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Discovering New Antibiotics: Bacterial Extracts Separated by Thin-Layer Chromatography Inhibit the Growth of Staphylococcus

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Bacterial Extracts Separated by Thin-Layer Chromatography Inhibit the Growth of *Staphylococcus*

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Abstract

Many bacteria have become resistant to commonly used antibiotics because of antibiotic use in people and animals. Therefore, new antibiotics are needed that will inhibit these resistant bacteria. Bacteria found in soil are a likely source for new antibiotics because of the limited available nutrients found in the soil. We isolated soil bacteria and screened them for antibiotic production against *Staphylococcus epidermidis*. Methanol extracts were made from entire agar plates of the soil bacteria that inhibited *S. epidermidis*. These extracts were spotted on a lawn of *Staphylococcus aureus;* growth inhibition was measured to confirm that the extracts contained the antimicrobial compounds. The confirmed inhibitory extracts were then separated by thin-layer chromatography using a chloroform-methanol mobile phase. The separated compounds were individually suspended in methanol and spotted onto *S. epidermidis* or *S. aureus* to assess inhibitory ability. Whole cell metabolite extracts isolated from four soil bacteria were found to inhibit both *S. epidermidis* and *S. aureus*. Four TLC-separated metabolite compounds, one from Hargis and three from Jackson, were found to inhibit *S. epidermidis*. These compounds will be further assessed for viability as new therapeutically relevant antibiotic treatments.

INTRODUCTION

Antibiotics production has been observed in a variety of bacterial species. An antibiotic is any molecule that inhibits the growth of or kills a microorganism. Antibiotics are most often produced in response to nutrient limitation. Bacteria manufacture these molecules as a competition mechanism, inhibiting nearby microorganisms that may be consuming necessary and limited resources (Hibbing, Fuqua, Parsek, & Peterson, 2010). A bacterium has no way of "seeing" if its neighbors are consuming the resources. So instead, when a major nutrient is limited, bacteria go into the stationary phase of growth and begin to produce secondary metabolites, including antibiotics (Aharonowitz & Demain, 1978). This introduction into the stationary phase can be signaled by catabolite repression or the stringent response. In catabolite repression, the absence of glucose causes the production of cAMP, which functions as a signaling molecule to stimulate the production of a variety of secondary metabolites including antibiotics (Aharonowitz & Demain, 1978). In the stringent response, a lack of nitrogen and thus charged tRNA causes the production of guanosine tetraphosphate, which also can signal antibiotic production (Traxler et al., 2008).

In addition to nutrient starvation, crowding can also stimulate antibiotic production. Quorum sensing is when bacteria perform an action only when the population has reached an elevated level (Waters & Bassler, 2005). Bacterial cells may produce an autoinducer that diffuses out of the cell. If there are enough cells of the same species producing this autoinducer, it will begin to diffuse back into the cells. The autoinducer will then signal for upregulation of various genes, including antibiotic synthesis genes (Waters & Bassler, 2005). When quorum sensing is used in antibiotic production, antibiotics are produced and stored so that lethal quantities can be released quickly to kill or inhibit the target cells without time for the cells to acquire resistance (Hibbing et al., 2010).

