlikar et al., 2019).

Bacterial adhesion and biofilm formation predispose the development of dental caries; therefore, it is crucial to demonstrate silver (I) cyanoximates' effect on biofilm development

(Gerasimchuk et al., 2010). Within dentistry, biofilm prevention data is important for the incorporation of silver (I) cyanoximates in post-plaque removal applications and preventative care. However, the effects of silver (I) cyanoximates against established biofilms are equally as important. A good antimicrobial agent should be able to inhibit biofilm formation and destroy already-formed biofilms. Mechanical cleaning via tooth brushing and flossing are the most common methods of dental biofilm control; however, this is only effective in easily accessible areas of the oral cavity (Martinez-Hernandez et al., 2020). Anti-biofilm agents can be used in combination with preventative, mechanical care to further biofilm management at home and within dentistry.

The overarching goal of this research is to incorporate silver (I) cyanoximates or other silver-based compounds into dental materials and subcutaneous medical devices. One of the preliminary steps of this process is in vivo testing, specifically mammalian or insect studies. As previously described, *G. mellonella* larvae are a model system for human pathogens and antimicrobial sensitivity testing. This is because the larval immune response is analogous to the mammalian innate immune response due to their phagocytic blood cells, called hemocytes, which are involved in phagocytosis, encapsulation, and clotting (Tsai et al., 2016). Drug toxicity assays ensure the drug is not toxic to the living system, and treatment assays test the ability of our compounds to eliminate or limit a bacterial *S. mutans* infection. Depending on the administered dosage, Ag(ACO) and Ag(BCO) were not toxic in vivo and demonstrated antibacterial efficacy after *S. mutans* infection in *G. mellonella* larvae (Figures 6 and 7).

Despite these findings, there are several limitations to using *G. mellonella*; most significantly, unlike mammals, these larvae do not have an adaptive immune system. To mitigate this, treatment with the silver compounds was distributed one-hour post-injection. This

infection-treatment model allows us to focus on the first line of defense, the innate immune system, which is very similar in insects and humans (Cools et al., 2019). Innate immune system larval blood cells (hemocytes) are mechanistically similar to human macrophages in their phagocytic processes (Cools et al., 2019). Despite these limitations, *G. mellonella* larvae act as a rapid and cost-effective in-between step in drug development, providing valuable information for future in vivo studies involving more complex vertebrates (Tsai et al., 2016).

The next step to discovering the antimicrobial activity of silver (I) cyanoximates is a more in-depth biochemical mechanism of action of these compounds. The effectiveness of specific silver (I) cyanoximates is relatively unknown because chemical interactions and modes of action remain unexplored. Current assays (MIC, MBIC, HA disc, and treatment assays) do not reveal the mode of action of the compounds but rather their effect on bacterial growth. Based on the results of the assays presented, silver (I) cyanoximate compounds Ag(BCO) and Ag(ACO), demonstrate effective antimicrobial properties. However, the applications of this data are limited if the biochemical interactions between silver (I) cyanoximates and *S. mutans* in the oral cavity remain unknown. Further, if the mechanism of these silver compounds is known, more in-depth combinational antimicrobial strategies can be explored.

Overall, we observed that Ag(BCO) and Ag(ACO) have a negative effect on *S. mutans'* biofilm growth on hydroxyapatite discs and can be used as non-toxic, antimicrobial treatments that eliminate or limit *S. mutans* bacterial infection in vivo—depending on the administered dosage. Future directions, including determining the mode of action of silver (I) cyanoximates, hydroxyapatite disc biofilm disruption assays, and more complex in vivo studies, can further our understanding of silver (I) cyanoximates' antimicrobial effects on *S. mutans* biofilm growth. Ultimately, this data contributes to the overall understanding of *S. mutans'* activity in the

presence of silver (I) cyanoximate compounds and the emergence of dental treatments utilizing silver compounds in the management and prevention of dental caries.

