TWO PLANT EXTRACTS THAT CAUSE UNIQUE AGGREGATED PHENOTYPES IN *STAPHYLOCOCCUS AUREUS* AND COULD ACT AS AN ANTIBIOTIC ALTERNATIVE

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Dedication

For Avery, Maya, and Sean. Everything I do is for you. And, for Oshini Fernando and Shima Asghari without whom, I couldn't have done this.

Abstract

This thesis is about the abilities of two plant extracts to cause aggregation or "clumping" of *Staphylococcus aureus.* This project is in response to rising levels of antibiotic-resistant bacteria, and a lack of methods to control the dispersal of bacteria of agricultural origin. As the implementation of antibiotics in the agricultural sector has increased since the discovery of antibiotics, concerns regarding human health have arisen. The use of antibiotics acts as a selection pressure against bacteria that are susceptible to the antibiotic's effects. This allows for those bacteria that possess antibiotic resistance to reproduce, creating strains of mainly antibiotic-resistant bacteria. The purpose of this thesis is to investigate two clump-inducing plant extracts that are a potential tool to limit the dispersal of bacteria in solution (ex. Bacteria in agricultural wastewater). By promoting clumping in the bacteria, it is thought that the bacteria's dispersal will be limited, decreasing instances of infection, thereby indirectly reducing the need for antibiotic use. The two plant extracts studied here were selected from a library of bacterial clump-inducing plant extracts. They come from different botanical families and induce clump formation in *S. aureus* resulting in differing clump phenotypes. In addition to clump formation, both plant extracts cause a significant reduction in the growth of *S. aureus (*F (3, 72) = 4.022E+17, p < 0.001), (F (3, 58) = 185, p < 0.001). It is found that clumping molecules act in an adhesive manner and that the bacteria do not play an active role in clump formation. With this finding the first steps of affinity purification of clumping molecules were carried out for further identification of their chemical identities. This thesis outlines a procedure to study, compare and purify other clump-inducing plant extracts, many of which have not been investigated.

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List of Abbreviations

ATCC: American type culture collection

ARB: Antibiotic-resistant bacteria

ARGs: Antibiotic resistance genes

EPS: Extracellular polymeric substance

DMSO: Dimethylsulfoxide

DWTP: Drinking water treatment plants

HGT: Horizontal gene transfer

MH: Mueller Hinton

TSA: Trypticase soy agar

TSB: Trypticase soy broth

TFA: Trifluoroacetic acid

WWTP: Wastewater treatment plants

Chapter 1: Introduction

1.1 Antibiotics.

The discovery of antibiotics was inarguably a gateway to the advances of modern medicine. Antibiotics not only allow one to treat bacterial infections, but also prevent them, enabling modern medical procedures, such as C-sections, open-heart surgery, cancer treatments, or even wisdom tooth removal (Hutchings et al., 2019). In 1928 Alexander Fleming discovered the first commercially available antibiotic, Penicillin after a Petri dish of bacteria was contaminated with fungus *Penicillium notatum (*Kong et al., 2010). The discovery of Penicillin was the starting point for the development of new antibiotics. As seen in figure 1, the "golden age" of antibiotic discovery yielded many new antibiotic compounds. Fleming's discovery demonstrated a concept originally suggested by Louis Pasteur, antibiosis (Hutchings et al., 2019; Kong et al., 2010). This was used to describe the theory that microbes secrete chemicals that kill or inhibit the growth of other microbes (Kong et al., 2010).

Having seen through Penicillin's discovery that microbes produce antibiotics as a defence mechanism against other microbes they may encounter, Selman Waksman conducted a systematic study aimed at identifying antibiotics produced by microbes (Kresge et al., 2004). Fleming's observations demonstrated the ability of fungi to secrete antimicrobial compounds. Waksman's work broadened the search to include antimicrobial compounds produced by bacteria and fungi (Kresge et al., 2004). Throughout his time, he developed a method to identify naturally occurring antibiotic properties and compounds, which would allow him to discover more than twenty natural inhibitory compounds including streptomycin (Kresge et al., 2004).

Once an antibiotic compound has been discovered, it may be chemically modified to better suit therapeutic use, or to create compounds suitable to antibiotic-resistant bacteria (Hutchings et al., 2019). Dorothy Hodgkin's discovery of the beta-lactam structure of Penicillin was a breakthrough that allowed for semi-synthetic derivatives of Penicillin to be produced (Hutchings et al., 2019). This discovery led to the development of other Penicillin class antibiotics and contributed to the Golden

Age of antibiotic discovery (Kong et al., 2010). By modifying naturally occurring compounds secreted by microbes, the area of exploration into new antibiotics widened (Kong et al., 2010). For example, Methicillin was the first semi-synthetic compound to be commercially developed, in 1960. The benefit of using Methicillin over penicillin is that it bypasses Penicillin resistance mechanisms in bacteria (Kong et al., 2010).

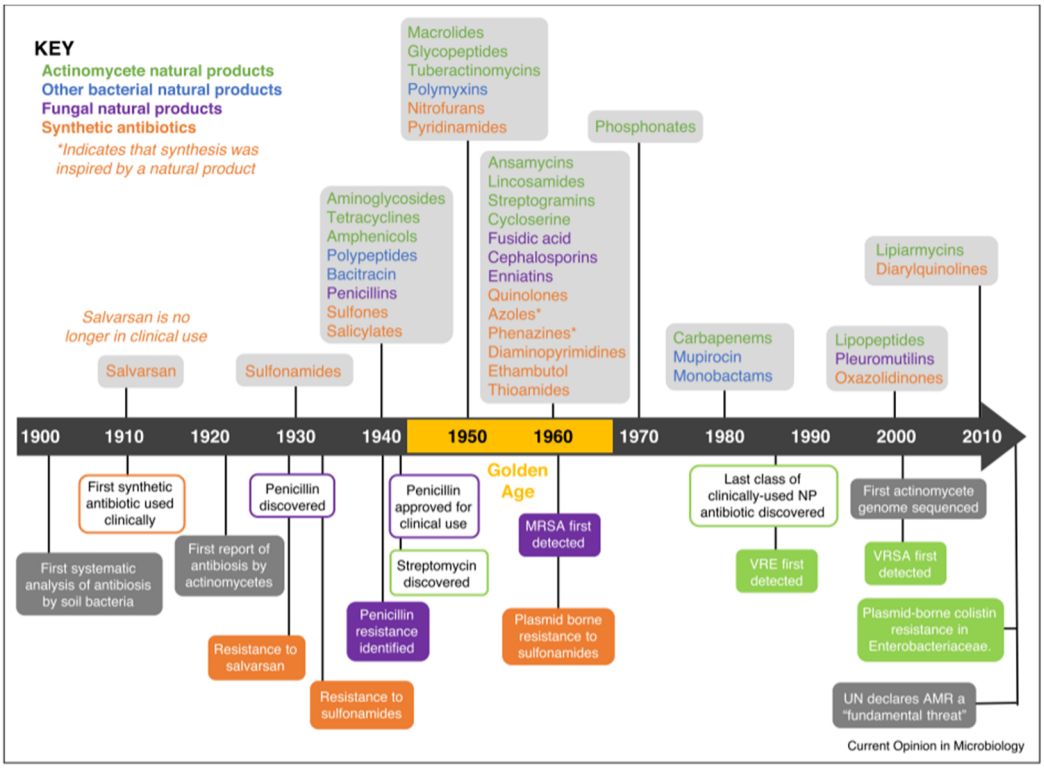

Figure 1. Timeline of antibiotic discovery in the 20th and early 21st centuries. Figure is from Hutchings et al., (2019), pg. 73. Figure is covered by a Creative Commons license.

Identifying antibiotics with diverse mechanisms of action is crucial not only for effectively targeting various bacterial strains but also for mitigating the emergence of antibiotic-resistant strains of bacteria (Donadio et al., 2010; Hutchings et al., 2019; Santajit & Indrawattana, 2016). For example, Lantibiotics such as Merscidin and Cinnamycin are post-translationally modified peptides originating from the bacterial ribosome. Isolated from Gram-positive species of bacteria such as *Firmicutes* and *Actinobacteria,* they are only effective against Gram-positive bacteria they act by targeting the cell wall (Nagao et al., 2006). Other antibiotic mechanisms of action include targeting cell wall synthesis, targeting protein synthesis by interrupting the ribosome, inhibiting of metabolic pathways, or targeting nucleic acid synthesis (Santajit & Indrawattana, 2016).

As seen in figure 1, there was a decline in the rate of antibiotic discovery in the late 1960s. The number of new antibiotic compounds from microbial sources being discovered began to dwindle; as economic investments switched to medical procedures made possible by the development of antibiotics, including tumor-removal, open heart surgery, caesarian sections, etc. (Hutchings et al., 2019). Currently, all commercially available antibiotics are derived from microorganisms or from partially or completely synthesized molecules made in a laboratory (figure 2). It is important to acknowledge that sources of natural product antibiotics may still exist in undiscovered microbes. Based on anecdotal evidence from Indigenous peoples' traditional medicine, and additional scientific evidence, it is expected that plants hold the potential to serve as the source for the next generation of antibiotics (Hutchings et al., 2019; Kong et al., 2010; Kostikova & Petrova, 2021).

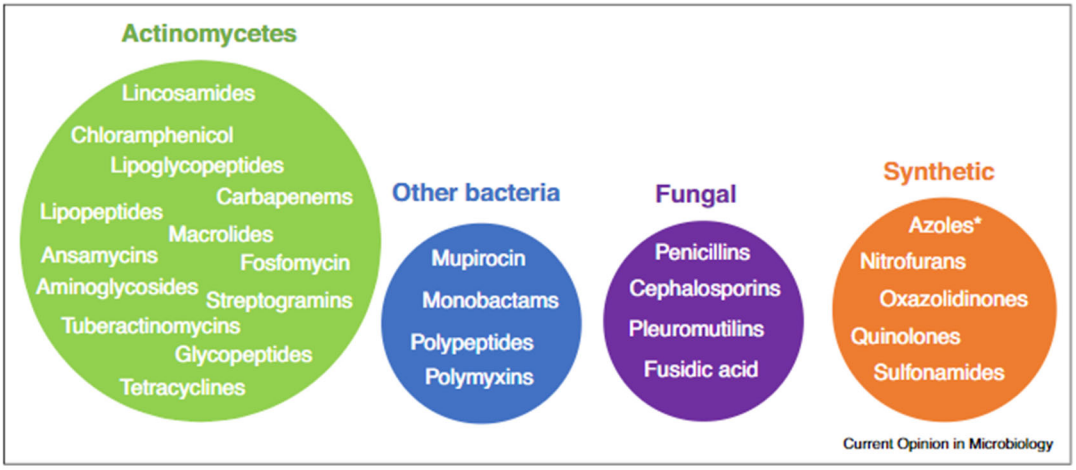

Figure 2. Origin of commercially available antibiotic compounds. Figure is from Hutchings et al., (2019), pg. 74. Figure is covered by a Creative Commons license.

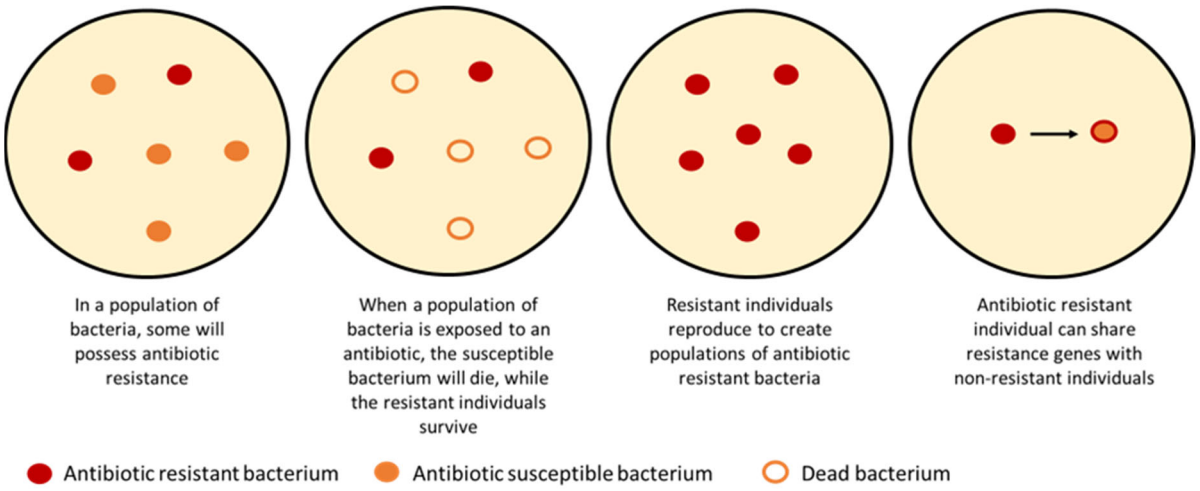

Figure 3. Treatment of a population of bacteria with an antibiotic selects for antibiotic-resistant bacteria.

1.2 Antibiotic resistance.

Antibiotic resistance naturally occurs in bacteria as a defence mechanism against antibiosis, developed through natural selection. The mechanisms of antibiotic resistance include the production of compounds that counteract the effect of an antibiotic, upregulation of efflux pumps which remove antibiotics from the cells, modifying the target structure of the antibiotics, or bypassing metabolic pathways (Santajit & Indrawattana, 2016). When a population of bacteria is exposed to an antibiotic, the individuals that are susceptible to the antibiotic will die, leaving individual bacteria that are resistant to the antibiotics to reproduce, creating strains of bacteria that are mainly antibiotic-resistant to the specific antibiotic (Hong et al., 2013). This concept is demonstrated in figure 3.

When Fleming discovered Penicillin, it was clear to him that antibiotic-resistant bacteria (ARB) existed and would increase following the usage of antibiotics and he warned of its use. Despite Fleming's warnings, the use of antibiotics in medicine and agriculture has been implemented. Because of the rampant antibiotic use, the number of strains of bacteria that are antibiotic-resistant has increased drastically (Chen et al., 2019; Hong et al., 2013; Kong et al., 2010; Liu et al., 2019). The World Health Organization (WHO) has declared antibiotic resistance as a global health concern (WHO, 2020). Without any action, it is expected that 50 million people will die every year from diseases caused by antibiotic-resistant microbes (WHO, 2020). In response, the WHO has outlined several initiatives to combat the prevalence of infections due to antibiotic-resistant bacteria. These initiatives include finding new antibiotic compounds, limiting the use of currently available antibiotics, and finding alternative strategies to antibiotic use to control the proliferation of bacteria (WHO, 2020).

1.3 Horizontal gene transfer.

Bacteria can exchange genetic material with one another, a process known as horizontal gene transfer (HGT) (figure 3) (Fall et al., 2007). HGT is considered one of the driving forces of bacterial genome evolution (Sun, 2018). Because of the ongoing exchange of genetic material among bacteria of different genera or species, the number of ARB is increasing. There are three methods by which bacteria may acquire foreign DNA: conjugation, transduction, and transformation (Burmeister, 2015; Sun, 2018). HGT allows bacteria to acquire new genes from other bacteria, allowing them to accumulate ARGs, resulting in the emergence of multi-drug resistant bacteria (MDR) (Sun, 2018).

1.3.1 *Staphylococcus aureus* and the ESKAPE pathogens.

When discussing ARB, it is important to note the *ESKAPE* pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species.* These bacterial species frequently exhibit antibiotic resistance, posing significant challenges for the healthcare system, as they are often responsible for nosocomial infection.

In this thesis the effects of clump-inducing plant extracts on *Staphylococcus aureus* ATCC 6538 will be studied. *S. aureus* is an opportunistic pathogen to both humans and animals, making the bacteria medically and agriculturally relevant (Papadopoulos et al., 2018). Many strains of *S. aureus* exhibit antibiotic resistance. Methicillin-Resistant *S. aureus* (MRSA) is resistant to all beta-lactam antibiotics. This and its ability to infect humans and animals make the bacteria particularly concerning (Papadopoulos et al., 2018). Also, because *S. aureus* grows in clustered association, it is an ideal model to demonstrate the effects of clump-inducing plant extracts (Cushnie et al., 2007; Koyama et al., 1977). In the future, it would be ideal to extend this work to other bacterial species with a special focus on bacteria from the ESKAPE group.

1.4. Agricultural sector.

1.4.1 Agriculture in Alberta.

The province of Alberta is a major contributor to Canada's agricultural industry, particularly in the production of food animals like cows, pigs, and chickens. In 2019, the Alberta agricultural industry produced 40% of Canada's total cow population (total = 4 915 000 individuals, encompassing dairy, beef cows, and calves), and 11% of Canada's pig population (total = 1 515 000 individuals, encompassing pigs of all sizes) (Alberta GOV, 2019). Alberta stands as a major contributor to Canada's food animal production (Alberta GOV, 2019).

1.4.2 Antibiotic use in agriculture.

The use of antibiotics is not only limited to the human population (Hong et al., 2013). Antibiotics are used in the production of livestock at subtherapeutic levels to control disease (prophylaxis) and to promote animal growth (Hong et al., 2013). In 2017 the government of Ontario reported that approximately 85% of all antibiotics used in Canada go towards use in agriculture. As a result, the presence of antibiotics in water systems and soil bodies that are near sites of livestock production has been widely reported; this includes water systems that provide drinking water to humans (Bird et al., 2019; Sura et al., 2022;Wang et al., 2020). Indeed, natural water and soil bodies that are in proximity to livestock production systems have been found to prominently contain ARB (Hong et al., 2013; Sura et al., 2022).

1.4.3 Antibiotics and antibiotic-resistant bacteria in agriculture wastewater.

As water disseminates through its natural systems, bacteria and potentially ARGs are dispersed into natural and human environments (Sura et al., 2022). Due to potential cross-resistance of bacteria to antibiotics used in both human and agricultural settings, the dispersal of ARB and ARGs is of great concern to human health. The need for alternative solutions to control the dissemination of ARB and ARGs is exemplified when the internal environment of waste-water treatment plants (WWTP) is analyzed.

WWTP acts as a vector for dispersal of both ARB and ARGs (Luprano et al., 2016). This is due to the conventional activated sludge process (CAS), which acts by combining wastewater with microorganisms to biologically degrade organic contaminants (Luprano et al., 2016). Its primary mechanisms employed for the removal of organic contaminants include microbial degradation, and sorption to particulate matter. CAS supplies favourable conditions for the proliferation of bacteria required for biological degradation. However, such conditions are ideal for facilitating horizontal transfer of ARGs amongst microbes. The conditions include high levels of nutrients, dense microbial populations, and persistent presence of antibiotics (Luprano et al., 2016). Boopathy (2017) confirmed the presence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in water before and after treatment in a WWTP. Indeed, the presence of ARGs has been reported in both influent and effluent from a WWTP in Alberta (Beukers, 2018). Water systems act as a method of ARB dispersal depositing ARB in environments (Bird et al., 2019).

In recent years, the issue of water scarcity has become apparent, prompting the implementation of water reuse. In agriculture, reusing water is beneficial for nutrient recovery possibilities, more rational allocation and conservation of freshwater resources, control of pollution and increase in food production in water-scarce areas (Luprano et al., 2016). For reuse, water must go through a WWTP to remove inorganic and organic contaminants. This is true for both municipal and agricultural wastewater. However, current methods of treating wastewater do not effectively reduce the influx of bacteria from agricultural origin, rather they provide ideal conditions for these bacteria to proliferate.