MECHANISMS OF ANTIBIOTIC RESISTANCE

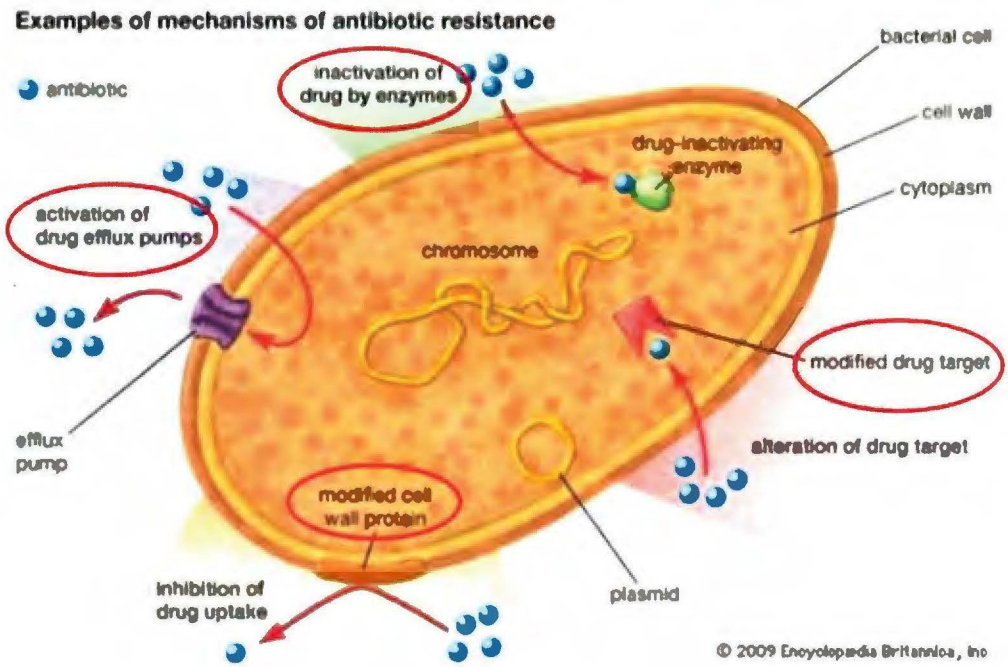


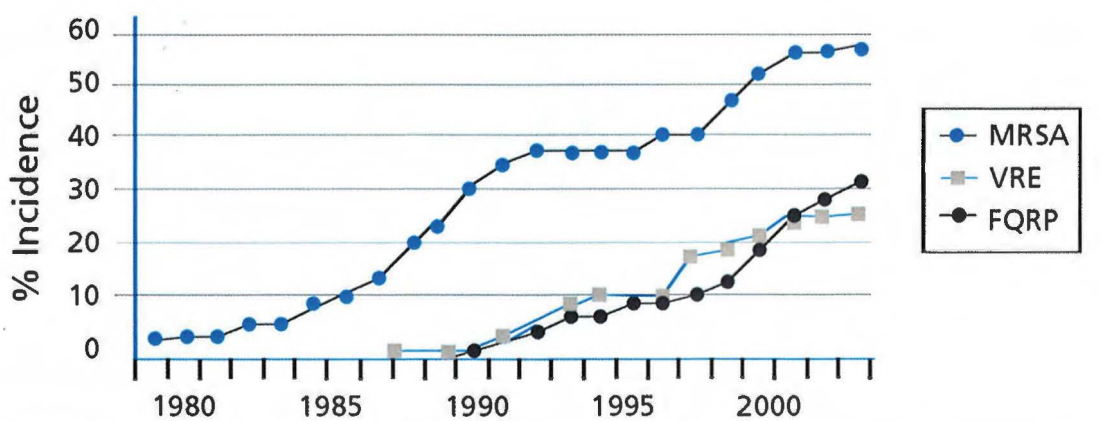
Figure 1: Mechanisms of antibiotic resistance.

This figure shows the most common mechanisms of bacterial resistance against antibiotics.

Mutations and acquisitions of adventitious physiological changes are common in bacteria because of their short generation time. When a population of bacteria is exposed to low levels of an antibiotic, resistance may develop as the individual cells that have a mutation protecting them from the antibiotic are able to survive and pass on the genes for that trait (Hibbing et al., 2010). Figure one indicates how bacteria can become resistant to antibiotics by decreasing production of porins that import the antibiotic, by producing efflux pumps to remove the antibiotic from within the cell, by producing an

enzyme to degrade or inactivate the antibiotic, or by acquiring mutations in the binding target of the antibiotic (Morier, 2016).

Antibiotic resistance in pathogenic bacteria is a huge problem in the medical field. Every year, in the United States alone, more than 2 million people become infected with bacteria that are resistant to at least one commonly used antibiotic ("Antibiotic Resistance", 2013). Figure two illustrates that methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and fluoroquinolone-resistant *Pseudomonas aeruginosa* have been on the rise since the early 1980s, only 40 years after the first antibiotic, penicillin, was produced for commercial use ("Alexander Fleming", 1999). Widespread resistance to penicillin and similar β-lactams such as methicillin is now a large problem because of overuse of these early antibiotics. Many bacteria were exposed to the antibiotic and resistant strains survived and proliferated, causing population growth of the resistant strains. Exposure to antibiotics may come through human ingestion and passage to waste water or through animal prescriptions ("Antibiotic use", 2014) (Rizzo et al., 2013).



MRSA = methicillin-resistant *Staphylococcus aureus*, VRE = vancomycin-resistant enterococci, FQRP = fluoroquinolone-resistant *Pseudomonas aeruginosa*

Infectious Diseases Society of America. 2004. Bad Bugs, No Drugs.

Figure 2: Rise of antibiotic-resistant bacteria since 1980. This graph shows three antibiotic resistant bacteria and the increase in percent incidence over the past 35 years.

Mass production and funding for research for the discovery of new antibiotics has been slowing down, even as bacterial resistance has been on the rise. Many drug companies have stopped producing antibiotics, because of the poor financial investment return. The problem is that antibiotics do their job. They kill the bacteria, stop the infection, and are no longer needed. Drug companies are more interested in drugs for long-term use and chronic illnesses ("Race against time", 2011). These drugs will continue to sell because they are required for the rest of a patient's life. Another problem affecting production of new antibiotics is lack of novelty in antibiotic mechanism. The World Health Organization reports that in a 2008 study, only 15 of 167 antibiotics being researched displayed potential to combat multidrug resistant pathogens ("Race against time", 2011).

In light of the increase of antibiotic resistant bacteria and decreasing number of effective commercial antibiotics, new antibiotic discovery is imperative. In this project I screened soil bacteria for antibiotic production. Once antibiotic producers were identified, I used two—Bubbles and Hargis-- as models to optimize antimicrobial extraction and purification methods. I then assayed whole cell extracts and isolated compounds for activity against *Staphylococcus*.

A Need for New Antibiotics

Acquisition of antibiotic resistance in bacteria found in livestock:

Antibiotic resistance in pathogenic bacteria is an increasingly important problem in the medical field over the past few decades. In 2014 the Centers for Disease Control and Prevention (CDC) stated that an estimated 2 million people per year become infected with antibiotic resistant bacteria and at least 23,000 people die each year due to these infections (Antibiotic Resistance, 2014). These pathogens become resistant when they are repeatedly exposed to the antibiotic through human or animal use. The bacteria can defend itself against antibiotics by a variety of traits including diminished production of porins, increased production of efflux pumps, inactivation or destruction of the antibiotic, or mutation of the binding target. Once any one of these traits is acquired, the susceptible bacteria which are exposed to the antibiotic quickly die, leaving the resistant ones alive and thriving, often without other bacteria to compete with for a food source.