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Figures

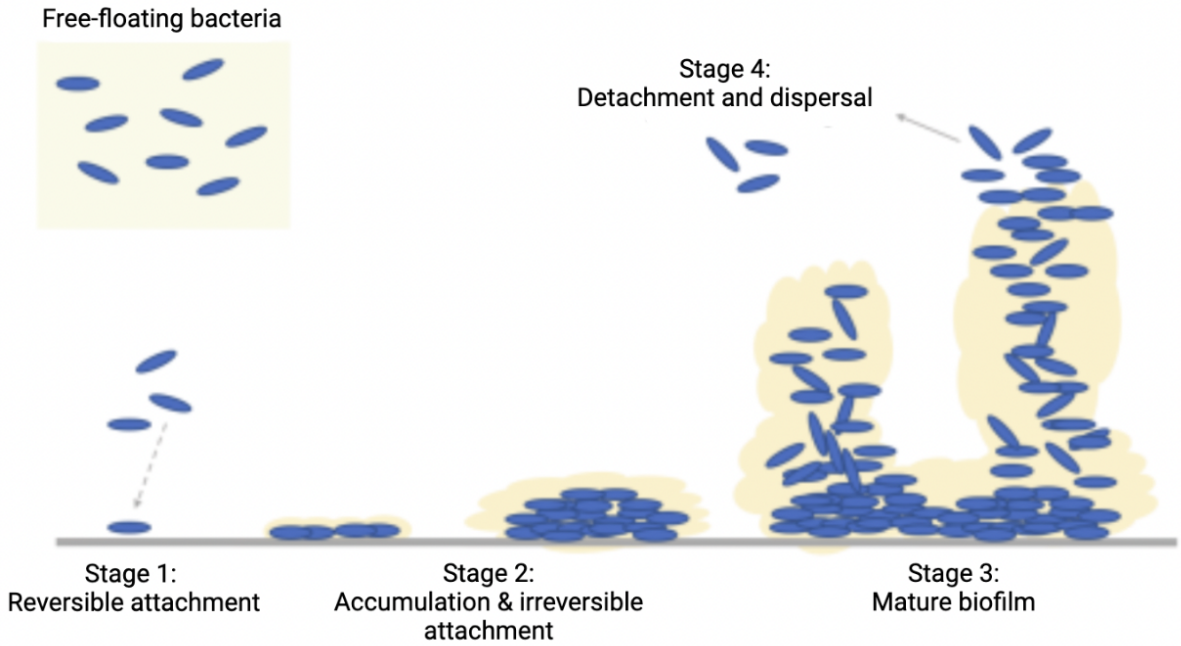

Figure 1. Stages of Biofilm Development. Planktonic cells reversibly attach to a surface and quickly accumulate bacteria. This accumulation forms a monolayer and a matrix whose interactions result in irreversible attachment. A mature biofilm forms a mushroom-like structure that contains channels for the distribution of nutrients and the expulsion of metabolic waste products. After a mechanical or chemical force, quorum sensing signals cells to detach and disperse to colonize new surfaces. Figure created with BioRender (adapted from Vasudevan, 2014).

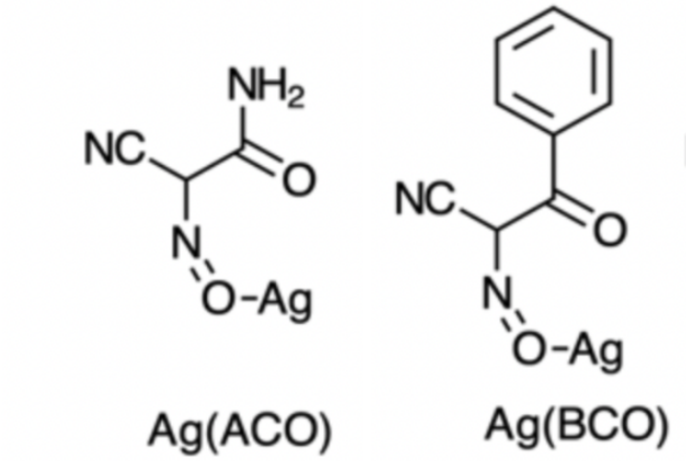

Figure 2. Chemical Structure of Silver (I) Cyanoximates of Interest. Silver (I) Nitrosodicyanomethanide (Ag(ACO)) and Silver (I)α-Oximido-(2-benzoyl)acetonitrile (Ag(BCO)).