1.4.4 Antibiotics and antibiotic-resistant bacteria of agricultural origin in soil bodies.

It was found that soil bodies near livestock production sites contain increased levels of antibiotic-resistant bacteria (Hong et al., 2013). Healthy soils are crucial for the production of high-quality plants needed to feed humans and other animals (Jansson & Hofmockel, 2018). Microbial diversity is a key indicator of soil health (Gatica & Cytryn, 2013). Undisturbed, natural soils contain bacteria that have evolved *in situ* (Gatica & Cytryn, 2013). Alteration of the soil microbiome through an influx of

bacteria from agricultural origin could change the microbial community and negatively affect soil health, and crop yield (Gatica & Cytryn, 2013). This is especially pertinent due to the presence of other contaminants in agricultural wastewater such as dissolved salts that could change salinity or pH, and the presence of veterinary pharmaceuticals such as antibiotics (Gatica & Cytryn, 2013; Sura et al., 2023).

Bougnom and Piddok (2017) demonstrated the presence of ARB in agricultural wastewater, as well as the ability of bacteria from agricultural origin to colonize the environment into which they have been introduced. They examined the presence of ARB in fields irrigated with agricultural wastewater compared to those irrigated with non-wastewater in three African cities. The prevalence of ARB in soils that were irrigated with agricultural wastewater was 72% higher than in soils irrigated with freshwater (Bougnom et al., 2020). It is noted that the influx of foreign bacteria and chemicals from agricultural sources could lead to a widespread change in the soil's microbiome (Gatica & Cytryn, 2013). Zhang et al., (2022) show the ability of ARGs of agricultural origin to persist in soils.

1.4.5 Reaction to the antibiotic crisis in agriculture.

The obvious method to reduce the spread of ARB is to reduce the use of antibiotics in agriculture. In Scandinavian countries, Sweden, Denmark, Norway and Finland, the use of antibiotics as growth promotors in livestock was banned in 1986 (Bengtsson & Wierup, 2006). Bengtsson and Wierup (2006), looked at the levels of ARB in swine feces in 2004, 18 years after antibiotic use was banned for use as a growth promotor. Reduced levels of ARB were found in all countries (Sweden (65%), Denmark (47%), Norway (40%) and Finland (27%)) (Bengtsson & Wierup, 2006). In 2018, restrictions have been put in place in Alberta that requires livestock producers to acquire a veterinary prescription to purchase antibiotics (BRC, 2018).

In Canada, members of the poultry industry are taking steps independent from the government to reduce the proliferation of antibiotic-resistant bacteria (Diarra & Malouin, 2014). They look at several alternative methods to antibiotic use. These alternatives include the use of probiotics (live microbial

supplements that improve gut microbiome), prebiotics (non-digestible food ingredient that promotes beneficial microbial growth), nutraceuticals (ex. Herb extracts, spices, aromatic oils), acidifiers (ex. Organic and/or inorganic acids) and minerals (ex. Zinc, copper) (Diarra & Malouin, 2014).

1.5 Use of synthetic chemical and natural coagulants in wastewater treatment.

Currently, there are not widely accepted methods to reduce the dispersal of bacteria from agricultural origin through water systems. Current WWTP acts as a vector for the distribution of ARGs and the proliferation of antibiotic-resistant bacteria. While steps are being taken to reduce the negative impacts of antibiotic use in agriculture (restriction of access to antibiotics, use of alternative strategies), the discovery of new alternative solutions to antibiotic use is pertinent.

One way in which contaminants are removed from industrial wastewater is with synthetic and natural coagulants/flocculants (El Bouaidi et al., 2022). Coagulants and flocculants promote the agglomeration of particles to the compound, creating flocs. Flocs settle into a sludge and are removed from wastewater effluent. Coagulants/flocculants are used to remove turbidity and biological contaminants from wastewater (El Bouaidi et al., 2022). While synthetic chemical contaminants have been used in the past, some evidence suggests that certain synthetic chemical coagulants (ex. Aluminium salts, and ferric chloride) could have detrimental effects on human health, and on the environment (should sludge not be properly disposed of). For example, residual alum that is left in drinking water has been shown to have detrimental effects on human health (El Bouaidi et al., 2022).

Due to the drawbacks of synthetic coagulant/flocculant use, there has been a push for the discovery of coagulants/flocculants from natural sources such as plants, bacteria, and fungi (El Bouaidi et al., 2022). One study looks at the ability of plant *Moringa oleifera* (common name: drumstick tree) to cause coagulation in *Microcystis aeruginosa* in drinking water treatment plants (DWTPs). It was found that the coagulant removes approximately 80% of bacterial cells (Ribau Teixeira et al., 2017). It has also been shown that *Moringa oleifera* can cause coagulation/flocculation

of organic matter pollutants such as chlorophyll a, and other chemical pollutants (Cardoso Valverde et al., 2018; El Bou Aidi et al., 2022; Ribau Teixeira et al., 2017).

1.6 Plant-based compounds with bioactive capacities.

Plants represent a source of bioactive molecules through the form of primary metabolites (compounds produced by the plant that are vital for its growth such as carbohydrates, proteins, nucleic acids, and chlorophyll) or secondary metabolites (compounds that are produced by the plant that are not vital to its growth such as flavonoids, anthraquinones) (Molina et al., 2022; Erb & Kliebenstein, 2020; Kernéis et al., 2015). Healthy soils contain a diverse community of microorganisms (Jansson & Hofmockel, 2018). Some soilborne bacteria (strains of *Pseudomonas*) are known to benefit plants by growth promotion, or suppression of plant-pathogenic bacteria (Raaijmakers & Mazzola, 2012). Several soilborne bacteria are known to be pathogenic towards plants, including *Agrobacterium, Clavibacter, Erwinia, Pantoea, Pseudomonas, Ralstonia, Streptomyces, Xanthomonas*, and *Xylella* genera with a range of species (Raaijmakers & Mazzola, 2012). Secondary metabolites are produced by plants in response to microbial soil communities; potentially as defence mechanisms against other organisms such as bacteria, or herbivores (Erb & Kliebenstein, 2020). As such, it is expected that some plants contain molecules that could alter specific bacteria, such as through growth promotion, inhibition, or aggregation.

The plant *Rumex vesicarius* has been shown to exhibit antibiotic effects against *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyogenes, Klebsiella pneumoniae (*El-Bakry & Alam, 2011). In fact, several plant-based compounds have been shown to exhibit antibiotic effects, despite the lack of commercially available antibiotics of plant origin (Cushnie et al., 2007; Jantová et al., 2000; Nikitina et al., 2007).

1.7 Instances of bacterial aggregation.

1.7.1 Biofilm formation.

Biofilms are defined as a layer of microbial communities existing together in a secreted extracellular polymeric substance (EPS) (Le & Otto, 2015; Xu et al., 2016; Zhou et al., 2015). Hostile

environments such as the presence of antimicrobials may trigger biofilm formation (Zhou et al., 2015). The formation of biofilms offers a protected method of growth for microbes, as well as vector for the dispersal of ARGs through HGT (Zhou et al., 2015). Bacteria adhere to a biotic or abiotic surface and secrete EPS as they divide.

1.7.2 Proteins and carbohydrates as plant-based coagulants.

Plant-based coagulants used to reduce turbidity in natural and industrial water are most often proteins or polysaccharides (Kabir et al., 2015; Kabir & Reza, 2014; Liu et al., 2010; Yin, 2010). Certain lectins (plant-based carbohydrate-binding proteins) from the *Kaempferia rotunda* rhizome have been shown to cause aggregation in *S. aureus* and *E. coli* (Kabir & Reza, 2014).

Some cacti from the *Opuntia* genus have been shown to contain plant-based coagulants. It is proposed that the mechanism of coagulation is a result of the plants mucilage (viscous substance containing complex carbohydrates) (Yin, 2010).

1.7.3 Flavonoid causing bacterial aggregation.

The flavonoid, galangin was found to cause an aggregatory phenotype in *S. aureus,* similar to what is observed by the plant extracts studied in this thesis (Cushnie et al., 2007). The Polygonaceae plant family is characterized by having high levels of flavonoids (Yunuskhodzhaeva & Eshbakova, 2010). Flavonoids have also been readily found in plants of the Rosaceae family (Jantová et al., 2000).

Galangin has been isolated from the African plant *Helichrysum aureonitens,* which shows aggregatory effects in *S. aureus.* The plant has long been used by Indigenous peoples in South Africa for the treatment of wounds (Afolayan and Meyer, 1997). Galangin has been studied for the myriad of effects it exhibits including antimicrobial, antibiotic, antiviral, antioxidant, aggregatory, and anti-cancer effects (Cushnie et al., 2007; Fang et al., 2019; Rampogu et al., 2021). Cushnie et al. (2007) look at the aggregatory effects of Galangin on *Staphylococcus aureus.* The authors note an inhibitory

effect exhibited after treatment of the bacteria with Galangin, however, they suggest that this effect is not a bactericidal one, but rather a consequence of aggregation (Cushnie et al., 2007).

1.8 Structure, function, and bioactivity.

It is well known that the structure of a molecule relates to its biological function. Theoretically, by attempting to alter the structure of potential molecules in a sample, their function should in turn be altered. This notion will be used in this thesis to either identify or rule out potential chemical identities of plant-based clumping molecules. Within this thesis a method is suggested by which plant extracts are treated in a way that would degrade or denature specific molecules potentially present in the sample. The performance of the treated plant extracts will allow for inferences to be made on the potential chemical identity of clumping molecules.

Exposure of polysaccharides to an acid can cause degradation of the polysaccharide (Ayadi et al., 2016; Liu et al., 2010 Rumpel & Dignac, 2006; Zhao & Monteiro, 2008). Heat treatment of proteins causes their denaturation (Cavagnero et al., 1998; Matsuura et al., 2015). Should the structure of biologically active compounds be altered, it is expected that their function would be inhibited.

1.8.1 Affinity purification of clumping molecules.

Affinity chromatography is a method by which specific molecules or groups of molecules may be purified from a complex mixture (Urh et al., 2009). It relies on specific biological interactions between two molecules (ex. Enzyme and receptor) (Urh et al., 2009).

While this thesis does not identify the chemical structure of clump-inducing plant extracts, it will be suggested that clumping molecules can be purified from plant extract-induced bacterial clumps, similar to affinity chromatography.

1.9 Objectives.

As a result of the large amounts of bacteria found in agricultural wastewater (many of which are strains with antibiotic resistance) and the lack of effective methods to control the dissemination of these bacteria into water and soil bodies, there is a need for alternative strategies to manage

dissemination of bacteria from agricultural origin. It is proposed that the plant extract-induced clumping of bacteria which will be studied in this thesis could be used to limit the dispersal of bacteria through water systems by clearing bacteria from solution. This process is similar to a chemical or natural coagulant/flocculant. This could have applications in the agricultural industry for the treatment of wastewater. This thesis investigates the biological activity and chemical nature of two plant extracts that induce clump formation in *S. aureus.* Due to the lack of coverage in the literature regarding this phenotype, this is a pilot project. New methods of studying this phenotype will be suggested and some inferences will be made partially on anecdotal evidence.

Part One: Biological activity of clump-inducing plant extracts

Specific Objectives:

1. Qualitatively and quantitively assess the clump phenotype observed in *S. aureus* by two plant extracts
2. Qualitatively and quantitively assess the presence of an inhibitory effect exhibited by clump-inducing plant extracts on *S. aureus*
3. Assess the reversibility of plant extract-induced bacterial clumping

Part Two: Chemical characteristics of clump-inducing plant extracts

Specific Objectives:

1. Determine the ability of plant extracts to induce clumping in dead *S. aureus*
2. Qualitatively determine the presence of proteinaceous and carbohydrate components in clump-inducing plant extracts
3. Purify clumping molecules for further identification

Chapter 2: Materials and Methods

2.1 Plant collection.

Plant material used in this thesis was sustainably collected. The plants are found in Southern Alberta near Lethbridge (Molina et al., 2022). Plant taxonomy was identified using documents based on "The flora of Southern Alberta" and verified against University of Lethbridge Herbarium samples. A pressing of the plant was prepared with information including collection date, site description, and location.

Table 1. Information on the collection of plants used in this thesis.

Plant Code	Harvesting Date	Location
GP95B	2018	East of Lethbridge, AB
GP2215B	August 8th, 2022	Near Vulcan, AB

Table 2. Information on the plants studied in this thesis.

Plant scientific name	Common name	Plant Family	Lab Code
Potentilla norvegica	Rough cinquefoil	Rosaceae	GP95B
Persicaria amphibia	Longroot, smartweed, water smartweed	Polygonaceae	GP2215B

2.2 Plant extraction.

Collected plant material was allowed to dry at room temperature (approximately 1 month); and then separated into flowers, leaves, stems, and roots. Using a grinder, the material from either the whole plant or one portion of the plant was ground into a powder. Plant material was submerged in freshly prepared, 75% ethanol at a w/v ratio of 1:10. The solvent was chosen for its polarity (Poojar et al., 2017). The extraction was performed at room temperature for 24 hours in the dark with agitation. The suspension was filtered using a Whatman Number 1 filter. The filtrate was placed in a dark 33°C incubator to allow the complete evaporation of ethanol. The dried material was collected, weighed, placed in vials, labelled, given a code number, and stored in darkness at room temperature until use. Extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/mL, stock solution (Barnes et al., 1992). Aliquots were prepared to allow single usage of the plant extract and to avoid any impact of freeze-thaw cycles. Dissolved extracts are kept at -20 °C.

2.3 Bacterial culture.

Staphylococcus aureus ATCC 6538 stock cultures purchased from Cedarlane are kept frozen in glycerol at -20°C. *S. aureus* is inoculated onto trypticase soy agar (TSA) plates and incubated at 37°C for 24 hours. Cultures of *S. aureus* on TSA were inoculated onto fresh TSA weekly. After 12 inoculations of the original culture, cultures are discarded, and fresh cultures are started from the frozen stock (Simione and Brown, 1991). For experiments, trypticase soy broth (TSB) is inoculated with *S. aureus and* then incubated for 18 hours at 37°C with shaking (Gera & McIver, 2013).

2.4 Inhibitory and clumping activity of GP95B and GP2215B.

2.4.1 Microdilution technique.

Using GP95B, and GP2215B (plant extracts that have been shown to induce clumping in *S. aureus*), a serial dilution was performed in TSB to obtain the following final concentrations; 1, 0.5, 0.25, and 0.125 mg/mL.

The wells of a 96-well plate were loaded with a mixture of 50 µL of a 1/1000 overnight culture *S. aureus* solution in TSB and 50 µL of plant extract at various concentrations (resulting in

final plant extract concentrations of 1, 0.5, 0.25, 0.125, or 0.0625 mg/mL) (Concentrations are based on previous experiments in the laboratory). For the controls, the wells were filled with 50 µL 1/1000 *S. aureus* solution and 50 µL of media (either Tryptic Soy Broth (TSB) or a 1/50 dilution of Dimethyl Sulfoxide (DMSO) in TSB, yielding a final DMSO concentration of 1/100). As for the negative controls, 50 µL of plant extracts in TSB with 1/100 DMSO were added to wells containing 50 µL of TSB alone. Each experimental condition was plated in triplicate.

The plate was placed in a Synergy plate reader for 18 hours at 37ºC under medium agitation. The optical density (OD) was measured every 30 minutes over 18 hours at a wavelength of 630 nm. The average and standard deviation of each condition was calculated for each time point. The optical density values from each condition plated with TSB (TSB, 1/50 DMSO, or plant extract), were used to normalize each value obtained from samples containing *S. aureus.* The normalized averages and the standard deviations were used to generate the graph on Excel. A *t*-test ($p = 0.05$) was used to compare the average optical density of *S. aureus* at 18 hours treated with each concentration of the plant extract with one another and with the control. Clumping was assessed visually. Photos were taken of the plate using the Ziggi HD camera, and IPEVO image software.

2.4.2 Crystal violet assay.

The purpose of this experiment is to estimate the sizes of *S. aureus* clumps induced by GP95B and GP2215B. Crystal violet will stain the clumped bacteria. Using acetic acid, the crystal violet can be removed, and the intensity of the colour can be read to quantify clump size; allowing for comparison of clump size between samples. This technique is based off Xu et al., (2016) that allows for the quantification of biofilms using crystal violet staining.

1/1000 overnight culture *S. aureus* in TSB was treated via the protocols described in section 2.4.1 with 1 and 0.5 mg/mL GP95B, GP2215B. 50 µL of each plant extract concentration will be added to 6-9 wells containing each 50 µL of bacteria. The plate was incubated in a plate reader for 18 hours at 37ºC with agitation. While the optical density was not read, the plate reader was used for

incubation to keep the same conditions used when inducing clumps in previous experiments. After incubation, photos were taken of the plate using the Ziggi HD camera and IPEVO image software.

The surrounding solution was carefully removed from the wells using a micropipette. Clumps were resuspended in 125 µL of 0.8% crystal violet in water. The clumps were incubated at room temperature for 15 minutes. Samples were combined into tubes separated by extract and extract concentration. Samples were vortexed, and then centrifuged at 10 000 rpm for 5 minutes. The supernatant was removed. The samples were washed using 1 mL of sterile dH2O. After removing the water, 0.5 mL of 30% acetic acid was added to the samples. Samples were incubated at room temperature for 15 minutes, after which they were vortexed and centrifuged at 10 000 rpm for 5 minutes. The supernatant was collected and 100 µL of each sample was added to as many wells of a 96-well plate as the volume would allow. 100 µL of 30% acetic acid was added to three wells of the plate. A single optical density reading of the plate was taken at 550 nm using the Epoch plate reader. Optical densities obtained were averaged and normalized using the average optical density of 30% acetic acid. Regarding statistical analysis, a Two-Way ANOVA ($p = 0.05$) and Tukey-Kramer HSD test were completed.

2.4.3 Viable plate count assay.

The viable plate count technique was performed as described by (Clais et al., 2015) to quantify the clumping and inhibitory capacities of GP2215B and GP95B at 0.5 mg/mL.

1/1000 dilution of overnight culture *S. aureus* was treated via the protocols described in section 2.4.1 with GP95B and GP2215B at 0.5 mg/mL. Each condition was added to 6 wells.

In one well of the 96-well plate, plant-extract induced clumps were resuspended in the surrounding solution using a micropipette for one minute. 10 µL of the resuspended clumps in surrounding solution was added to 10 mL TSB, diluting it to 10^{-3}; the sample was then diluted to 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. This technique was repeated using 10 µL of the solution surrounding the clump, then 10 µL of nontreated *S. aureus.* This was repeated at least twice per experiment.

100 µL of each dilution was plated on TSA in triplicate. Plates were incubated overnight at 37°C. The number of colonies was counted on plates that contained between 30 and 300 colonies.