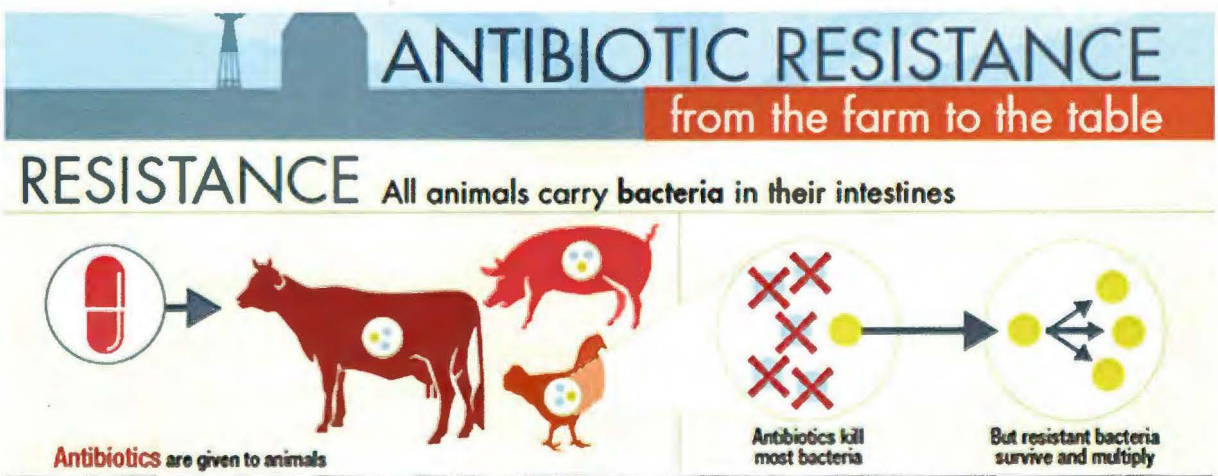


Figure 3: Spread of Antibiotic Resistant Bacteria from Livestock.

Antibiotic use in livestock can select for resistant bacteria that can then be spread to humans via meats, animal products, and vegetables. This image was published by the CDC.

When animals are fed antibiotics, resistant bacteria survive and reproduce, permitting livestock to act as a reservoir for antibiotic resistant bacteria (NARMS, 2015). While it is difficult to directly compare the amount of antibiotics being used in livestock and in humans, several studies have indicated that about 75% of antibiotics sold in the US are sold into the livestock industry (2013 Summary, 2015). While this antibiotic usage in livestock arguably helps animals and farmers, it contributes to the rise of antibiotic resistant bacterial infections in humans.

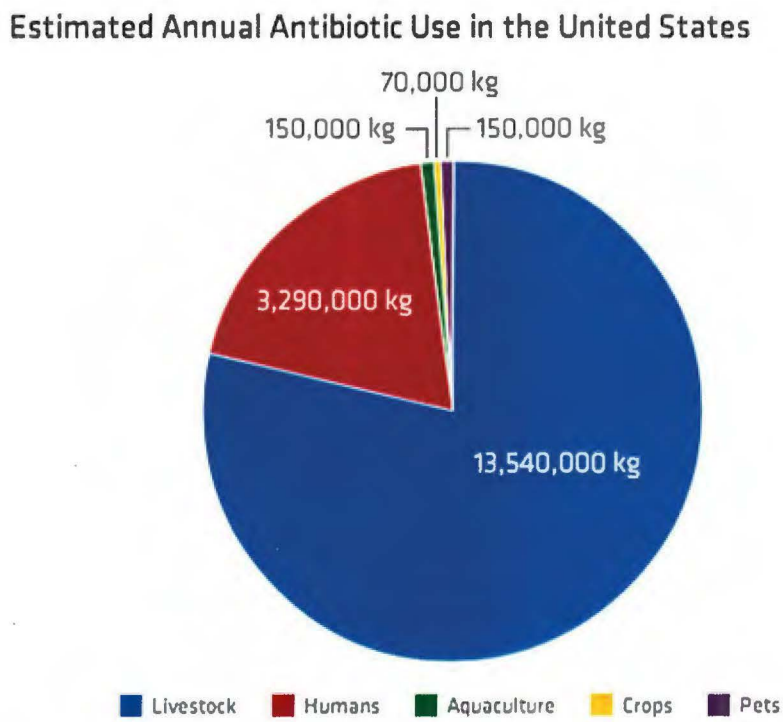


Figure 4: Distribution of Antibiotic Use per Year.

This graph shows the estimated percentage of antibiotics used per year in kilograms for livestock, humans, aquaculture, crops, and pets. This image was produced by Aidan Hollis and published in *The New England Journal of Medicine.*

Transmission of resistant bacteria from livestock to humans:

The resistant bacteria cultured in antibiotic-treated animals can be passed to humans in one of three ways: consumption of undercooked meat, consumption of crops grown in manure fertilizer, and water runoff from farms and agricultural environments (NARMS, 2015). Many intensively farmed food animals, such as chickens and turkeys, are routinely fed antibiotics during growth. The consequent resistant bacteria can be transmitted to humans if the meat is not cooked properly. Further, fertilizers made with animal fecal matter can contain the resistant bacteria and contaminate fields of fruits and vegetables. Water runoff from agricultural environments, like livestock ranches and farms, can contain the resistant bacteria as well (Fig. 5)(NARMS, 2015). An example of a bacteria transmitted in this way is *Salmonella*, which causes an estimated 1.2 million infections in the United States each year. Of these, about 23,000 are hospitalized, and 450 die from their infections (NARMS, 2015). It is very important that pathogenic bacteria like these are exposed to the least amount of antibiotics possible to reduce proliferation of resistant strains.