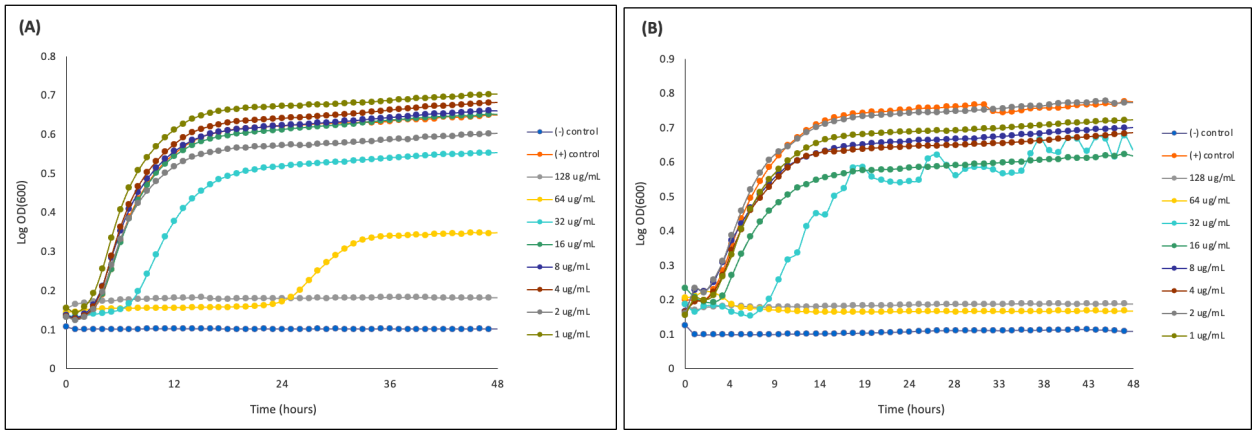

Figure 3. Ag(BCO) shows no change in its effect on *S. mutans'* growth in aerobic or anaerobic conditions. Growth curves were performed using 100-well honeycomb plates inoculated with increasing concentrations of Ag(BCO) from 1 µg/mL to 128 µg/mL. Anaerobic plates were inoculated with mineral oil, and both (A) aerobic and (B) anaerobic plates were incubated at 37℃. Absorbance values (OD_{600}) were recorded every hour for a 48-hour time period. OD_{600} values were plotted against time to generate growth curves.

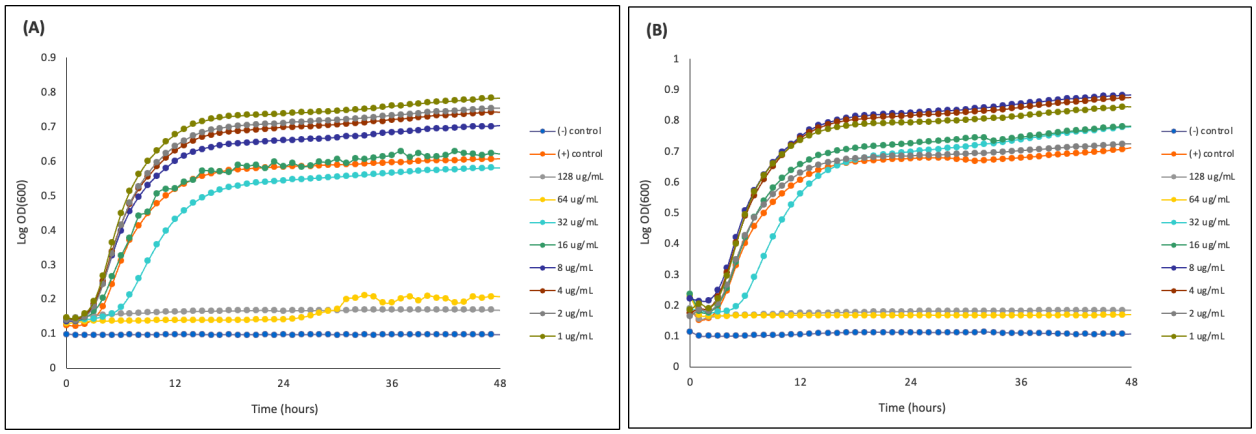

Figure 4. Ag(ACO) shows no change in its effect on *S. mutans'* growth in aerobic or anaerobic conditions. Growth curves were performed using 100-well honeycomb plates inoculated with increasing concentrations of Ag(ACO) from 1 µg/mL to 128 µg/mL. Anaerobic plates were inoculated with mineral oil, and both (A) aerobic and (B) anaerobic plates were incubated at 37℃. Absorbance values (OD_{600}) were recorded every hour for a 48-hour time period. OD_{600} values were plotted against time to generate growth curves.

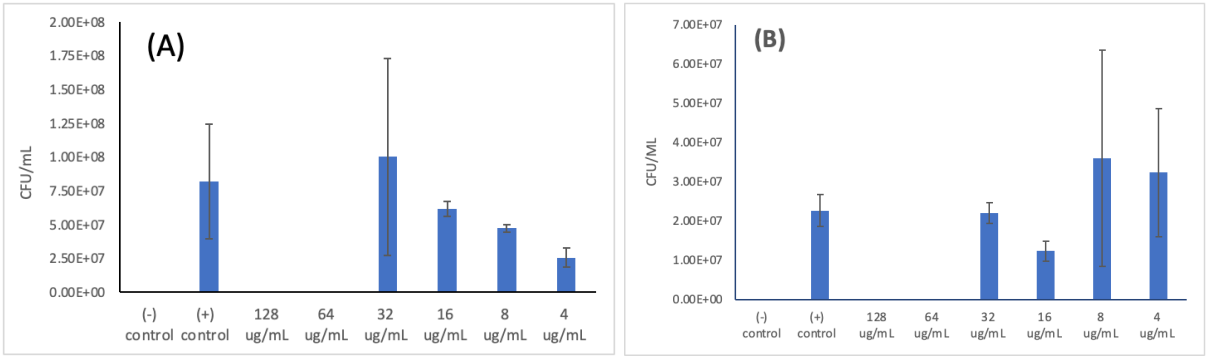

Figure 5. Higher concentrations of Ag(BCO) and Ag(ACO) demonstrated negative effects on *S. mutans'* biofilm growth on hydroxyapatite discs. Hydroxyapatite (HA) disc assays were performed using hydroxyapatite discs soaked in human saliva and absorption buffer (1:1). Two 24-well plates were inoculated with increasing concentrations of (A) Ag(BCO) and (B) Ag(ACO) from 4 ug/mL to 128 ug/mL and incubated at 37℃ for 24 hours. HA discs were scraped, and biofilm-forming bacteria were serially diluted and plated. Plates were incubated at 37℃ for 24 hours, and CFU/mL was calculated.