The following equation was used to determine the bacterial concentration:

Equation 1.

$$Bacterial\ concentration\ \left(\frac{CFU}{mL}\right) = \frac{number\ of\ colonies\ on\ plate\ (CFU)}{quantity\ of\ solution\ plated\ (mL)}\ x\ dilution\ of\ sample$$

The concentration of bacteria in the clumps was determined using the following equation:

Equation 2

$$Concentration\ of\ bacteria\ in\ clumps\left(\frac{CFU}{mL}\right)$$

$$= Total\ concentration\ of\ bacteria\left(\frac{CFU}{mL}\right)$$

$$-\left(\frac{CFU}{mL}\right)\ Concentration\ of\ bacteria\ in\ solution\ surrounding\ clumps$$

The following equations were used to determine log reduction

Equation 3

$$Percent\ reduction = \frac{B}{A} \times 100$$

Where A = total number of viable microorganisms without treatment

B = total number of viable microorganisms after treatment

Statistical analysis was used to determine if the difference in bacterial concentration is significant between bacteria treated with plant extracts to the control (nontreated *S. aureus*). This was

done using a One-Way ANOVA ($p = 0.05$), and Tukey's HSD. In subsequent replicates of the experiment, only the dilutions that were shown to produce 30 – 300 colonies when plated on TSA were prepared. The percent reduction of *S. aureus* after treatment with either plant extract was determined for each replicate, and averaged. The average percent reduction of *S. aureus* was compared between plant extracts using an *F*-test for variance, and *t*-test for statistical significance ($p < 0.05$). The percentage of bacteria found in the clump after treatment with either plant extract was evaluated in the same way as the percent reduction.

2.4.4 Microdiffusion technique.

The method of microdiffusion technique was developed in our laboratory based on the Kirby-Bauer Disk Diffusion Technique described by Biemer et al., (1973). This technique will allow to quantify the inhibitory effects of clumping plant extracts. A lawn of *S. aureus* was prepared using overnight culture. Using a medium-sized pipette tip, four wells were created in two TSA plates. Clump-inducing plant extracts were added to the wells: GP2215B 50 mg/mL, GP95B 50 mg/mL (20 µL of 100 mg/mL stock solution in DMSO diluted in 20 µL of water) GP2215B 25 mg/mL, GP95B 25 mg/mL (20 µL of 50 mg/mL dilution in 20 µL of water). On the second plate, 40 µL of water, DMSO, and 5 mg/mL Kanamycin were added to individual wells. Plates were incubated at 37°C for 24 hours; after which the zones of clearing were measured with a ruler in millimeters.

2.5 Reversibility of clump formation.

2.5.1 Treatment of previously clumped *S. aureus.*

It could be suggested that clump-inducing plant extracts may select for certain genotypes of bacteria in the clump, by only clumping those bacteria. If this is the case, then by treating previously clumped *S. aureus* with the plant extract that had previously induced clumping, it would be expected that re-clumping previously clumped *S. aureus* would produce larger clumps than clumping fresh *S. aureus.*

To determine whether this is the case, clumps were induced in *S. aureus* with one plant extract at 0.5 mg/mL over 18 hours. The solution surrounding the clumps was removed, and the

clumped bacteria was collected. The clumped bacteria were vortexed thoroughly, to remove the clumping molecules, and washed three times in distilled water. Again, the sample was vortexed. The previously clumped bacteria were diluted in TSB to an optical density of 0.86. This is the optical density of 1/1000 *S. aureus* overnight culture in TSB that is seen at the beginning of the previous experiments (time = 0) (therefore, the approximate bacterial concentration).

Once this optical density was reached (1/100 dilution of previously clumped *S. aureus* in TSB), the diluted, previously clumped bacteria was treated in the wells of a 96-well plate. To each well, 50 µL of previously clumped *S. aureus* was added with 50 µL of fresh TSB, or fresh plant extract (1 mg/mL plant extract for a final concentration of 0.5 mg/mL in the wells) (whichever plant extract originally clumped the bacteria). This was repeated with a fresh preparation of 1/1000 overnight culture *S. aureus* in TSB. For the controls, 50 µL of TSB was added to the wells with 50 µL of TSB or the plant extract used. Each condition was done in triplicate. The plate was incubated in the plate reader for 18 hours at 37ºC with agitation. The optical density of the wells was read every 30 minutes at a wavelength of 630 nm.

After incubation, photos were taken of the plate using the Ziggi HD camera and IPEVO image software. The presence of clump and their sizes were assessed visually. The average optical density and standard deviation at each 30 minutes for interval of each sample was determined and plotted against the time.

2.6 Chemical characteristics of GP95B and GP2215B.

2.6.1 Brightfield microscopy.

To quickly assess the clumping abilities of a plant extract, 10 µL of GP95B or GP2215B was added to a microscope slide along with 10 µL of overnight culture *S. aureus.* The solution was carefully resuspended on a microscope slide, using a 10 µL pipette. 10 µL of *S. aureus* was also combined with 10 µL of TSB on a microscope slide as a control. The microscope slides were incubated at room temperature for 5-10 minutes, to allow the solution to fully evaporate. The slides

were heat-fixed, and then stained with methylene blue (12 mg/mL) for 1 minute. The slides were viewed under brightfield microscopy using oil immersion on the Olympus BX53 Microscope. Photos were taken of the preparation using the Olympus SC50 camera on cellSens Standard Software.

2.6.2 Chemical fixation of *S. aureus*.

The fixation of *S. aureus* was performed as described by Kniggendorf et al., (2011) with some modifications. *S. aureus* was grown in TSB overnight at 37° with agitation. 1 mL of overnight culture *S. aureus* was centrifuged at 10 000 rpm for 5 minutes. The supernatant was discarded and replaced with 1 mL 75% ethanol in water (Kniggendorf et al., 2011). The sample was vortexed thoroughly and then centrifuged at 10 000 rpm. The sample was then washed twice with water then centrifuged at 10 000 rpm for 5 minutes and replaced with 1 mL of fresh water. 10 µL of fixed *S. aureus* was added to a microscope slide along with 10 µL of clump-inducing plant extract at 1 mg/mL in TSB (Section 2.8). The samples were mixed on the microscope slide using a micropipette. The time at which clumping occurred was assessed visually. The slides were heat-fixed, then stained with methylene blue. The slides were viewed under brightfield microscopy using oil immersion on the Olympus BX53 Microscope. Photos were taken of the preparation using the Olympus SC50 camera with cellSens Standard Software.

2.6.3 Heat treatment of extracts.

When heated at 90ºC for 30 minutes, it is expected that proteins will be denatured (Pinto et al., 1991). To determine whether clumping molecules in GP95B and GP2215B are proteins or have proteinaceous components, plant extracts were heat shocked, denaturing any proteins present in the sample. After which, *S. aureus* were treated with the heat-shocked plant extracts.

To prepare extracts for heat treatment, 28 µL of 100 mg/mL plant extract stock solution was resuspended in 1372 µL of TSB for a concentration of 1 mg/mL. The extracts were split between two tubes (700 µL in each tube). 700 µL TSB was added to two tubes. One tube of each sample (GP2215B 1 mg/mL, GP95B, 1 mg/mL, TSB) was placed into a 90ºC water bath, for 30 minutes. The

other three samples were incubated at room temperature for 30 minutes and acted as a negative control.

Overnight culture of *S. aureus* was vortexed thoroughly. 10 µL of overnight culture of *S. aureus* was added to a microscope slide along with 10 µL of one heat-treated sample (GP95B 1 mg/mL, GP2215B 1 mg/mL, TSB) and 10 µL of methylene blue stain. The sample was mixed on the microscope slide using a micropipette. The sample was left at room temperature to allow full evaporation (5 min). This was repeated with the other two heat-treated samples and with the non-heat-treated controls. The slides were viewed under brightfield microscopy using oil immersion on the Olympus BX53 Microscope. Photos were taken of the preparation using the Olympus SC50 camera with the cellSens Standard Software.

2.6.4 Trifluoroacetic acid treatment of plant extracts.

Trifluoroacetic acid (TFA) was diluted from stock concentration to 2 M in sterile water. Fourteen microliters (14 µL) of a stock solution containing plant extracts GP95B and GP2215B at a concentration of 100 mg/mL, along with DMSO (used as a negative control), were individually dispensed into separate 5 mL Eppendorf tubes. 500 µL of 2 M TFA was added to each tube.

The tubes were closed and left to incubate at room temperature for 24 hours. The tubes were then opened and left in a fume hood, allowing the TFA to evaporate. After one week of evaporation in the fume hood, each sample was washed with 1 mL of TSB, and then resuspended in 686 µL of TSB. The pH of the solution was read using pH paper. If the pH of experimental samples was lower than 6, then the solution was washed again with TSB. On a microscope slide, 10 µL of fresh overnight culture of *S. aureus* was mixed with 10 µL of a TFA-treated plant extract (GP95B or GP2215B), or TFA-treated DMSO, and 10 µL of methylene blue. A cover slip was added. The slides were viewed under brightfield microscopy using oil immersion (Olympus BX53 Microscope). Photos were taken of the preparation using the Olympus SC50 camera with cellSens Standard Software.

2.6.5 Clumping molecule purification process.

The objective of this experiment is to concentrate the molecules causing clump formation (clumping molecules) from our selected plant extracts to pursue their isolation.

A 1/1000 diluted overnight culture of *S. aureus* in TSB was treated with the plant extract in a 96-well plate for 18 hours at 37ºC with agitation (as described in section 2.4.1). Solution surrounding plant-extract induced clumps was carefully removed using a micropipette and discarded. Clumps were resuspended in 75 µL of sterile dH_2O, per well. Resuspended clumps were collected and placed into a single Eppendorf. Samples from clumps induced by the two different plant extracts were kept in separate tubes. Tubes were vortexed thoroughly then centrifuged for 5 minutes at 10 000 rpm. The supernatant was poured into a new tube (the pellet was set aside for the experiment in section 2.5.1), and then filtered through a 0.45 µm filter using a 3-c.c. syringe and a hypodermic needle. The filtered supernatant was placed into a clean tube, evaporated for a week in a 30ºC incubation, then resuspended in 500 µL of sterile dH_2O.

To determine whether clumping molecules have been collected, 50 µL of a 1/1000 *S. aureus* in 50% TSB was treated in a well of a 96-well plate with 50 µL of concentrated clumping molecules, or 50% TSB as a control. A reduced concentration of TSB was used to reduce the growth of *S. aureus* thereby accommodating the unknown concentration of clump molecules. Additionally, 50 µL of clumping molecules was added to a well with 50 µL of TSB as a negative control. The plate was incubated in a plate reader for 18 hours at 37ºC with agitation. The presence of clumping was assessed visually, and photos were taken using the Ziggi HD camera on IPEVO image software.

Chapter 3: Results

Chapter 3, Part 1: Investigation of the effects of GP95B and GP2215B on *S. aureus.*

3.1.1 Analysis of clump-inducing plant extracts.

Previously, Megan Puchbauer in our laboratory observed a clump formation while testing the plant extracts for their antibiotic properties (2019). It was found that plant extracts from different plant families were causing clumping of various bacteria: *S. epidermidis, E. coli, C. freundii,* or *S. aureus.* Table 3 is an analysis of the results obtained in our laboratory with plant extracts that were tested for their capacity to clump at least one species of bacteria. Out of the 104 plant extracts tested from this plant library, 50 of them have been tested for their clumping activities. 26 plant extracts were found to cause clumping in at least one species of bacteria (Table 3). It is noted that the amount of clumping induced in each sample varies drastically between extract and bacterial type and is not indicated here.

Table 3. Analysis of plant extracts' clumping activity in bacteria. Underlined extracts chosen for further analysis.

Extracts	Family	Clumped species	Species not clumped
GP2A	*Amaranthaceae*	*C. freundii, S. aureus, S. epidermidis*	*E. coli*
GP2B	*Amaranthaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP2D	*Amaranthaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP6A	*Boraginaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP10A	*Polygonaceae*	*C. freundii, E. coli, S. epidermidis*	
GP10B	*Polygonaceae*	*S. epidermidis, C. freundii*	*E. coli*
GP15A	*Polygonaceae*	*C. freundii, E. coli, S. aureus, S. epidermidis*	
GP15B	*Polygonaceae*	*C. freundii, E. coli, S. aureus, S. epidermidis*	
GP30A	*Chenopodiaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP30B	*Chenopodiaceae*	*C. freundii, S. epidermidis*	*E. coli*
GP58	*Brassicaceae*	*C. freundii, S. epidermidis*	*E. coli*
GP72A	*Solanaceae*	*C. freundii*	*E. coli, S. epidermidis*
GP72B	*Solanaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP95A	*Rosaceae*	*E. coli, S. aureus, S. epidermidis*	
GP95B	*Rosaceae*	*C. freundii, E. coli, S. aureus, S. epidermidis*	
GP101A	*Euphorbiaceae*	*E. coli, S. aureus, S. epidermidis*	
GP101B	*Euphorbiaceae*	*C. freundii, S. epidermidis*	*E. coli*
GP105B	*Verbenaceae*	*C. freundii, S. epidermidis*	*E. coli*
GP106A	*Asteraceae*	*C. freundii, S. epidermidis*	*E. coli*
GP110A	*Asteraceae*	*C. freundii, S. epidermidis*	*E. coli*
GP117B	*Asteraceae*	*S. epidermidis*	*E. coli, C. freundii*
GP146A	*Asteraceae*	*C. freundii, S. epidermidis*	*E. coli*
GP146B	*Asteraceae*	*C. freundii, S. epidermidis*	*E. coli*
GP147B	*Asteraceae*	*S. aureus*	*C. freundii, E. coli, S. epidermidis*
GP151A	*Asteraceae*	*S. epidermidis*	*E. coli*
GP250B	*Caprifoliaceae*	*S. epidermidis*	*C. freundii, E. coli*
GP2215A	*Polygonaceae*	*S. aureus*	
GP2215B	*Polygonaceae*	*S. aureus*	

The results of table 3 indicate that several plant extracts from plants of different families induce clump formation. The profile of bacterial species in which each plant extract causes clumping differs between extracts.

To choose the plant extracts for our study on clump-inducing activity, we initiated our selection process by assessing the clumping phenotype. This preliminary evaluation guided us to identify GP95B and GP2215B as the most suitable candidates for studying clumping activity. We chose to use *Staphylococcus aureus* as our bacterial model. Our subsequent analysis confirmed that these plant extracts exhibited more pronounced clumping effects when interacting with this specific bacterium. To diversify our research, we deliberately selected two plant extracts from different botanical families. This strategic choice enabled us to compare their activities.

The clumps induced by GP95B and GP2215B in *S. aureus* are notably distinct in appearance when observed with the naked eye. GP95B-induced clumps appear more compact, with the surrounding solution being cloudier in contrast to GP2215B. In the case of GP2215B, the clumps induced in *S. aureus* seem to be comprised of smaller units. Our project's primary objective is to analyze the clumping phenotype. Consequently, the exploration of plant extracts that elicit discernible differences in clumping effects on *S. aureus* will enable us to conduct a comprehensive analysis of the variability among clump-inducing plant extracts.

3.1.2 Bacterial clumps are more visible with 0.5 mg/mL of plant extracts.

S. aureus were treated with decreasing concentrations of GP95B and GP2215B for 18 hours. In GP95B treated *S. aureus* samples, there are aggregations of the bacteria present at all the concentrations tested, which are not present in the control (*S. aureus* alone) (Figure 4). If clumping is present in *S. aureus* samples treated with each concentration of GP95B, the size of the clumps decreases and the solution surrounding the clumps gets cloudier as the concentration of GP95B decreases (Figure 4). The most pronounced clumping is seen in *S. aureus* treated with GP95B at 0.5 mg/mL (Figure 4).

In GP2215B treated *S. aureus* samples, there are aggregations of the bacteria at the two highest concentrations (1 mg/mL and 0.5 mg/mL) (Figure 4). The *S. aureus* clumps induced at 1 mg/mL appear small and compact, compared to those induced at 0.5 mg/mL which appear composed of compact, uniform units of clumped bacteria (Figure 4). The clumps get smaller as the concentration decreases below 0.5 mg/mL. The solution surrounding the clumps appears clear in samples of *S. aureus* treated with 1 mg/mL and 0.5 mg/mL GP2215B (Figure 4). The cloudiness of the surrounding solution increases as the concentration of the plant extract decreases (Figure 4). As with GP95B, GP2215B at 1 and 0.5 mg/mL produces the more defined clumps with the clearest surrounding solutions when compared to the other concentrations (Figure 4). The study of the bacterial clump phenotype will be pursued with the concentration of 1 and 0.5 mg/mL of the two plant extracts selected.

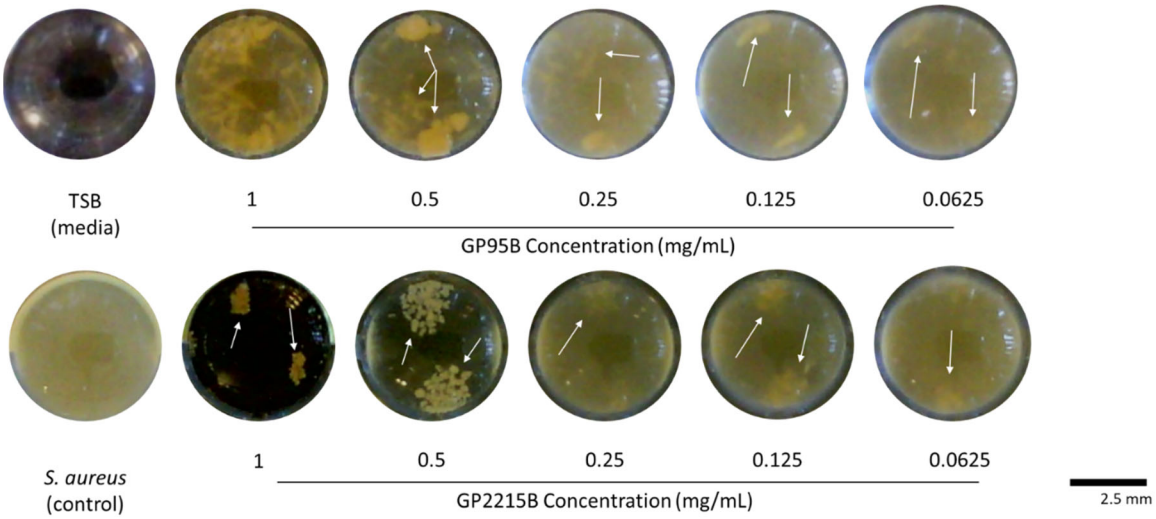

Figure 4. *S. aureus* treated with decreasing concentrations of GP95B and GP2215B for 18 hours. Photos were taken of the wells from one experiment. Experiment was repeated three times.

3.1.3 Indirect measurement of clump size using crystal violet.

While qualitative assessment was important in the initial observation of *S. aureus* clumps, it is necessary to measure the size of *S. aureus* clumps, to compare clumping abilities between extracts at different concentrations. To do so an assay was developed that would allow to compare *S. aureus* clump sizes. After the clumps were stained with crystal violet, the stain was extracted, and the intensity of the stain was read.