Antibiotic resistant bacteria can also be passed from person to person. People carrying these bacteria can transmit the infection through everyday contact in social environments. Some of the places bacteria are most likely to spread are medical environments like hospitals and nursing homes (Fig. 6).

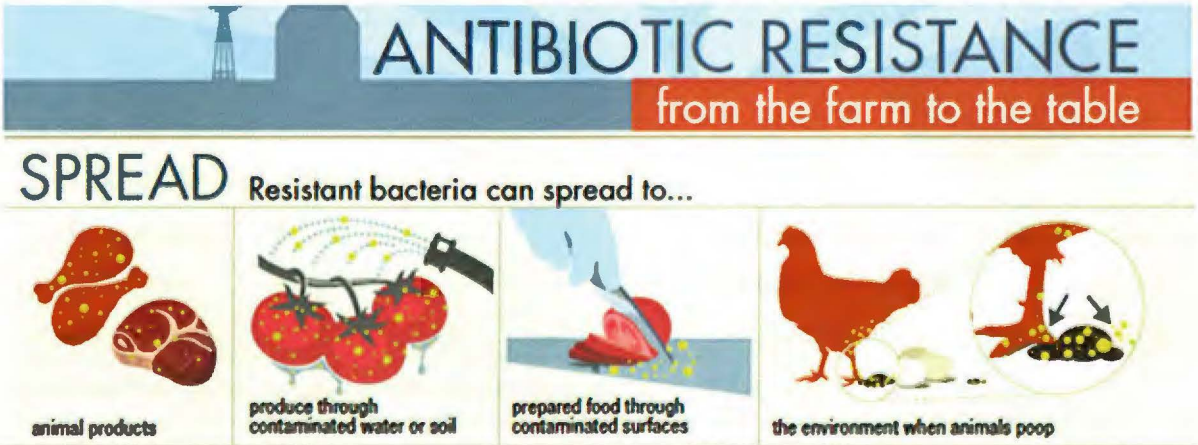


Figure 5: Spread of Antibiotic Resistant Bacteria to Humans.

Bacteria can be transferred to humans when we eat animal products or products that have been contaminated by bacteria in the environment, soil, or water. Published by the CDC.

Misuse of antibiotics and promotion of livestock growth:

Resistant bacteria result from widespread, repeated use of antibiotics. These drugs are not just used to treat sick animals in the agriculture industry. Antibiotics are often used fed to animals that live in close quarters to keep them healthy and to promote their growth (Fig. 6). Antibiotic use has been shown to increase animal growth by inhibiting the normal gut flora, which allows the animal to better utilize nutrients, as there are less microbes to consume the nutrients (Gaskins, Collier, & Anderson, 2002). Since antibiotic-treated animals have fewer microbes in their gut, the animal is able to spend less energy maintaining the balance of gut flora, which also increases the size of the animal (Gaskins et al., 2002). The development of antibiotic resistance in bacteria has been expedited by the concurrent use of similar antibiotic in humans and animals. Figure 6 shows antibiotic classes prescribed to animals. All antibiotic classes used in livestock are also prescribed to humans except ionophores. Bacteria that are resistant to one antibiotic are often also resistant to other antibiotics that work by a similar mechanism. Therefore, the use of antibiotics with similar mechanisms in humans and animals increases the potential for resistance development (Fig. 6).

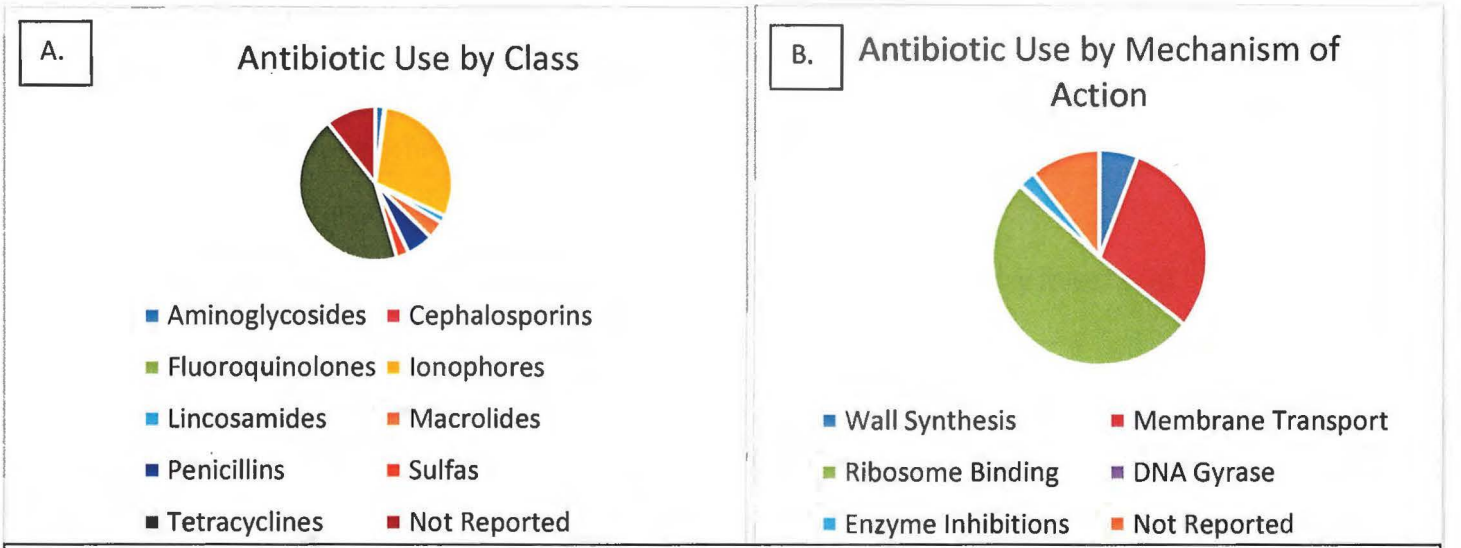


Figure 6: Antibiotic drug classes currently used in the treatment of livestock in kilograms.