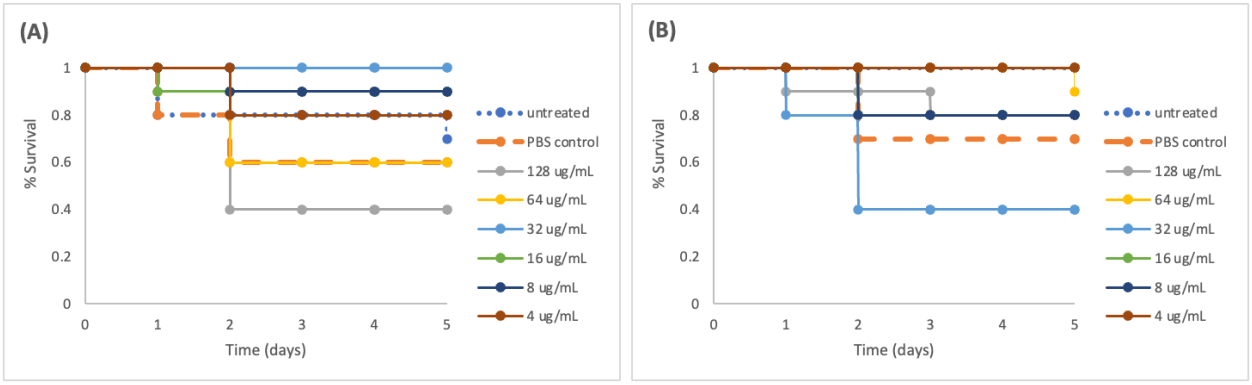

Figure 6. Ag(ACO) and Ag(BCO) are not toxic to *Galleria mellonella* larvae. Survival of untreated larvae after treatment with different concentrations of Ag(ACO) and Ag(BCO). (A) Survival of larvae treated with Ag(ACO). (B) Survival of larvae treated with Ag(BCO).

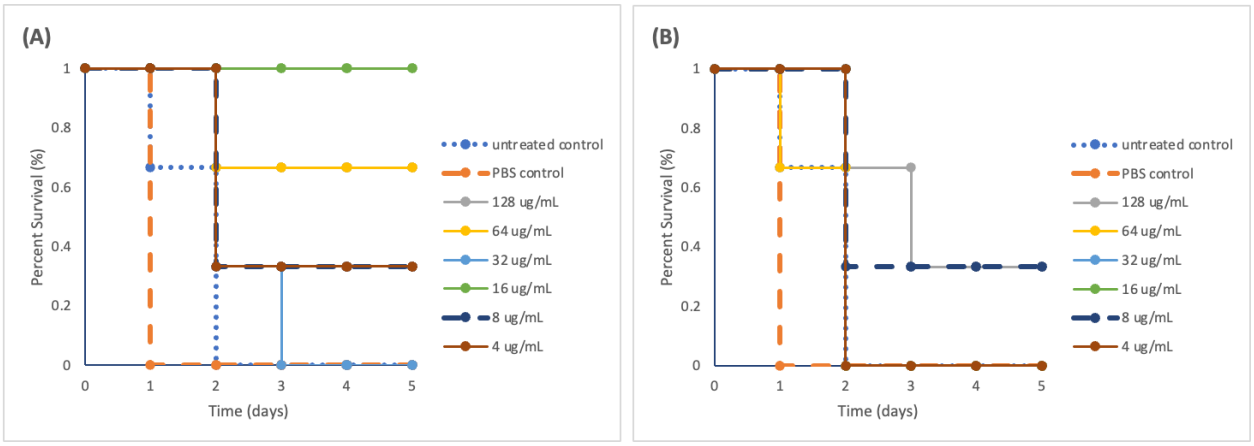

Figure 7. Higher concentrations of Ag(ACO) and Ag(BCO) demonstrate in vivo antibacterial effectiveness against *S. mutans* infection in *G. mellonella* larvae. In vivo treatment assay on *Galleria mellonella*. (A) survival of larvae treated with Ag(ACO) after *S. mutans* UA159 infection; (B) survival of larvae treated with Ag(BCO) after *S. mutans* UA159 infection.