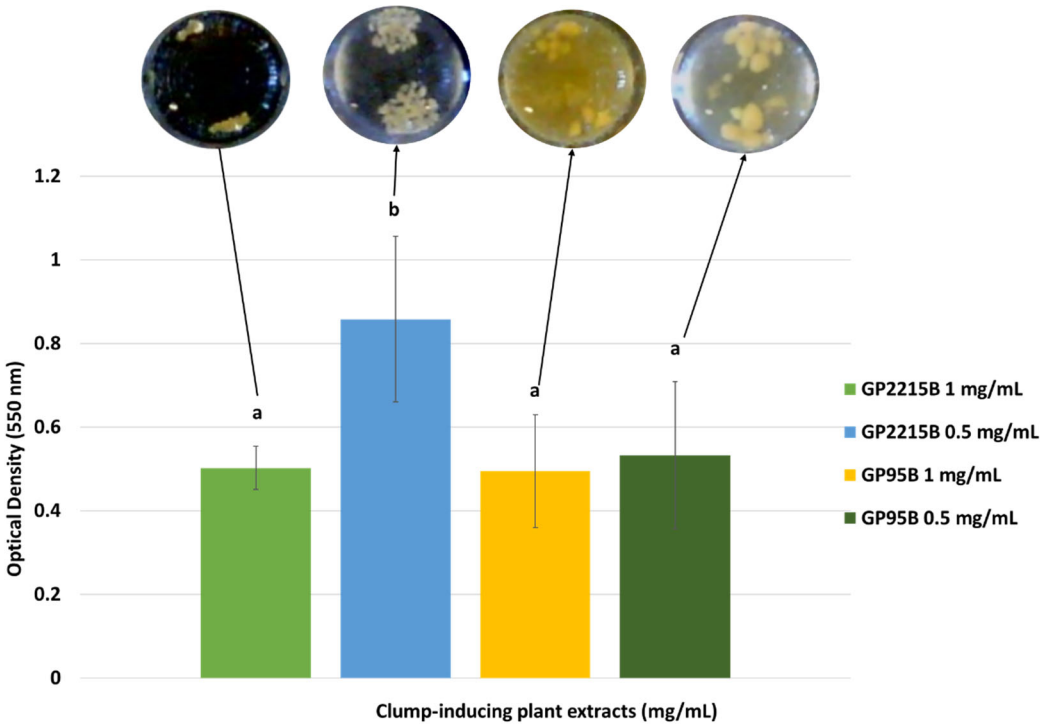

Figure 5. Quantity of crystal violet extracted from *S. aureus* clumps formed after treatment with plant extracts, GP2215B or GP95B at two concentrations (1, 0.5 mg/mL) for 18 hours.

Experiment was repeated three times. Two-Way ANOVA (p = 0.05) and Tukey's HSD were used to assess significance, which is denoted by "a, b."

The largest amount of crystal violet extracted came from *S. aureus* clumps obtained with GP2215B at 0.5 mg/mL ($M = 0.858$ $SD = 0.0392$) (Figure 5). There was significantly more crystal violet extracted from *S. aureus* clumps obtained by GP2215B at 0.5 mg/mL, compared to clumps induced with GP95B at 1 mg/mL ($M = 0.521$ $SD = 0.0181$) 0.5 mg/mL, ($M = 0.566$ $SD = 0.0312$) and GP2215B at 1 mg/ml ($M = 0.502$ $SD = 0.00264$) (F (3,52) = 17.0, $p < 0.01$) (Figure 5). There is no significant difference in the value of crystal violet extracted from *S. aureus* clumps caused by GP95B 1 mg/mL, GP2215B 1 mg/mL, and GP95B 0.5 mg/mL (Figure 5) (F (3,52) = 17.0, $p < 0.01$) (Figure 5). Analyzing the photos depicted in Figure 5, GP2215B at 1 mg/mL induces clumps that appear smaller than those induced by GP2215B at 0.5 mg/mL, matching the crystal violet quantification of the clump sizes. *S. aureus* clumps induced by GP95B at 1 mg/mL appear smaller than those induced by the extract at 0.5 mg/mL. However, it was found that *S. aureus* clumps induced by GP95B at 1 or 0.5 mg/mL do not differ in size (F (3,52) = 17.0, $p < 0.01$) (Figure 5). It is challenging to visually compare the clump sizes of GP95B and GP2215B induced clumps, because of the differences in their phenotypes. GP95B clumps induced by 1 or 0.5 mg/mL appear larger than those induced by GP2215B at 1 mg/mL. However, it was found with this technique, that their sizes are not significantly different (F (3,52) = 17.0, $p < 0.01$).

3.1.4 Bacterial clumps obtained with GP95B and GP2215B contain living bacteria.

Despite being able to measure clump sizes using crystal violet, it is necessary to measure the quantity of living bacteria in and around the clump. To achieve this, we used the viable plate count technique. Living bacteria in experimental samples were quantified (those contained in the clump and solution surrounding the clump). Separately, bacteria in the solution surrounding the clump were quantified. The quantity of bacteria in the clump was determined by subtracting the number of bacteria in the solution surrounding the clump, from the number of bacteria in the whole sample.

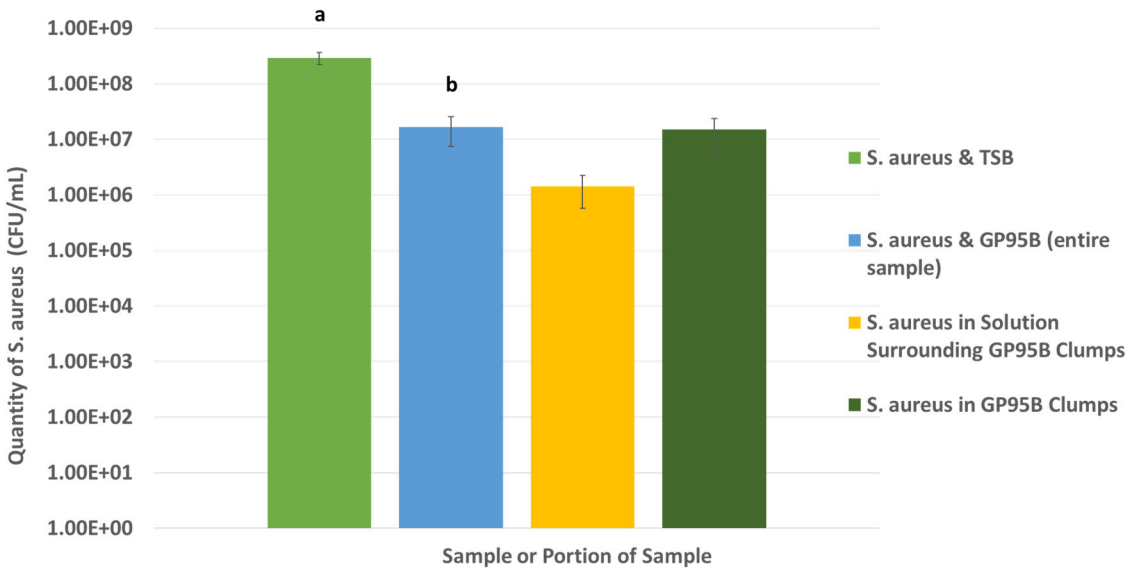

Figure 6. Quantity of *S. aureus* (CFU/mL) in each portion (entire sample, solution surrounding clumps, in clump) of samples treated with GP95B 0.5 mg/mL, compared to *S. aureus* in TSB (control).

One-Way ANOVA (p = 0.05) and Tukey's HSD was used to assess significance between the control and entire sample. Significance is denoted by "a, b."

Figure 6 shows the quantification of non-treated *S. aureus* and *S. aureus* treated with GP95B after 18 hours of incubation with agitation. Using the viable plate count technique, the total quantity of *S. aureus* in samples treated with GP95B 0.5 mg/mL was determined. Separately, the technique was used to quantify the bacteria in the solution surrounding the clump induced by GP95B. Using these values, the quantity of *S. aureus* in the clump was determined. Statistical analysis was used to determine whether the reduction in *S. aureus* growth was significant. There is significantly more *S. aureus* in non-treated samples (M = 2.92E+08 SD = 5.55E+15) compared to samples of *S. aureus* treated with GP95B 0.5 mg/mL(M = 1.64E+07 SD = 8.19E+13), showing that the extract significantly reduces the growth of the bacteria (Figure 6) ($F(3, 72) = 4.022E+17$, $p < 0.001$) (Figure 6).

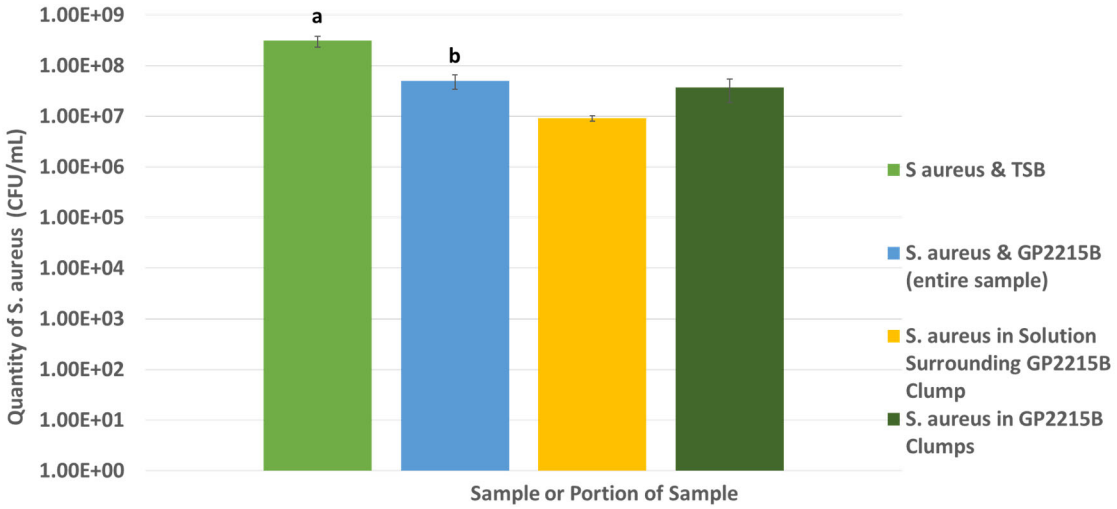

Figure 7. Quantity of *S. aureus* (CFU/mL) in each portion (entire sample, solution surrounding clumps, in clump) of samples treated with GP2215B 0.5 mg/mL, compared to *S. aureus* in TSB (control).

One-Way ANOVA (p = 0.05) and Tukey's HSD was used to assess significance between the control and entire sample. Significance is denoted by "a, b."

Figure 7 shows the quantification of non-treated *S. aureus*, and after treatment with GP2215B at 0.5 mg/mL for 18 hours. The largest number of bacteria was found in non-treated *S. aureus* (Figure 7). The number of bacteria in the non-treated *S. aureus* (M = 3.09E+08 SD = 5.520 E+15) samples is significantly greater than the quantity in samples treated with GP2215B 0.5 mg/mL (M = 5.02E+07 SD = 2.56953E+14); showing that the extract significantly reduces the growth of the bacteria (F (3, 58) = 185, $p < 0.001$) (Figure 7).

Table 4. Portion of *S. aureus* found in clumps, and percent reduction after exposure to plant extracts for 18 hours.

Plant extract	Plant extract concentration	Portion of *S. aureus* found in clump (%)	Percent reduction (%)
GP95B	0.5 mg/mL	88	94
GP2215B	0.5 mg/mL	74	84

GP95B at 0.5 mg/mL is causing effective clumping of *S. aureus*, with 88% of viable bacteria being found in the clump (Table 4). This is more than the percentage of bacteria found in GP2215B [0.5 mg/mL] induced clumps, which is 74% (Table 4). By comparing the quantity of bacteria in plant extract-treated *S. aureus* to non-treated *S. aureus,* it was determined that GP95B and GP2215B caused a 94%, and 84% reduction of *S. aureus,* respectively, after 18 hours of incubation (Table 4). Both extracts caused a significant decrease in the number of viable *S. aureus* ($F(3, 58) = 185$, $p < 0.001$) (Figure 7) ($F(3, 72) = 4.022E+17$, $p < 0.001$) (Figure 6). The results of the viable plate count experiment show that there is both clumping and inhibitory effects displayed by GP95B and GP2215B on *S. aureus.*

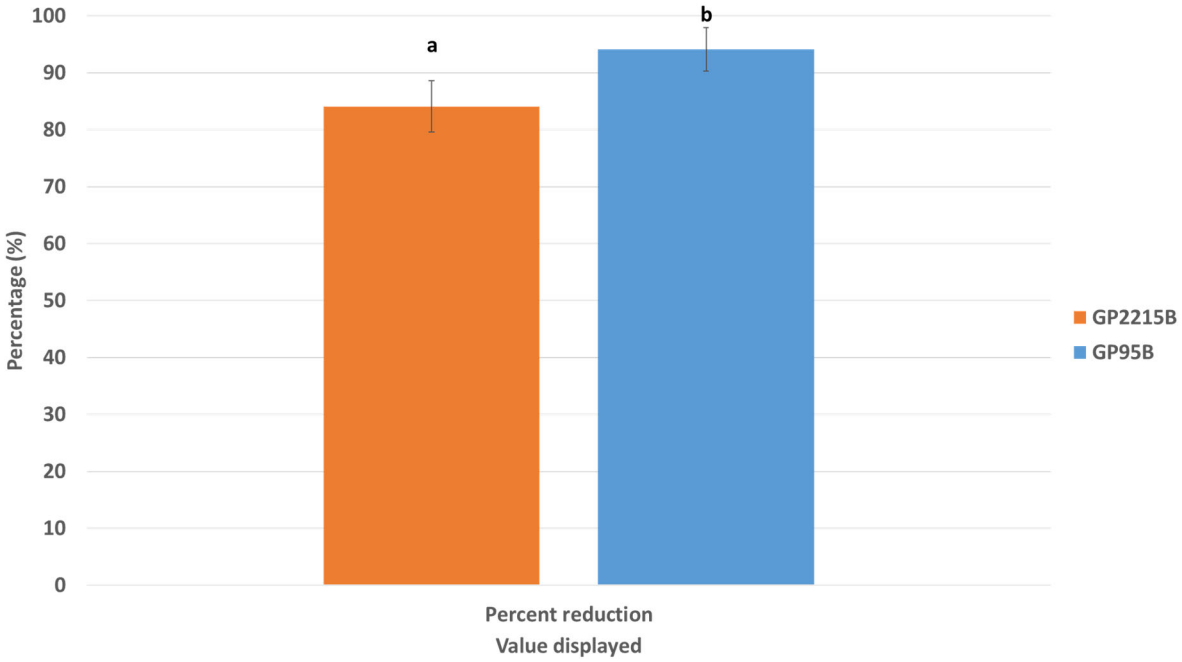

Figure 8. Percent reduction of *S. aureus* after treatment with GP95B or GP2215B (0.5mg/mL) as determined by viable plate count, after 18 hours (*t*-test, $p = 0.05$).

Significance is denoted by "a, b." The data used were compiled from three independent replicates of the viable plate count technique.

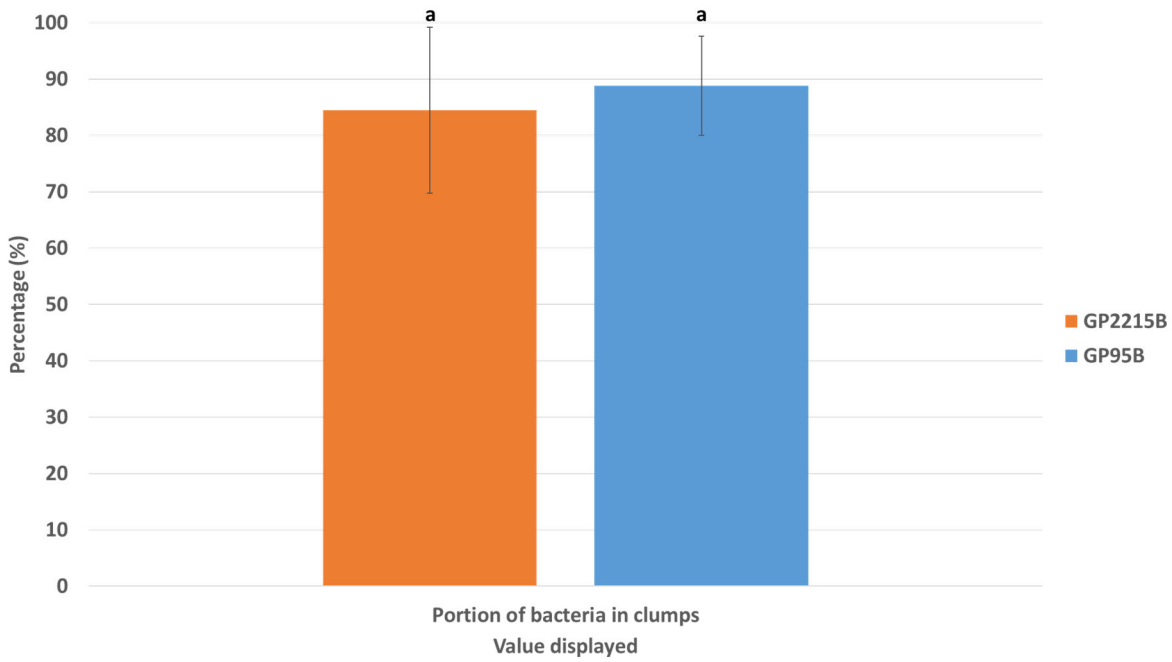

Figure 9. Percentage of *S. aureus* found in the clump after treatment with GP95B or GP2215B (0.5 mg/mL) after 18 hours as determined by three replicates of the viable plate count (*t*-test, $p = 0.05$).

Significance is denoted by "a."

Figure 8 compares the percent reduction in number of viable bacteria after treatment with GP95B or GP2215B 0.5 mg/mL (as determined by the viable plate count). A significantly greater reduction in the concentration of living *S. aureus* was found in samples treated with GP95B (M = 84.06 SD = 20.4) compared to GP2215B (M = 94.1 SD = 14.7), t (16) = -6.96 $p < 0.001$. GP95B is significantly more effective at reducing the growth of *S. aureus* compared to GP2215B.

Figure 9 compares the percentage of living bacteria located in the clump after treatment with GP95B or GP2215B for 18 hours. This was determined using the results of the viable plate count assay. It was found that the clumping abilities of GP95B (M = 88.8 SD =77.4) and GP2215B (M = 74.3 SD = 856) do not differ significantly t (21) = -2.07, $p > 0.05$ (Figure 9).

3.1.5 GP95B and GP2215B inhibit the growth of *S. aureus.*

In our laboratory, several plant extracts have exhibited antibacterial activity. In this experiment, we focused on studying the effects of GP95B and GP2215B in inhibiting the growth of *S. aureus* over an 18-hour period. This investigation employed the microdilution technique.