Bacteria that acquire resistance to one antibiotic often also have resistance to antibiotics that work through the same or similar mechanisms. Chart 6A shows different classes of antibiotics used in livestock. Chart 6B shows antibiotics separated by mechanism. Charts adapted from information published in the "2013 FDA Annual Report on Antimicrobial Sold or Distributed for Use in Food-Producing Animals."

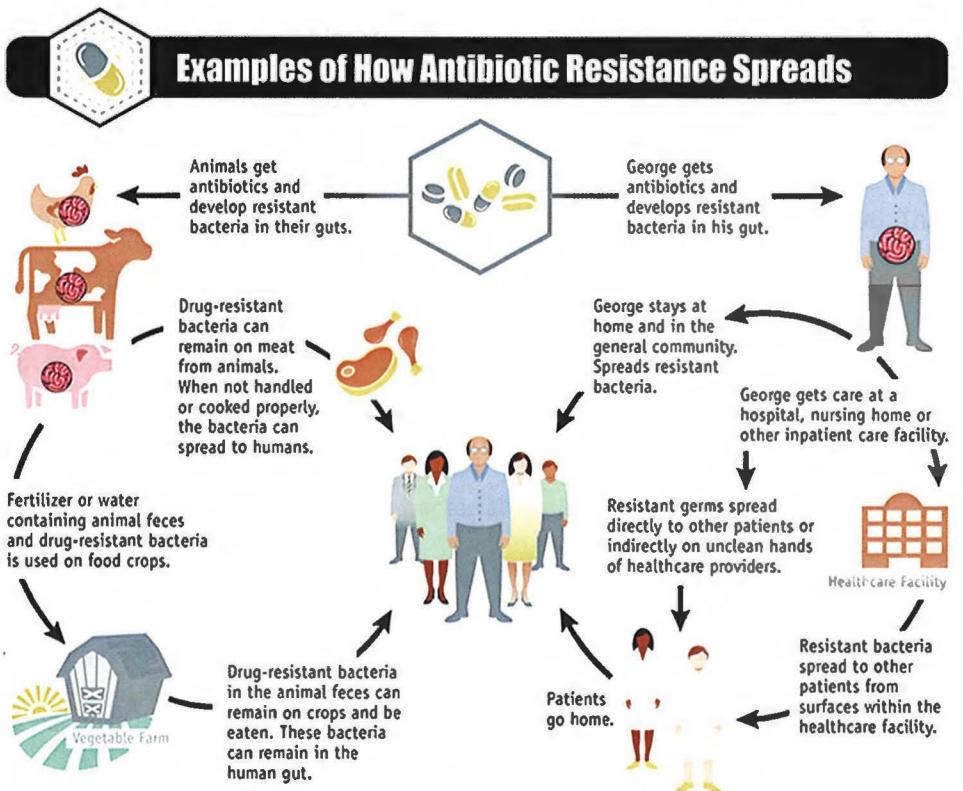


Figure 7: Spread of antibiotic resistant bacteria among animals and humans.

Bacteria that are resistant to antibiotics can be spread from animals to people. From there these bacteria can be spread through everyday contact or through healthcare facilities like hospitals and nursing homes.

Changes being made to decrease resistance in bacteria carried by livestock:

The Generic Animal Drug and Patent Restoration Act (GADPTRA), passed in November of 1988, was one of the first laws that brought information about livestock-approved antibiotics into the public eye. This act requires information on all drugs approved by the Food and Drug Administration (FDA) for use in the treatment of animals to be made available to the public in a publication known as the Green Book (Green Book, 2016). The Green Book contains information on drug trade names, active ingredients,

sponsor companies, and species it has been approved for use on (Green Book, 2016). The information made available in the Green Book aids scientists in their research on antibiotic resistant bacteria.

The National Antimicrobial Resistance Monitoring System (NARMS) is a collaboration of the CDC, FDA, and the U.S. Department of Agriculture (USDA) that was launched in 1996. This system tracks antibiotic resistant bacteria, such as *Salmonella* and *Campylobacter,* which are known to infect humans through food (NARMS, 2015). The system also identifies control points that can reduce the transfer of antibiotic resistant bacteria to food, soil, and water from agricultural locations. NARMS tests humans, livestock, and retail meat for resistant bacterial strains to monitor trends and outbreaks, helping them better understand the emergence and spread of antibiotic resistance. NARMS also provides information to federal agencies, policymakers, agricultural industries, and the public on how to reduce resistance among bacteria in food-producing animals and helps the FDA make decisions regarding safe antibiotic use for animals (NARMS, 2015). One of NARMS primary recommendations is that antibiotics be used only for therapeutic purposes and that they be reserved for treatment of animal and human health needs and only under the supervision of a qualified health professional (NARMS, 2015).

Animal producing industries are getting pressure to moderate antibiotic use both from regulatory agencies and consumers. The increase in antibiotic resistant bacterial infections has made consumers more aware of the dangers of misusing antibiotics. Many foodservice companies are letting concerns about antibiotic resistance drive their purchase of meats. According to a report published in 2012, sales for meats without routine use of antibiotics were up 25 percent over the three prior years, despite an overall decline in U.S. per capita meat consumption of beef, pork, chicken and turkey over the same time period (Perrone, 2012).