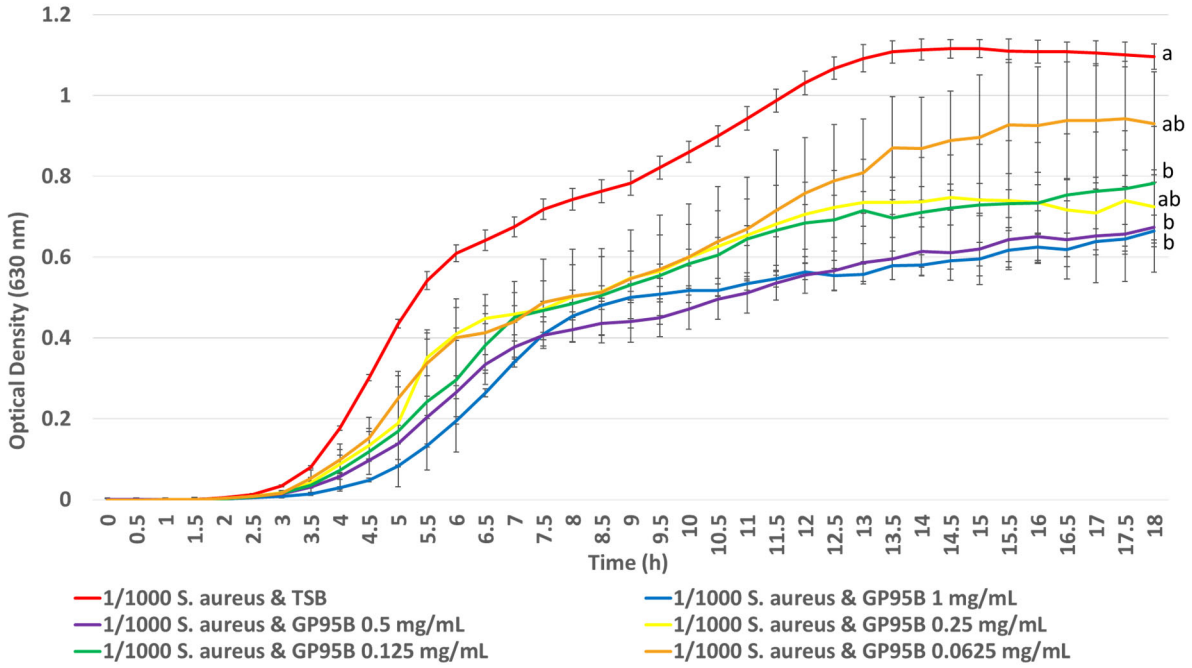

Figure 10. Quantification of *S. aureus* growth over 18 hours with serial dilutions of plant extract GP95B. Plotted values are the average ± standard deviation.

Experiment was repeated three times. Optical density of each sample at 18 hours was compared via One-Way ANOVA (p = 0.05) and Tukey's HSD. Significance is denoted by "a, b."

In figure 10, a change in the optical density of *S. aureus* treated by any concentration of GP95B can be observed when compared to the non-treated sample. The average optical density of each sample at 18 hours was compared using a One-Way ANOVA and Tukey's HSD; revealing that the inhibitory effect of GP95B on *S. aureus* at 18 hours is significant (F (5,12) = 5.62, p = 0.007). Tukey's HSD post-hoc test indicates that samples treated with GP95B 1 mg/mL *(M = 0.754 SD = 0.00155),* 0.5 mg/mL (*M = 0.738 SD = 0.0124*), and 0.125 mg/mL (M = 0.8103 SD = 0.0196) had significantly lower optical densities at 18 hours compared to non-treated *S. aureus* (M = 1.096 SD = 0.001025). Samples of *S. aureus* treated with GP95B at 0.25 mg/mL (M = 0.854 SD = 0.00833) and 0.0625 mg/mL (M = 0.949 SD = 0.0161) did not significantly reduce the maximum optical density of *S. aureus* compared to the non-treated sample.

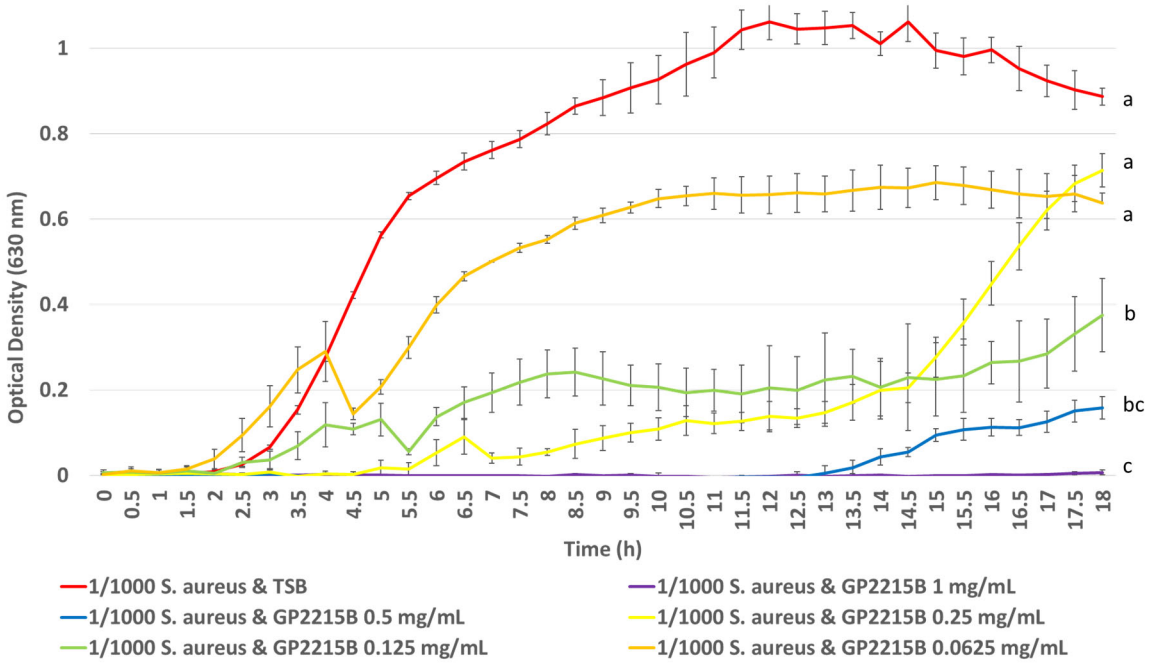

Figure 11. Quantification of *S. aureus* growth over 18 hours with serials dilution of plant extract GP2215B. Plotted values are the average ± standard deviation.

Experiment was repeated three times. Optical density of each sample at 18 hours was compared via One-Way ANOVA (p = 0.05) and Tukey's HSD. Significance is denoted by "a, b, c."

The growth of *S. aureus* treated with GP2215B at a range of concentrations was assessed (Figure 11). A One-Way ANOVA assessed the optical density of *S. aureus* treated with GP2215B concentrations compared to non-treated *S. aureus* at 18 hours and indicated significance (F (5, 12) = 59.1, $p < 0.001$). Using the Tukey's HSD post hoc test it was found that the average optical density at 18 hours in samples treated with GP2215B at 1 mg/mL (M = 0.00767 SD = 3.03E-05), 0.5 mg/mL (M = 0.191 SD = 0.000704) and 0.125 mg/mL (M = 0.397 SD = 0.00741) is significantly less than the optical density of non-treated *S. aureus* ($M = 0.877$ $SD = 0.00764$) (F (5, 12) = 59.1, $p < 0.001$) (Figure 11). GP2215B at 1 and 0.5 mg/mL reduced the final optical density to the same extent when compared to non-treated *S. aureus* (F (5, 12) = 59.1, $p < 0.001$) (Figure 11). The final optical density of *S. aureus* treated with GP2215B at 0.125 mg/mL is significantly different than that of *S. aureus* treated with the extract at 1 mg/mL and not significantly different from the value obtained with GP2215B at 0.5 mg/mL. The optical density of *S. aureus* treated with GP2215B at 0.25 mg/mL (M = 0.746 SD = 0.001561) and 0.0625 mg/mL (M = 0.649 SD = 0.000589) is not significantly lower than non-treated *S. aureus* (Figure 11).

3.1.6 Inhibitory effects of GP95B and GP2215B can be demonstrated visually.

Using the microdilution technique, the inhibitory activity of GP95B and GP2215B on *S. aureus* was quantified. However, since these extracts clump bacteria, it was important to validate the previous results using the microdiffusion technique based on the Kirby Bauer Method with specific modifications to fit our research question (Biemer, 1973). This method allows the assessment of the isolated inhibitory activity of the plant extracts without interference from their clumping activities.

In brief, a bacterial lawn was inoculated on Mueller Hinton (MH) Agar, and plant extracts were introduced into wells within the agar. Subsequently, the molecules diffused through the Mueller Hinton (MH) agar, establishing a concentration gradient. If the plant extracts possessed an inhibitory activity, a zone of inhibition that formed around the well would be observed. It is to be noted that the

diameter will not be proportional to the intensity of inhibition as it depends on the chemical properties of the molecules responsible of the antibacterial activity.

In this study, we examined GP95B, GP2215B, Kanamycin (a well-known antibiotic), and DMSO 1.1 g/mL (the solvent used to dissolve the plant extracts). The zones of inhibition were measured and are presented in figure 12.

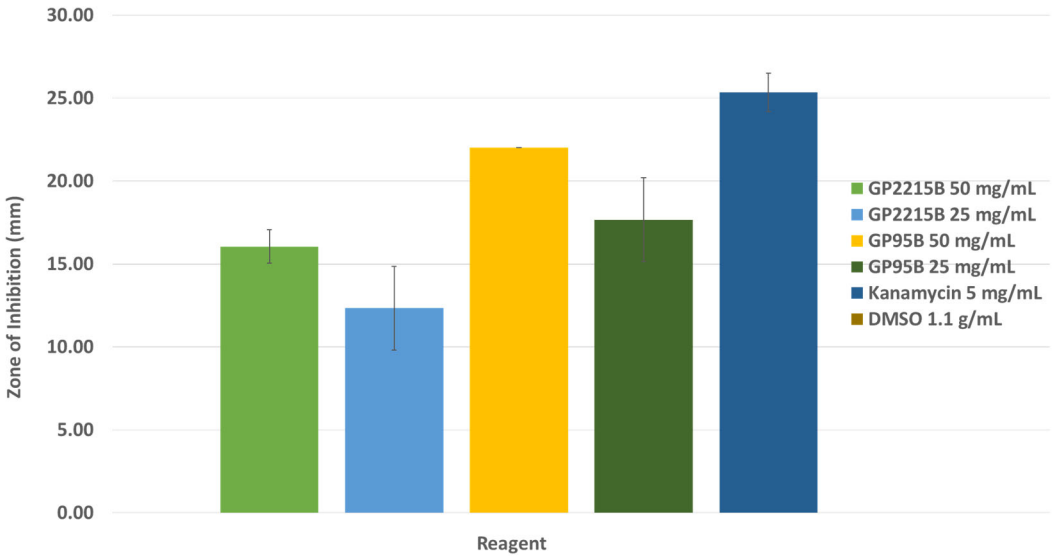

Figure 12. Zone of clearance (mm) in *S. aureus* on Mueller-Hinton agar after treatment with clump-inducing plant extracts (GP95B, GP2215B at 50 and 25 mg/mL). Value for DMSO = 0.

GP2215B and GP95B at 50 and 25 mg/mL, and Kanamycin at 5 mg/mL all produced a zone of clearing with *S. aureus.* The only compound that did not produce a zone of clearing was DMSO at the concentration used to resuspend the plant extract. Both plant extracts are less effective at inhibiting *S. aureus* at 25 mg/mL than at 50 mg/mL (Figure 12).

3.1.7 GP95B and GP2215B are not selective of *S. aureus* subtypes.

One of the questions that remained was to know if the plant extracts would clump a subpopulation of *S. aureus* and if we could increase the clumping activity by using the previously clumped bacteria.

To do so, the solution surrounding the clumps was removed and the clumped bacteria were collected and washed two times, then diluted to 1/1000 in TSB. The previously clumped bacteria were incubated with GP95B at 0.5 mg/mL. The optical densities of these bacterial treatments were read through a kinetic of 18 hours and the following results were obtained.

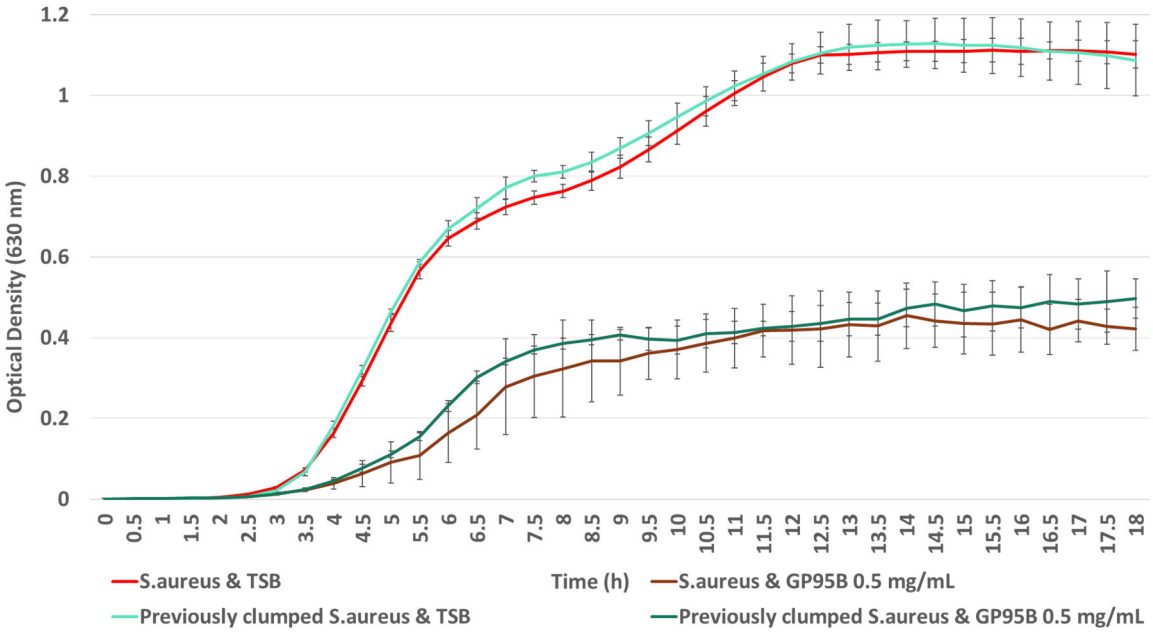

Figure 13. Optical density curve of fresh and previously clumped *S. aureus* treated with GP95B 0.5 mg/mL for 18 hours. Plotted points are the average +/- standard deviation. Experiment was repeated three times.

Previously clumped *S. aureus* were incubated over 18 hours and treated with GP95B at 0.5 mg/ mL in figure 13. The growth of non-treated *S. aureus* and the growth of previously clumped non-treated *S. aureus* follow the same optical density curve. When previously clumped *S. aureus* is treated with GP95B at 0.5 mg/mL, and the optical density curve matches that of *S. aureus* treated with GP95B at 0.5 mg/mL. Having been previously clumped does not affect the ability of *S. aureus* to grow after being removed from experimental conditions.

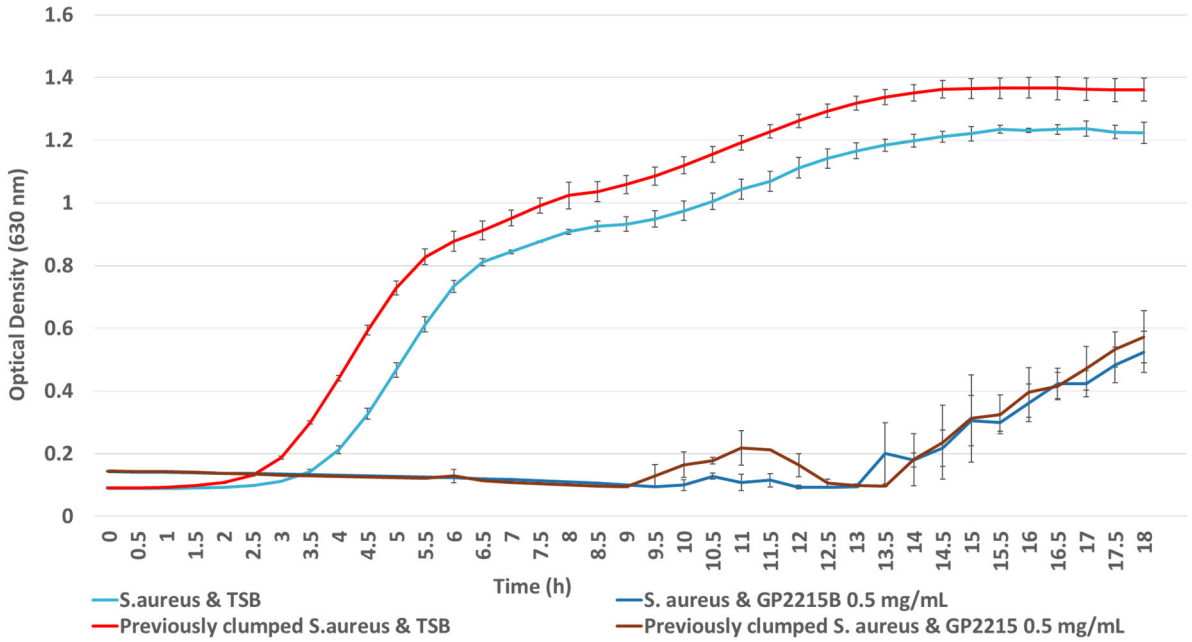

Figure 14. Optical density curve of fresh and previously clumped *S. aureus* treated with GP2215B 0.5 mg/mL for 18 hours. Plotted points are the average +/- standard deviation. Experiment was repeated three times.

There is a small reduction of the lag phase of previously clumped *S. aureus* with GP2215B 0.5mg/mL which could be explained by a higher number of viable bacteria in the previously treated sample (Figure 14). Despite this, previously clumped *S. aureus* non-treated follows the typical growth curve that is expected in *S. aureus.* After treatment of previously clumped *S. aureus* with GP95B at 0.5 mg/mL, the growth of the bacteria follows the growth curve of *S. aureus* treated with GP95B at 0.5 mg/mL (Figure 14).

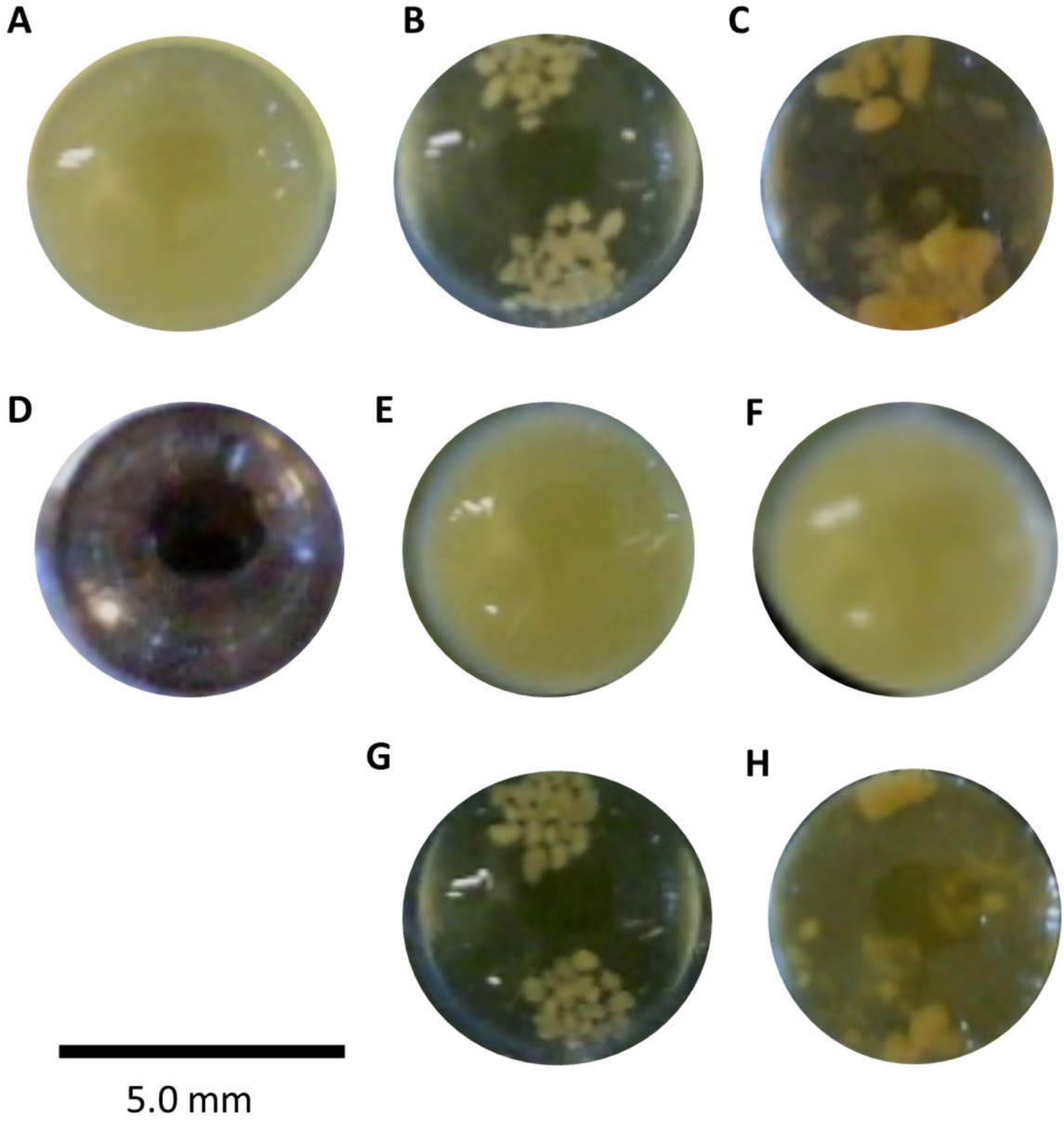

Figure 15. Fresh and previously clumped *S. aureus* treated with GP95B and G2215B 0.5 mg/mL for 18 hours.

A) Fresh *S. aureus* & TSB, B) Fresh *S. aureus* & GP2215B 0.5 mg/mL, C) Fresh *S. aureus* & GP95B 0.5 mg/mL, D) TSB, E) Previously clumped (with GP2215B) *S. aureus* & TSB, F) Previously clumped (with GP95B) *S. aureus* & TSB, G) Previously clumped (with GP2215B) *S. aureus* & GP2215B 0.5 mg/mL H) Previously clumped (with GP95B) *S. aureus* & GP95B 0.5 mg/mL.

Clumping is present in fresh and previously clumped *S. aureus* samples treated with GP95B or GP2215B (Figure 15). Clumping is not present in samples of fresh and previously clumped *S. aureus* that were not treated (Figure 15). Clumps appear the same in fresh and previously clumped bacteria (Figure 15). If clumps were larger in samples of previously treated *S. aureus*, then the results would suggest that GP95B and GP2215B select for *S. aureus* with specific genotypes, because it would be expected that only previously clumped *S. aureus* with the selected genotype would be present in the sample (Figure 15). However, this is not the case, so the results suggest that a specific genotype of *S. aureus* is not being selected by GP95B and GP2215B during clump formation (Figure 15).

Chapter 3, Part 2: Towards the identification of chemical properties of the clumping factors of GP95B and GP2215B.

In the next experiments, manipulations will be made to the bacteria or plant extracts to better understand the mechanism of action of the clumping activity and the chemical property of the clumping factors.

3.2.1 Evidence of rapid *S. aureus* clumping with GP95B and GP2215B.

In the prior experiments, 18-hour kinetic assessments were conducted, allowing for the observation of the antibacterial activity of our extracts over the 18-hour mark as well as the clump formation at 18 hours. In the ongoing quest to identify the clumping factors, the aim was to investigate whether clumping could occur within a shorter period to facilitate the study of the chemical properties of the clumping factors.

The method described in section 2.8 was developed to quickly assess clump formation in *S. aureus.* In brief, the bacteria were incubated with the plant extracts on a microscope slide, at room temperature for five minutes. The samples were heat fixed and stained with methylene blue and viewed under brightfield microscopy.

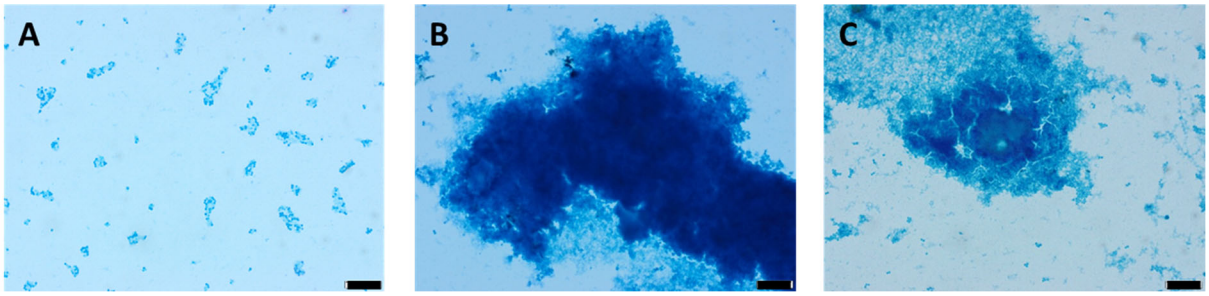

Figure 16. Overnight culture of *S. aureus* with no treatment (control) or treated with GP95B or GP2215B for 5 minutes at room temperature.

Samples were heat-fixed, stained with methylene blue and are viewed using brightfield microscopy, under oil immersion. Scale bar = 10 mm.

A.) Non-treated *S. aureus* B.) *S. aureus* & GP95B 0.5 mg/mL C.) *S. aureus* & GP2215B 0.5 mg/mL.

As can be seen in Figure 16, there is a clear aggregation of the bacteria after exposure with the plant extracts; this amount of aggregation is not present in the non-treated *S. aureus* samples. As expected, it can be observed in the non-treated sample a clustered grape-like formation that is characteristic of the genus *Staphylococcus* (Koyama et al., 1977). These results indicate that it is possible to observe clumps after only five minutes.

3.2.2 Chemically fixed *S. aureus* can be clumped by GP95B and GP2215B.

To elucidate the mechanism of action of clump-inducing plant extracts, it is important to determine if clump formation requires the bacteria to be alive. To determine whether bacteria are involved in the clump formation process, *S. aureus* were killed by chemical fixation and treated with GP95B or GP2215B for 5 minutes on a microscope slide. The slides were viewed under brightfield microscopy, and the images displayed in Figure 17 were taken.

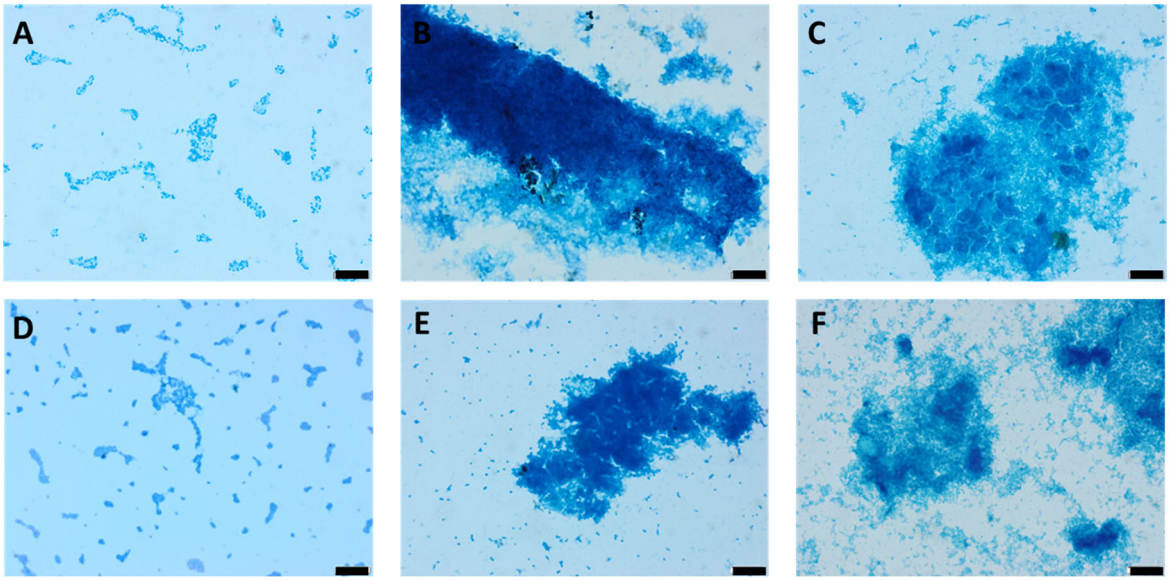

Figure 17. *S. aureus* fixed, and non-fixed treated with clump inducing plant extracts (GP95B, GP2215B) after five-minute incubation at room temperature, methylene blue stain, scale bar = 10 mm *S. aureus* fixed, and non-fixed treated with clump inducing plant extracts (GP95B, GP2215B) after five-minute incubation at room temperature, methylene blue stain, scale bar = 10 mm, bright field microscopy.

A.) Non-treated *S. aureus*, B.) *S. aureus* & GP95B 0.5 mg/mL, C.) *S. aureus* & GP2215B 0.5 mg/mL, D.) Non-treated Fixed *S. aureus*, E.) Fixed *S. aureus* & GP95B 0.5 mg/mL, F.) Fixed *S. aureus* & GP2215B 0.5 mg/mL.

GP95B and GP2215B caused clumping in both fixed and non-fixed *S. aureus* (Figure 17). Clumping is not seen in non-treated *S. aureus* in both the fixed, and non-fixed samples (Figure 17). These results suggest that bacteria are not involved in clump formation, rather clumping is the result of a physical, "adhesive" effect of the plant extract.

3.2.3 The clumping factors of GP95B and GP2215B are non-proteinaceous.

To continue elucidating the chemical nature of the clumping factors of GP95B and GP2215B, the plant extracts were heated to 90^{o}C for 30 minutes. At this temperature, for this duration, it is expected that the three-dimensional structure of proteins will be lost through heat denaturation (Pinto et al., 1991). *S. aureus* were treated with the heated extracts, or heated media, for five minutes on a microscope slide. *S. aureus* were treated in the same way with extracts that were not heated. The slides were viewed under brightfield microscopy.

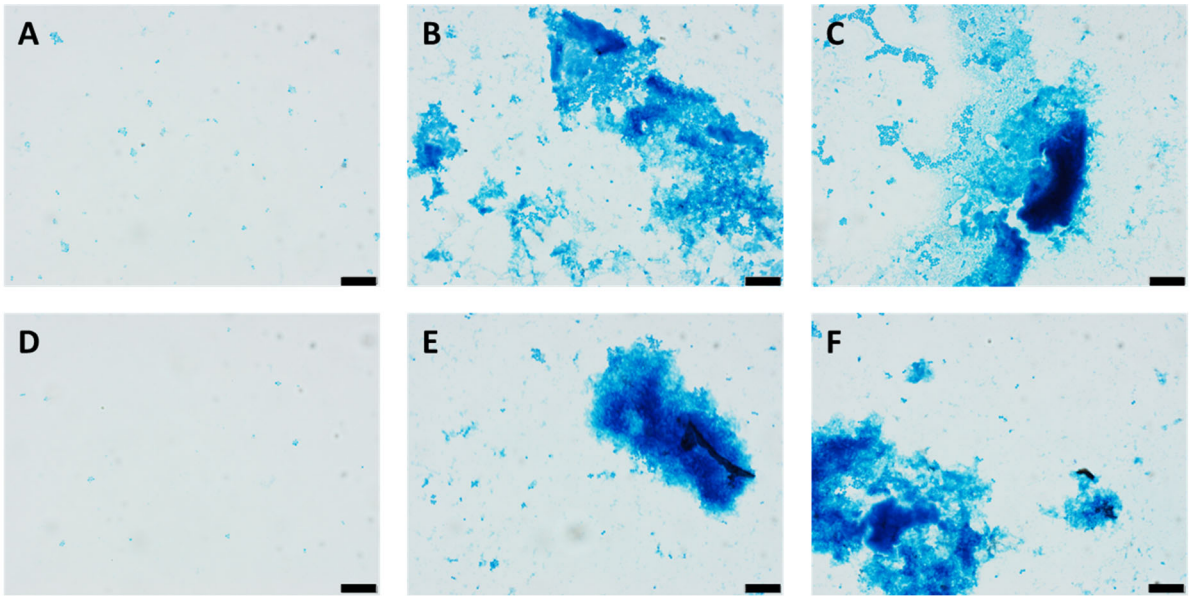

Figure 18. *S. aureus* treated with heat-treated plant extracts GP95B, GP2215B and non-treated extracts for five-minute incubation at room temperature, methylene blue stain, scale bar = 10 mm, bright field microscopy.

A.) Non-treated *S. aureus*, B.) *S. aureus* & GP95B 0.5 mg/mL, C.) *S. aureus* & GP2215B 0.5 mg/mL, D.) *S. aureus* & heat-treated DMSO, E.) *S. aureus* & heat treated GP95B 0.5 mg/mL, F.) *S. aureus* & heat-treated GP2215B 0.5 mg/mL.

As can be observed in figure 18, heat-treated plant extracts GP95B and GP2215B can clump *S. aureus* after 5 minutes in a comparable manner as the one observed with *S. aureus* treated with non-heat-treated extracts. Clumping is not seen in non-treated *S. aureus,* and *S. aureus* combined with heat-treated media (Figure 18). These results suggest that the clumping factors of GP95B and GP2215B are non-proteinaceous.

3.2.4 Carbohydrate structures do not play a role in the clumping activities of GP95B and GP2215B.

Trifluoroacetic acid (TFA) is known to degrade carbohydrates (Ayadi et al., 2016; Rumpel & Dignac, 2006; Zhao & Monteiro, 2008). To determine whether clumping molecules contain carbohydrate components, GP95B and GP2215B were treated with 2M TFA for 24 hours. After the acid had evaporated, and the treated extracts were resuspended in TSB, *S. aureus* were treated with the TFA-treated plant extracts, on a microscope slide for five minutes.

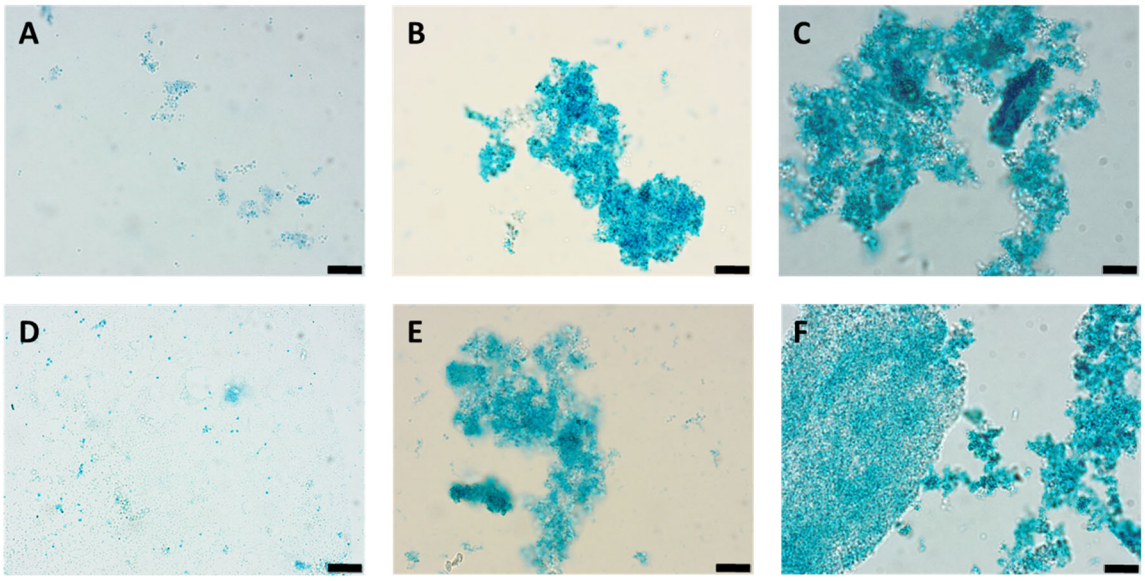

Figure 19. *S. aureus* treated with TFA-treated plant extracts GP95B, GP2215B. Five-minute incubation at room temperature, methylene blue stain, bright field microscopy observation, scale bar = 10 mm.

A.) Non-treated *S. aureus*, B.) *S. aureus* & GP95B 0.5 mg/mL, C.) *S. aureus* & GP2215B 0.5 mg/mL, D.) *S. aureus* & TFA-treated DMSO, E.) *S. aureus* & TFA-treated GP95B 0.5 mg/mL, F.) *S. aureus* & TFA-treated GP2215B 0.5 mg/mL.

As shown in Figure 19, clumping is present in *S. aureus* samples with GP95B and GP2215B that had been treated with TFA. The clumps observed are remarkably similar to the ones obtained with the non-treated extracts GP95B and GP2215B (Figure 19). No clumping was observed in the control non-treated *S. aureus* (Figure 19). This indicates that clumping activities of GP95B and GP2215B do not require carbohydrate components.

3.2.5 Clumping molecules from GP95B and GP2215B induced clumps can be collected and concentrated.

The final step of this project will be to determine the chemical identity of clumping molecules found in plant extracts GP2215B and GP95B. To do so it is important to purify the clumping factors.

Clumping molecules were collected from *S. aureus* clumps induced by GP95B or GP2215B. *S. aureus* were treated with the concentrated clumping molecules from GP2215B or GP95B induced clumps, or with the whole plant extracts. The plate was incubated for 18 hours then the presence of clumps was assessed.

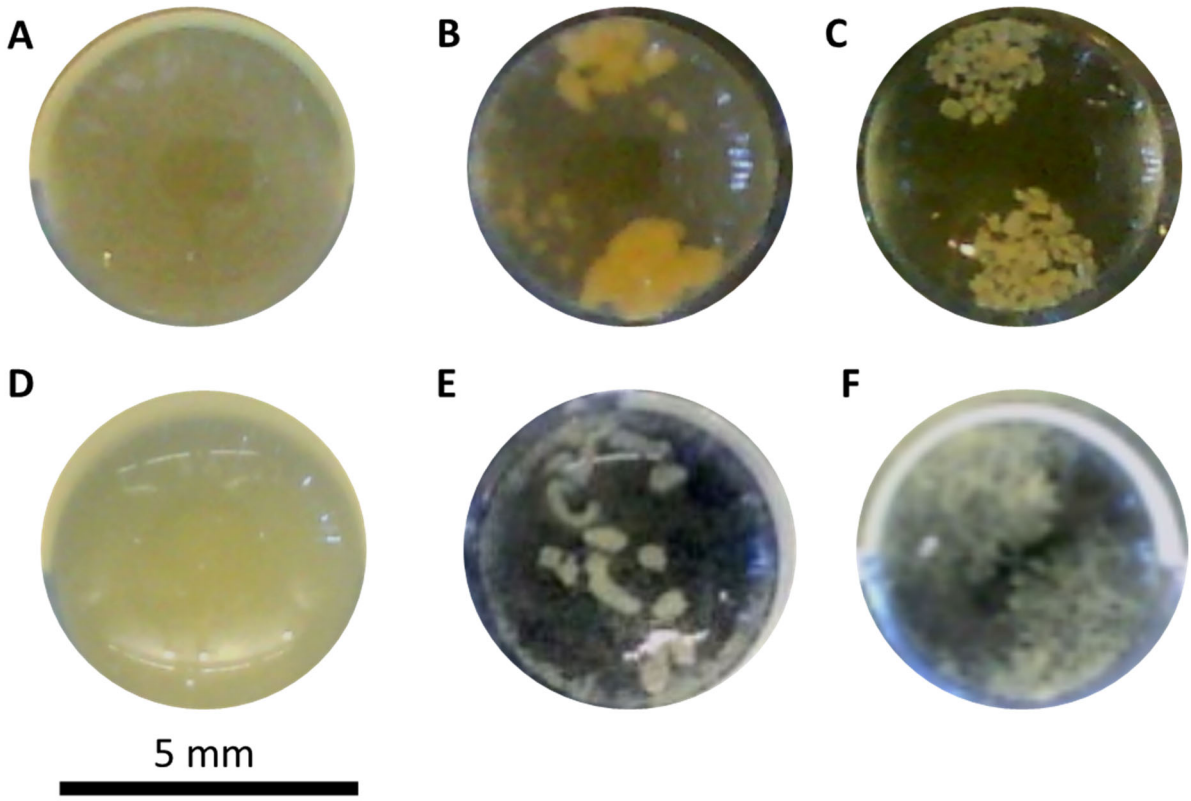

Figure 20. *S. aureus* treated with GP95B or GP2215B whole extracts at 0.5 mg/mL, or clumping molecules isolated from GP95B or GP2215B. Scale bar = 5.0 mm. Experiment was repeated three times.