In addition to these consumer concerns and a growing demand for safer animal products, on April 11th, 2012 the FDA published three documents that called for steps to reduce antibiotic use in livestock

(US Food and Drug, 2012). *The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals* is a guide for the agricultural industry that recommends phasing out use of medically important drugs for promotion of livestock growth and phasing in veterinary oversight in the use of these antibiotics for therapeutic purposes. A draft guidance was also published which requested that drug companies voluntarily remove production uses of antibiotics from their FDA-approved product labels and add veterinary oversight to the marketing status. The final document released by the FDA on the topic was an outline of ways that veterinarians can authorize the use of certain animal drugs in feed, which can make the needed veterinary oversight efficient and practical (US Food and Drug, 2012).

Due to this pressure from both the FDA and consumers, companies like McDonalds have promised to decrease the use of unnecessary antibiotics in their food-producing animals. McDonalds has a plan to completely eliminate the use of antibiotics important to human medicine from their chickens by March of 2017 (McDonalds, 2015). Panera Bread chicken and turkey products are both 100% antibiotic free as of 2015 (Yohannan, 2015). This trend continues to rise as many restaurant chains vow to use antibiotic-free meat products. Cable Network News (CNN) has published a list of food service companies who are making changes in their policies on meats raised with antibiotics. The list reports that Panera Bread and Chipotle's are doing the best, serving almost all of their meats raised without routine antibiotic use Tinker, 2015). Chick-Fil-A is also making changes to their meats in regard to antibiotic use. Dunkin' Donuts and McDonald's are planning to make changes, but as of yet do not have working policies fully in place (Tinker, 2015).

MATERIALS AND METHODS

Bacteria and Media:

Bacteria. Hargis, Jackson, and Small are antibiotic-producing bacteria that were isolated at Ouachita Baptist University in Fall 2014. Additional bacteria were isolated from soil during this research as described in the Small World Initiative protocols (Data Collection, 2015). *Staphylococcus epidermidis* and *Staphylococcus aureus* were used as screening bacteria to assess the antibacterial efficacy of antibiotic-producing soil bacteria and cell extracts.

Media. All streak plates and spread-patch assays were performed on potato dextrose agar (PDA) plates. Most broths used were 100% brain heart infusion (BHI) broths. Hargis bacterium was grown in Luria-Bertani (LB) broth.

Isolation of Soil Bacteria:

Isolating Bacteria from Soil Sample. The purpose of plating the soil sample is to grow isolated bacterial colonies from the soil. Three grams of soil were diluted with phosphate buffered saline (PBS) to 30mL and mixed to form the 10^{-1} dilution. One milliliter of supernatant was taken from the 10^{-1} dilution and diluted with PBS to 15mL and mixed to form the 10^{-2} dilution. One hundred microliters of the 10^{-2} dilution were added to 900µL of PBS to make the 10^{-3} dilution. This process was repeated to create dilutions

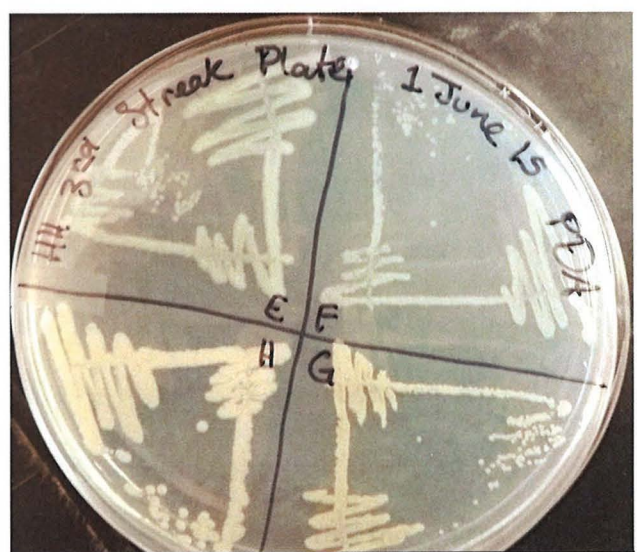


Figure 8: Quad-streak plate method

Four different soil bacteria can be isolated on a quartered agar plate using this method.

to 10^{-8}. One hundred microliters of each dilution was spread evenly with a sterilized spreading rod onto PDA plates. Plates were allowed to incubate inverted for 48hrs at 25°C.

Quad-streak plate. The purpose of the streak plate is to produce increasingly isolated colonies, each grown from a single bacterium. An agar plate was divided into quarters and labeled for bacteria to be streaked. A flamed loop was used to pick an isolated colony and streak the bacteria back and forth onto the appropriate quadrant of the agar plate. The loop was again flamed and pulled through the center of the previous streak to collect some bacteria and in a continuous motion, a second streak was made. This process was repeated 2 more times to produce 4 streaks with increasingly isolated bacterial cells (Fig. 8).

Screening for Antibiotic Production:

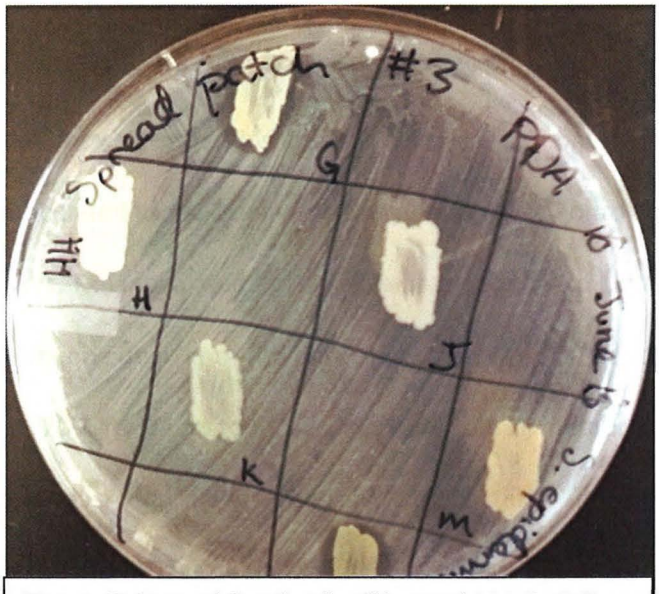


Figure 9: Spread Patch of soil bacteria against *S. epidermidis*.

Zones of inhibition can be seen around the patches marked G (Buttercup) and N (Blossom).

Spread Patch Method. This method was adapted from the Small World Initiative (Small World Initiative, 2012). A 4x4 grid was drawn onto the bottom of an agar plate. Squares were labeled for isolated soil bacteria and spaced so that bacteria were diagonal to each other, with at least one space in between. A maximum of 6 soil bacteria were applied to each plate. This was to ensure that the zone of inhibition could be clearly seen and measured for each individual bacterium. A sterile Q-tip was dipped into a broth of *Staphylococcus* and wrung out by pressing all surfaces of the Q-tip firmly to

the side of the tube. The screening bacteria was spread onto the agar plate by streaking the Q-tip back and forth to cover the entire agar surface, then turning the plate 45° and re-streaking with the same Q-tip. This process was repeated 3 times for maximum coverage of screening bacteria. The screening was allowed 3-5 minutes to dry onto the surface of the agar before soil bacteria was streaked on. This was to prevent the soil bacteria from running in the excess broth on the top of the agar. A flamed loop was used to pick an isolated colony of soil bacteria and then this bacteria was streaked into the center of the labeled square to form a patch, making each patch a uniform size. Spread-patch plates were allowed to grow at least 2 days at 25°C, then the zone of inhibition was measured (Fig. 9).

Isolation of Antimicrobial Metabolites:

Whole Cell Metabolite Extraction. This method was adapted from The Small World Initiative (Small World Initiative, 2012). The purpose of antibiotic extraction is to obtain maximal antibiotics produced by the bacteria for testing and thin-layer chromatography (TLC) separation. Two hundred microliters of inoculated broth, grown for 2 days, was spread onto an agar plate and allow to grow at 25°C for 2-4 days. The inoculated agar plate was chopped into $1cm^2$ pieces and both the agar and the bacterial lawn from one quarter of the plate was transferred into each of four glass scintillation vials. Vials were frozen at -20°C for 24hrs to lyse cells and release all metabolites produced. Three milliliters of methanol was added to each vial of frozen agar and bacteria. The vials were shaken at 120 to 170 rotations per minute (rpm) at 37°C for 24hrs to allow organic solvent to extract soluble components from the bacterial lawn and agar. All liquids were transferred from each of the vials into one clean vial. Organic solvents were allowed to evaporate by directing a weak stream of air onto the surface of the liquid for 24 to 48 hours. Whole cell extracts were resuspended in 80µL of methanol.

TLC Separation. Each resuspended extract was spotted onto a TLC plate using a capillary pipette, making a spot of about 0.5 cm in diameter. This was repeated five times, for a total of 6 droplets applied to the same location on the TLC plate, to ensure that adequate extract had been added to the plate. The TLC plate was run in a mobile system of 62 parts chloroform to 35 parts methanol to 3 parts water. Plates were viewed under both long and short wave UV light for visualization of separation.

Confirmation of Antibiotic Presence:

Spot inhibition assay. Two types of spot inhibition assays were performed: whole cell extract spot inhibition assays and TLC plate spot inhibition assays. For the whole cell extract spot inhibition assays, 10μL of resuspended whole cell extract was spotted onto a PDA plate and allowed to dry. This was repeated 3 times for a total of 30μL of extract per spot. A mixture of 7mL of 1/2x PDA top agar and 200μL of 2 day incubated *S. epidermidis* broth was poured over the spotted, dry, PDA plate. Spot inhibition assay using 2 day incubated *S. aureus*-inoculated 1/4x PDA top agar was also performed. Following a 48 hour incubation period, zones of inhibition were measured to determine antimicrobial activity.

For the TLC plate spot inhibition assays, TLC plate spots were cut out and the silica containing the spot was removed from the plate and transferred into a microcentrifuge tube. Sixty microliters of methanol was added to resuspend separated extracts. Five repetitions of 10μL spots of this suspension were pipetted onto a PDA plate and allowed to dry, for a total of 50μL. Two day incubated *S. epidermidis*-inoculated 1/2x PDA top agar was poured over the spotted, dry, PDA plate. Following a 48 hour incubation period, zones of inhibition were measured to determine antimicrobial activity.

Chloramphenicol and ampicillin were used as positive controls in both the whole cell extract spot inhibition assay and the TLC plate spot inhibition assay of *S. epidermidis* and *S. aureus*. Methanol was used as a negative control for both spot inhibition assays.

RESULTS

Spread Patch. I found six bacteria that inhibited growth of *S. epidermidis;* two of these six bacteria inhibited growth of *S. aureus*. Zones of inhibition for all isolated soil bacteria are reported in Figure 10. Of these antimicrobial-producing bacteria, I chose three that consistently produced clear zones of inhibition in the *Staphylococcus* and named them Buttercup, Blossom, and Bubbles (Fig. 10).