A.) *S. aureus* and TSB, B.) *S. aureus* and GP95B 0.5 mg/mL, C.) *S. aureus* and GP2215B 0.5 mg/mL, D.) *S. aureus* and distilled water, E.) *S. aureus* and concentrated GP95B, F.) *S. aureus* and concentrated GP2215B.

The results in figure 20 indicate that when *S. aureus* is treated with GP2215B or GP95B clumping molecules, clumping is present in the sample after 18 hours. Clumps induced by GP2215B purified clumping molecules appear different than those induced by the whole plant extracts but are clearly formed (Figure 20). Clumps induced by GP95B concentrated extracts appear more like those induced by the whole GP95B extract (Figure 20). The results of this experiment indicate that clumping molecules can be removed from GP95B and GP2215B induced *S. aureus* clumps and used to re-clump fresh *S. aureus.* Clumping is not present in non-treated samples (Figure 20).

Chapter 4: Discussion

This thesis is composed of a series of experiments that aim to gain insight into a novel phenotype: the bacterial clumps formed in the presence of plant extracts. Understanding this phenomenon is essential as its applications would facilitate the removal of bacteria in solution. This would serve economic sectors such as the livestock industry to treat runoff water from agricultural sites. Antibiotic use in the animal agriculture industry has resulted in ARB being found in agricultural wastewater. Even if wastewater is treated in a conventional WWTP, these treatment plants are not designed to remove bacteria, but rather inadvertently promote proliferation of ARB and ARGs (Liu et al., 2019; Gatica et al., 2013). ARB of agricultural origin are ultimately dispersed into water and soil systems (Beukers, et al., 2023). The influx of foreign bacteria into natural soil and water bodies has the potential to change their microbiome, enabling ARB to continue proliferating (Gatica et al., 2013). The dispersal of ARBs is not only a concern for human health but also for the environment. Clump-inducing plant extracts could act as a method to treat agricultural wastewater, by removing the bacteria (many of which are antibiotic-resistant) and limiting its dispersal, thereby reducing chances of infection.

Prior to my arrival at the Microbial Research Laboratory at Lethbridge College, a unique phenotype was discovered by the laboratory when screening Alberta prairie plant extracts for their antibacterial activities. This phenotype is characterized by visible aggregations of bacteria in liquid culture, which was termed by our laboratory ‘clumping.’ Under the supervision of Dr. Sophie Kernéis, I conducted a project intending to characterize the clumping activity exhibited by certain plant extracts. This project is the first to investigate Alberta prairie plants for their clumping activities on bacteria with a scientific approach, granting novelty to the project. It is important to note that this master’s thesis is a pilot project to understand and develop ways to study the clumping phenotype induced in bacteria by certain plant extracts. It is expected that the results collected here will be used to advise further research into other clump-inducing plant extracts and their effects on a wider range of bacteria, such as those listed in table 3.

My master's thesis is the continuation of the research I started during an independent study and an undergraduate thesis at the Microbial Research Laboratory. As noted by Cushnie et al., (2007) there are few standardized techniques that allow for this phenotype to be studied, due to its novelty. As a result, many of the techniques described here are ones that I developed in the laboratory. These techniques can now be used to further study this plant extract-induced bacterial clumps and to extend to the other clumping extracts to obtain potentially many different clumping factors for other relevant pathogenic bacteria than *S. aureus*. The importance of finding clumping factors that could act as alternatives to antibiotics is exemplified by rising levels of antibiotic resistant strains of bacteria. Using the methods and results presented, this project can be expanded to include other strains of bacteria.

4.1 Part 1: Bacterial clump formation promoted by plant extracts

4.1.1 Alberta prairie plant extracts cause clumping of bacteria.

The Antibiotic Alberta Prairie Plant Project focuses on describing the bioactive effects of plant extracts made from Alberta plants. The plant extracts that cause clumping in at least one bacterium are listed in Table 3. While the clumping effect is scarcely reported in the literature, our studies reveal that there are several botanical species, belonging to different families, exhibiting clumping activities with different species of bacteria. Interestingly, certain plant extracts will cause clumping in only specific bacteria and not others. For example, GP2B causes clumping of *S. epidermidis,* and does not cause clumping of *E. coli* and *C. freundii* (Table 3). GP10B causes clumping of *S. epidermidis* and *C. freundii* but not of *E. coli.* GP10A causes clumping of *E. coli, S. epidermidis* and *C. freundii* (Table 3). The differences in ability to clump different bacteria by different plant extracts could indicate the presence of distinct clumping molecules being found in the plant extracts. Plant extracts contain an unknown number of molecules due to their crude nature (Erb & Kliebenstein, 2020). It could be that clumping molecules are highly specific to a bacterial species and will only cause clumping in one species of bacteria. Alternatively, it could be that plant-derived clumping molecules are not very specific, and one type of clumping molecule may cause clumping in

several types of bacteria. Plant extracts that can cause clumping in more than one species of bacteria may contain several different clumping molecules.

Using the microdilution assay it has been shown that many plant extracts can clump bacteria (Table 3). It is important to note that the clumps formed vary in terms of their size, their appearance, and the optical density of the surrounding solution, in favour of having different mechanisms of action of the clumping factors present in the plants showing clumping activities. Table 3 reveals that there are several plant extracts causing clumping of different bacteria by potentially different mechanisms of action.

4.1.2 GP95B and GP2215B induce clumps with distinct phenotypes.

When considering the results obtained with the crystal violet assay (an assay considering dead and living bacteria), it is concluded that the number of bacteria present in the clumps formed with GP95B at 1 mg/mL ($M = 0.521$ $SD = 0.0181$) 0.5 mg/mL ($M = 0.566$ $SD = 0.0312$) and with GP2215B at 1 mg/ml ($M = 0.502$ $SD = 0.00264$) is the same (determined by Two-Way ANOVA and Tukey's HSD) (F (3,52) = 17.0, $p < 0.01$) (Figure 5). The clumps formed with GP2215B at 0.5 mg/mL ($M = 0.858$ $SD = 0.0392$) have a higher number of bacteria than for GP95B at both concentrations and for GP2215B at 1 mg/mL (F (3,52) = 17.0, $p < 0.01$) (Figure 5).

Taking the results obtained by the crystal violet assay (Figure 5) and the visual observation (Figure 4) of the clumps it is concluded that the composition of the clumps is dependent on the extract. The relationship between clump size and extract concentration is also dependent on the extract (F (3,52) = 17.0, $p < 0.01$) (Figure 5). These conclusions favor the presence of different clumping activities in GP95B and GP2215B, whether it is due to a different number of the same clumping molecules, or to the presence of different clumping molecules.

4.1.3 GP95B and GP2215B (concentration of 0.5 mg/mL) show a different inhibition on the growth of *S. aureus* at 18 hours.

To confirm the presence of an inhibitory activity in the chosen plant extracts, three techniques were utilized to investigate the antibacterial activity of the two plant extracts: the microdilution, microdiffusion and the viable plate count (VPC) techniques.

GP2215B at 1 mg/mL shows a complete inhibition of S. *aureus* growth, as seen as a flat line for the optical density of this sample over the 18 hours of incubation (Figure 11). At 0.5 mg/ml, GP2215B shows a complete inhibition up to 12 hours, with growth starting after this time. Statistical analysis comparing the optical density of non-treated *S. aureus* with decreasing concentrations of GP2215B at 18 hours revealed that the concentrations 1, 0.5 and 0.125 mg/mL result in the optical density of *S. aureus* at 18 hours decreasing significantly (F (5, 12) = 59.1, $p < 0.001$) (Figure 11).This observation demonstrates the presence of inhibitory molecules in GP2215B which explains the clarity of the surrounding solution in samples treated with both concentrations (Figure 4).

Unlike GP2215B, it is observed in the results of the microdilution technique that GP95B shows a partial inhibition of *S. aureus* over 18 hours at the concentrations tested (Figure 10). It was found using a One-Way ANOVA that GP95B at 1 (M = 0.754 SD = 0.00155) and 0.5 mg/mL (M = 0.738 SD = 0.0124), and 0.125 mg/mL (M = 0.8103 SD = 0.0196) significantly reduce the optical density of *S. aureus* at 18 hours compared to the value of non-treated *S. aureus*; indicating that GP95B exhibits an inhibitory effect on *S. aureus* at the two concentrations (F (5, 12) = 59.1, $p <$ 0.001) (Figure 11).

The final optical density values of *S. aureus* treated with GP95B at 1 and 0.5 mg/mL do not differ significantly from one another suggesting that the inhibitory effect exhibited by the plant extracts at these two concentrations does not differ, according to this technique.

The microdilution technique is a useful tool that allows for many plant extracts to be assessed at once for potential clumping and inhibitory effects. However, this technique is typically utilized to

measure the optical density of uniformly turbid solutions. Clump formation may interfere with the optical density reading due to the clumps' irregular shapes. Therefore, it is important to use additional techniques to properly characterize the inhibitory effects of GP95B and GP2215B.

The microdiffusion technique, based off the Kirby Bauer Disk Diffusion method, is commonly used in microbiology to determine the antibiotic-resistance of bacteria towards commercialized antibiotics. When comparing the zones of clearing produced by the two plant extracts, it is observed that GP95B produces at both concentrations a greater zone of clearing when compared to GP2215B (Figure 12). However, this technique is based on the diffusion of the molecules inside the agar medium and so the size of the zone of inhibition will not be proportional to the antibacterial activity of the molecules studied unless we consider the same ones (Nassar et al., 2019). Indeed, the Kirby Bauer method uses standardized tables to estimate the antibiotic-resistance of the bacteria tested against known antibiotics (Nassar et al., 2019). This technique is particularly useful in mitigating the interference caused by clumping activity from plant extracts, which could affect results when using the microdilution technique solely based on optical density measurements.

Finally considering the results obtained with the viable plate count, high inhibitory activities are confirmed for both plant extracts at 0.5 mg/mL. Both GP2215B (F (3, 58) = 185, $p < 0.001$) (Figure 7) and GP95B (F (3, 72) = 4.022E+17, $p < 0.001$) (Figure 6) significantly reduce the growth of *S. aureus* after 18 hours. GP95B shows a 94% reduction in living *S. aureus* at 18 hours while GP2215B shows an 84% reduction of living *S. aureus* at 18 hours (Table 4). GP95B reduces the growth of *S. aureus* at a significantly greater magnitude than GP2215B, t (16) = -6.96 $p < 0.001$ (Figure 8).

In conclusion, the results of these techniques show that both plant extracts have antibacterial activity that depends on the concentration of plant extracts considered. The clumping activities depend on the quantity of bacteria present therefore the magnitude of the inhibitory effect will impact the quantity of bacteria present in the clump. For example, it would be expected that increasing the

inhibitory effect on the bacteria would decrease the size of the clump. These conclusions show the intrinsic link between the clumping and inhibitory activities exhibited by the plant extracts.

4.1.4 Plant extracts have a bactericidal activity.

As discussed, in the previous paragraph both GP95B and GP2215B exhibit a high inhibitory activity on the growth of *S. aureus* (Table 4). The following question is to evaluate the mechanisms leading to this inhibition. By referring to known antibiotics we can distinguish different activities based on their mechanisms of action. For example, Penicillin and the beta-lactam antibiotics in general will inhibit the formation of peptidoglycan (Spratt, 1978). Peptidoglycan is one main component of the bacterial cell wall, and its inhibition will lead to osmotic shock of newly synthesized bacteria (Spratt, 1978).

Bacteriostatic antibiotics such as tetracycline will inhibit the proliferation of the bacteria. Tetracycline acts on the ribosome, affecting the protein synthesis of the bacteria, thereby making them incapable of growth or division, but allowing their cell morphology to be preserved (Chukwudi, 2016). In a clinical situation where a bacteriostatic antibiotic is used, it will be expected that the host's immune system removes the bacteria (Chukwudi, 2016). However, in an experiment, there is no host immune system to perform this task.

The microdiffusion techniques and the viable plate count only consider living bacteria, whereas the crystal violet technique indirectly considers living and dead bacteria as they will both be stained by crystal violet. It is known that the dead bacteria may be less stained than the living bacteria due to an alteration of their cell walls (Scherrer, 1963). Considering the results obtained for both extracts at 0.5 mg/mL with the crystal violet assay, there is a significantly greater quantity of crystal violet released for the clumps formed with GP2215B at 0.5 mg/mL in comparison to the release of crystal violet by the clumps formed with GP95B at 0.5 mg/mL (F (3,52) = 17.0, $p < 0.01$) (Figure 5). This is in favor of having more bacteria in the clumps formed with GP2215B (0.5 mg/mL) than in the ones formed with GP95B (0.5 mg/mL). This result does not align with the results obtained with the

viable plate count in which only 74 % of the bacteria are present in clumps formed with GP2215B at 0.5 mg/ml while GP95B has 88 % of the bacteria in the clumps (Table 4). This technique only considers living bacteria while the crystal violet assay counts the living and the dead bacteria. Considering the results of the two techniques, it can be concluded that clumps formed with GP2215B at 0.5 mg/mL contain more dead bacteria than the clumps formed with GP95B, both being obtained with 0.5 mg/mL of extracts.

Pursuing this analysis, it is observed that GP95B causes a cloudy surrounding solution of the clumps formed at both concentrations while for GP2215B at both concentrations the solutions surrounding the clumps are very to fairly clear depending on the concentration used (Figure 4). Considering the results obtained with the viable plate count that enumerates only the living bacteria after treatment with the extracts at 0.5 mg/mL, it can be concluded that the cloudy surrounding solution observed with 0.5 mg/mL of GP95B should contain mostly intact dead bacteria or a large amount of bacterial cell fragments. The percentage of bacteria found in the surrounding solution with the viable plate count is lower than that observed for the surrounding solution of the GP2215B surrounding solution at 0.5 mg/mL. It is hypothesized that the bacteria present in the surrounding solution of GP2215B at 0.5 mg/mL would be mostly alive.

To continue the interpretation of the proportion of living/dead bacteria present in GP2215B induced clumps two hypotheses are described; the first one in which for GP2215B the bacteria would be clumped alive and then killed while in the clumps, or the second being that bacteria are dead before being clumped. In this instance, the bacteria cells remain intact, and their outside surfaces would not be changed and would still be recognized by the clumping factors. These hypotheses aim to address the conclusion that GP2215B induced clumps contain living and dead bacteria.

For GP95B induced clumps it is hypothesized that the clumping factors are only clumping living bacteria because the dead bacteria would have altered outside surfaces that would not allow them to be clumped. A second hypothesis is that the clumping factors would be saturated by living

bacteria not allowing the dead bacteria to be in the clumps, this could suggest a higher affinity between clumping factors and the living bacteria, as opposed to dead bacteria. The hypothesis demonstrating that clump formation is based on the ratio of bacteria to clumping molecules is visualized in figure 22. To pursue these questions, it would be beneficial to perform vitality staining to determine in the surrounding solution and in the clumps the respective proportion of living versus dead bacteria for both extracts at 0.5 mg/mL.

Considering the experiments in which the bacteria were killed by fixing them with ethanol, it was shown for both extracts that even if *S. aureus* are fixed, they will be able to be clumped by the plant extracts (Figure 17). This result aligns with the idea that dead bacteria can be found in the clumps, should they be killed in a way that preserves their structure. The fact that GP95B would have few dead bacteria in the clumps can be in favor of the mechanism of inhibition damaging the cell wall of the bacteria reducing their capacity to be clumped. It is not clear the exact mechanisms of action of the clumping and inhibitory activities, it is strongly suggested that the two plant extracts should have distinct mechanisms of action.

4.1.5 Efficiency of clump formation is dependent upon the ratio of bacteria to clumping molecules.

As discussed, in the previous paragraph, it is intriguing to see a different clumping phenotype for both extracts at 0.5 mg/mL when treated with *S. aureus.* Despite the differences in the phenotypes induced by the two plant extracts, it is obvious that the concentration of the plant extract affects its clumping and inhibitory abilities. This can be seen with the microdiffusion technique where the results show a lower zone of inhibition for extracts when at 25 mg/mL compared to their inhibitory capabilities at 50 mg/mL (Figure 12). This suggests that because the inhibition of *S. aureus* growth is higher at 1 mg/mL than at 0.5 mg/mL, there will be fewer bacteria at the highest concentration reducing the number of bacteria in the clumps. This observation is demonstrated with GP2215B at 1 mg/mL in which the clumps are very small, and the solution is clear, at 0.5 mg/mL we can see clear surrounding solution with bigger clumps, the clumps appear very compact compared to the one

observed with GP95B at both concentrations (Figure 4). Regarding the observation of the wells for GP95B at both concentrations we can see that their surrounding solutions are cloudy at both concentrations suggesting the presence of bacteria. These bacteria based on the viable plate counts must be mostly dead as the quantification revealed only 12% of living bacteria present in the surrounding solution. Because with the microdilution technique, the two concentrations of GP95B show a similar inhibition of *S. aureus* (Figure 10), it is expected that there will be a similar number of living bacteria in the clumps induced by GP95B at both concentrations. This notion is further supported by the results of the crystal violet assay that shows clumps induced by GP95B at either concentration do not differ in size (Figure 4). Based on the viable plate count it is found that 16% of the living bacteria are in the surrounding solution of the clumps induced by 0.5 mg/mL GP2215B. For this extract, it is suspected that the clumping factor may have a similar affinity for *S. aureus* whether they are living or dead or that bacteria can be killed when inside the clumps. These hypotheses are summarized in figure 21.

Because we have been able to isolate the clumping molecules present in the clumps, we will be able to address the question to know if the clumping factor of GP2215B is responsible for this double activity (inhibition and clumping) on *S. aureus*.

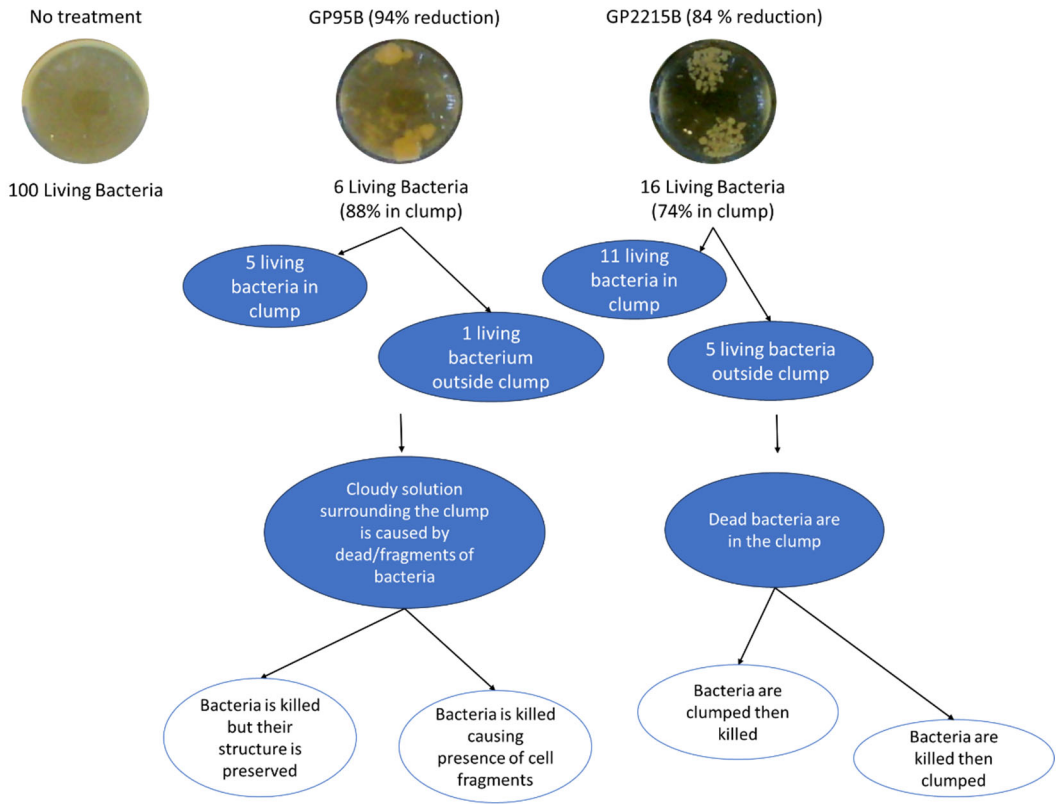

Figure 21. Visualization of the hypotheses addressing the inhibitory and clumping effects of GP95B and GP2215B on S. aureus.

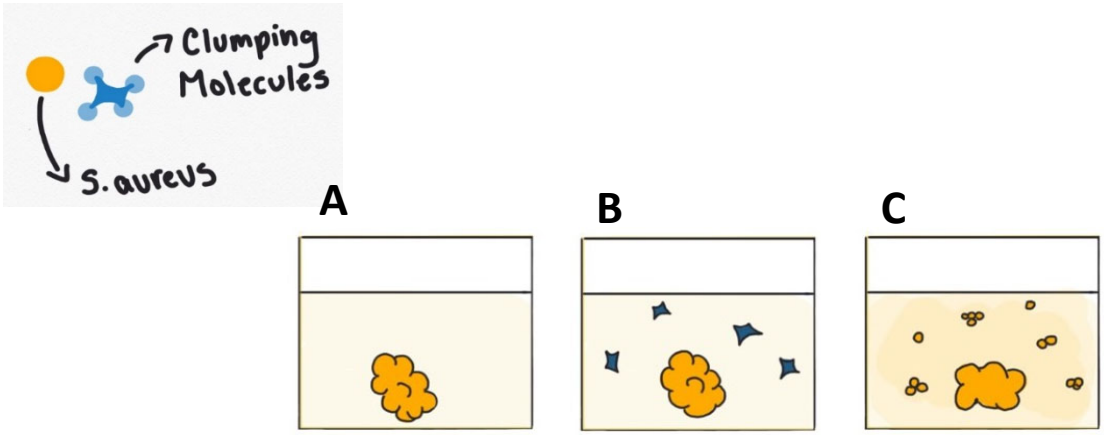

Figure 22. Visualization of the hypothesis stating that clumping efficiency is dependent upon the ratio of *S. aureus* to GP95B or GP2215B clumping molecules.

A) The ratio of clumping molecules to bacteria is ideal. There are no bacteria or clumping molecules in the solution surrounding the clump; B) The number of clumping molecules exceeds the amount required for clump formation. Excess clumping molecules are found in the solution surrounding the clump; C) The number of bacteria exceeds the concentration of clumping molecules. There are not enough clumping molecules to clump each bacterium, so there are excess bacteria in the solution surrounding the clump.

4.2 Part 2: Chemical characteristics of plant extracts GP95B and GP2215B

4.2.1 Characterization of clumping molecules in plant extracts GP95B and GP2215B.

To view *S. aureus* clumps induced by GP95B and GP2215B, the bacteria and one plant extract were added to a microscope slide, and clump formation occurred. It is possible to heat fix and stain the resulting *S. aureus* clumps so they can be viewed under brightfield microscopy (Figure 16). The results of this technique show clumping of *S. aureus* after exposure to GP95B or GP2215B 0.5 mg/mL after just five minutes. This observation will prove to be useful in this chapter, as this technique allows for the quick determination of clumping abilities of plant extracts. Using this technique, it can be quickly determined whether clump formation is possible after manipulations are made to *S. aureus* or the plant extracts.

This also indicates that clumping is occurring quickly demonstrating the fast-acting nature of these clump-inducing compounds. Not only are clumps visible on the microscope slide after five minutes (Figure 16), but they are still visible after 18 hours of incubation (Figure 4). This is interesting because clumps are forming quickly but maintaining their structure throughout the 18-hour incubation. This observation speaks to the stability of clumping molecules from GP95B and GP2215B.

4.2.2 Clump formation is not induced by *S. aureus*.

In figure 17, chemically fixed bacteria were incubated with GP95B or GP2215B at 0.5 mg/mL on a microscope slide. Fixing the bacteria using a chemical fixative such as ethanol kills the bacteria but keeps their structure intact (Kniggendorf et al., 2011). The results of this experiment indicate that GP95B and GP2215B 0.5 mg/mL can cause clumping of fixed *S. aureus.* This suggests that clumping is not induced by the bacteria in response to the presence of plant-derived molecules. There are many instances in which bacteria may respond to environmental stressors. Certain environmental conditions may trigger a biochemical change in bacteria (Le & Otto, 2015; Xu et al.,

2016). For example, the presence of certain environmental factors may cause the bacteria to respond defensively by aggregating or beginning biofilm formation (Le & Otto, 2015).

The results indicating that GP95B and GP2215B cause clumping of fixed bacteria shows that clump-formation is not a result of a biochemical reaction performed by the bacteria, such as biofilm formation, as fixed bacteria are not alive. Knowing that clump formation is not the result of a process initiated by the bacteria, it is suggested that clump formation is a result of a physical "adhesive" process, whereby clumping molecules would bind to multiple bacteria, causing them to clump together. For this to be the case, clumping molecules must be able to attach to more than one bacterium, otherwise, a clump would not form. These results show evidence of a potential mechanism of action. This hypothesized mechanism is visualized in figure 23.

The hypothesis suggests that clumping molecules interact with the outer surface of the bacterial cell. *S. aureus* is known to be covered in around 24 cell wall-anchored proteins (Foster, 2019). The exact number of cell wall-anchored proteins is dependent on the strain of *S. aureus* and the culture conditions (Foster, 2019). These proteins are important in host-pathogen interactions. For example, Clumping Factor Protein A (ClfA) is an important structure of the *S. aureus* cell wall; ClfA binds to fibrinogen as a method of immune evasion (Foster, 2019). Due to the adhesive nature of the clumping molecules in GP95B and GP2215B, it could be hypothesized that the clumping molecules are interacting with structures on the outer surface of *S. aureus.*

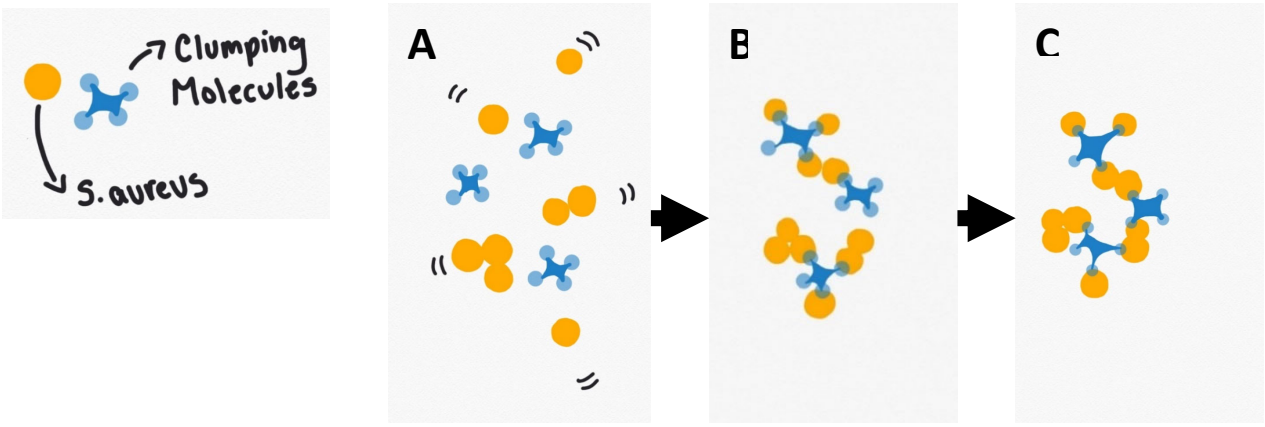

Figure 23. Visualization of the hypothesized mechanism behind clump formation in *S. aureus* with GP95B and GP2215B.

A) *S. aureus* & clumping molecules in solution with agitation; B) Clumping molecules begin to bind to at least one bacterium, forming small clumps, C) Small clumps continue to be bound together by the clumping molecules, forming a larger clump.

4.2.3 Clump-inducing plant extracts do not select variants of *S. aureus.*

Observing that plant extracts GP95B and GP2215B induce clumping in *S. aureus*, the question arises as to whether these clump-inducing plant extracts are favoring the selection of specific *S. aureus* genotypes. To test this, *S. aureus* were treated with GP95B or GP2215B at 0.5 mg/mL for 18 hours. The clumped bacteria were collected, washed, and inoculated into a fresh medium that was either non-treated or treated with GP95B or GP2215B (depending on which extract the bacteria were previously clumped with). The optical density analysis and resulting phenotype (clumping) of previously treated bacteria were compared to that of freshly prepared and treated *S. aureus.*

If a certain genotype of *S. aureus* were being selected by clump-inducing plant extracts, it would be expected that clumps of previously clumped bacteria would be larger than those of fresh bacteria, as most or all of the bacteria should have been found in the clump. This is not what was visualized in Figure 15. It seems that having been previously clumped, does not affect the clump size of the bacteria when in fresh medium, after treatment with GP95B or GP2215B 0.5 mg/mL. Therefore, it is concluded that clump-inducing plant extracts do not select for certain genotypes of bacteria in the clump. One of the drawbacks of this experiment is that the resulting clumps were not measured for their size (for example, using the crystal violet assay), but only assessed visually.

Figure 13 depicts the bacterial growth curve of fresh, and previously clumped bacteria treated with GP95B and non-treated. When visually assessed, the curves obtained for *S. aureus* previously clumped with GP95B 0.5 mg/mL do not differ from those of fresh *S. aureus,* when it is not treated, or treated with GP95B 0.5 mg/mL (Figure 13).

The same conclusions cannot be made with GP2215B as were made with GP95B regarding their effects on fresh and previously clumped *S. aureus.* As seen in figure 14, the optical density curves of previously clumped with GP2215B, and fresh *S. aureus* are similar. However, there is a slight increase of the lag phase for the fresh *S. aureus* compared to non-treated *S. aureus* that was previously clumped with GP2215B (Figure 14). It is unclear why we have a reduced lag phase for the

previously clumped bacteria with GP2215B. However, the results of this experiment indicate that using previously clumped bacteria does not increase the effectiveness of clump formation by either GP95B or GP2215B. This leads to the conclusion that clump formation does not select for a bacterium with a specific genotype during clump formation.

It is interesting to note that in previously clumped *S. aureus,* when grown with no treatment, clumping is not seen (Figure 13 & 14). Previously clumped *S. aureus* were washed before being reinoculated into fresh medium. This observation suggests that clumping molecules can be removed from *S. aureus,* quite easily in favor of a weak binding between the clumping molecules and the bacteria; in other words, clump formation is reversible. With this observation it is shown that clumping molecules could be removed from *S. aureus* clumps to facilitate chemical identification.

4.2.4 Clumping molecules can be removed from *S. aureus* clumps and used to clump fresh bacteria.

As described, the evidence in Figures 13 & 14 suggests that GP95B and GP2215B clumping molecules can be removed from *S. aureus* clumps and used to reclump fresh *S. aureus.* Clumping molecules were separated from *S. aureus* clumps induced by GP95B and GP2215B. The isolated compounds were added to fresh *S. aureus,* with a low quantity of media (because the quantity of clumping molecules in the sample is unknown, a low quantity of media will enable one to keep a higher concentration of clumping molecules). It was found that *S. aureus* clumps had formed after treatment with the concentrated compounds (Figure 20). The clumps appear different in phenotype to those induced by GP95B and GP2215B, this could be because the exact concentration of the clumping molecules is unknown and a result of a lower quantity of *S. aureus*. It is unlikely that only the clumping molecules were isolated, however, reducing the number of chemicals within the sample will allow for easier identification of the molecule(s) responsible for clump formation.

The data represented in Figure 20 is based off an experiment using a small amount *S. aureus* clumps induced by GP95B and GP2215B that allowed for the collection of clumping molecules. It

should be noted that this experiment can be replicated on a larger scale. This was done by adding 1 mL of 1/1000 dilution overnight culture of *S. aureus* in TSB to an Eppendorf with 1 mL of GP95B or GP2215B 0.5 mg/mL. By adding more bacteria and more plant extract, larger clumps were observed (data not shown). This will aid in the identification of clumping molecules, as a large quantity of the clumping molecules will be needed for further identification. This also shows that clump-inducing plant extracts can cause clumping of *S. aureus* when the scale of the experiment is increased.

4.2.5 Clumping molecules are not proteins or carbohydrates.

The results in figure 18 indicate that heat-treating the plant extracts does not inhibit clump formation. With this information, it can be hypothesized that clumping molecules found in GP95B and GP2215B are not proteins nor contain proteinaceous compounds important for their clumping activities. While it has been shown that some lectins cause aggregation of bacteria, it is suggested that GP95B and GP2215B induced clump formation is not the result of the presence of lectins or other protein compounds (Kabir et al., 2015; Kabir & Reza, 2014). While this can be hypothesized from these results, it could be further analyzed by protein extraction and by using an SDS-Page gel to determine the presence of proteins in concentrated clumping molecule fractions.

It is seen in figure 19 that treatment of GP95B and GP2215B with TFA does not inhibit their ability to cause clump formation in *S. aureus.* This indicates that the clumping molecules in GP95B and GP2215B are likely not polysaccharides, or molecules that contain polysaccharide components.

Further interpretation of these observations would benefit from a positive control. These two assays do not elucidate the exact chemical characteristics of clumping molecules found in GP95B and GP2215B. They do not possess chemical properties of proteins or carbohydrates. These observations will allow for easier determination of their identity.

This still leaves the question of the chemical structure of the clumping molecules. It was shown that clumping molecules can be isolated from *S. aureus* clumps. Using this technique, the chemical structure of the isolated clumping molecules can be determined by HPLC, NMR and Mass

spectrometry. It could be hypothesized that the clumping molecules are flavonoids. The plant extracts come from two different plant families both characterized by large amounts of flavonoids. Galangin, a plant-based flavonoid was found to have a similar aggregatory effect on *S. aureus* (Cushnie et al., 2007). Therefore, we could hypothesize that clumping molecules in GP95B and GP2215B could be flavonoids.

Chapter 5: Conclusions

The purpose of this thesis was to investigate and compare the novel clumping effects of plant extracts GP95B and GP2215B on *S. aureus.* This pilot project described techniques that I developed and can be used to continue the study of clump-inducing plant extracts.

In this thesis two plant extracts from different plant families were studied: GP95B and GP2215B. The two plant extracts were found to cause effective clumping in *S. aureus* by clumping 88% (GP95B) and 74% (GP2215B) of living *S. aureus.* Both plant extracts caused a reduction in the growth of *S. aureus* over 18 hours. GP95B had a significantly greater inhibitory effect, reducing *S. aureus* by 94%, while GP2215B caused an 84% decrease in *S. aureus.* Even though GP95B is showing to be more effective at inhibiting the growth of *S. aureus* both plant extracts exhibit effective clumping and inhibitory effects.

Clump formation by both GP95B and GP2215B is a result of physical interactions between clumping molecules and bacteria, rather than an interaction in which bacteria are actively involved. This was demonstrated through the ability of the plant extracts to cause clumping of fixed *S. aureus*. Therefore, it can be concluded that the clumping molecules act through an adhesive manner by "holding" onto more than one bacterium.

The mechanism proposed supports the hypothesis that efficiency of clumping is dependent on the ratio of clumping molecules to bacteria. The nature of the physical interaction between clumping molecules and bacteria favors this hypothesis because a clumping molecule should have a limit to how many bacteria it can interact with, simply due to the size of the bacteria and clumping molecules. Therefore, the number of clumping molecules needed to cause clumping in *S. aureus* is dependent upon the number of bacteria. This hypothesis is visualized in figure 22.

In terms of the larger application of this research, it has been shown that there are many Alberta plant extracts that cause clump formation in at least one bacterium (Table 3.). This thesis outlines protocols that allow for the clumping and inhibitory effects of the plant extracts to be

assessed, compared, and chemically purified for further identification. While this thesis focuses on *S. aureus* as a model organism, we showed that there is a larger number of plant extracts that induce clumping in different species of bacteria. These techniques may be applied to other plant extracts and other bacteria.

It is becoming increasingly important to find strategies to reduce or manage the proliferation of ARB (Bengtsson & Wierup, 2006; Bougnom & Piddock, 2017; Chen et al., 2019; Couch et al., 2019; Hong et al., 2013). Conventional WWTPs are known to promote the proliferation of antibiotic resistance. There are high levels of ARB in animal agriculture wastewater. Therefore, it is pertinent to find strategies to reduce the dispersal of ARB from agricultural origin. Plant-based coagulants/flocculants have been used to treat industrial wastewater (Cardoso Valverde et al., 2018). Similarly, clump-inducing plant extracts could be used to clump bacteria, limiting their dispersal, and reducing the possibility of interaction between bacteria and humans or animals. This could limit instances of infection by a bacterium, and reduce the need for antibiotic use, thereby reducing the potential selection of more ARB. Reducing the dispersal of ARBs from soil bodies, and from WWTP will reduce the proliferation of ARB and the transfer of ARGs between bacterium in these environments. The data collected here can be used in the future to find clumping molecules that interact with different bacteria from different environments.

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