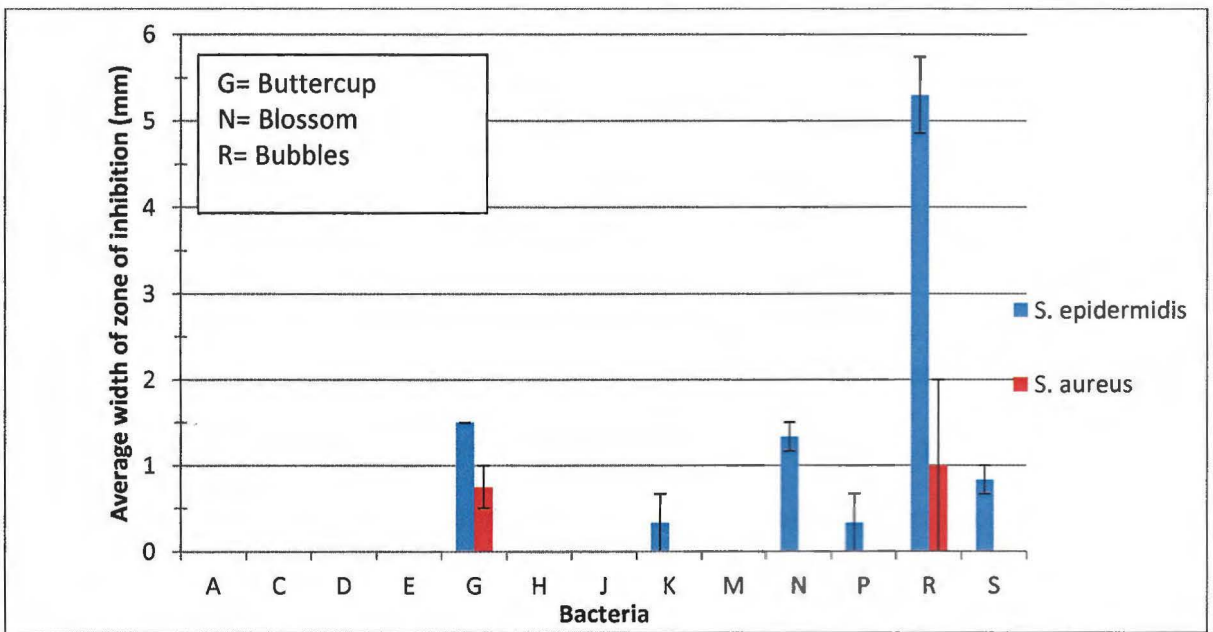


Figure 10: Zones of inhibition produced by soil bacteria against *S. epidermidis* and *S. aureus.*

Zones of inhibition were calculated by subtracting the width of the soil bacteria patch from the width of the zone of inhibition, divided by two. This calculation gives the width of the distance from the soil bacteria patch to the edge of the zone of inhibition.

Because Bubbles consistently produced larger and clearer zones of inhibition in both types of *Staphylococcus,* I chose this bacterium to perform the whole cell metabolite extraction (Fig.11).

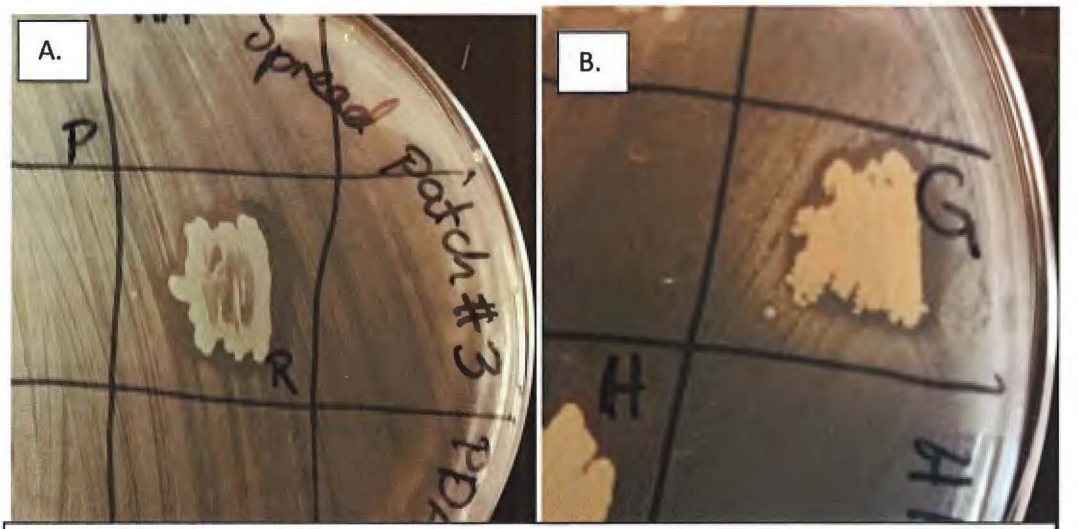


Figure 11: Zones of inhibition indicating antimicrobial compounds secretion from soil bacteria.

A. Bubbles inhibiting growth of *S. epidermidis.*

B. Buttercup inhibiting growth of *S. epidermidis*

Spot inhibition assay of Whole Cell Methanol Extract. In addition to Bubbles, three bacteria—Hargis, Jackson, and Small-- were chosen for whole cell methanol extraction and their extracts were screened againt *S. aureus* and *S. epidermidis* (Fig. 12). Commercial antibiotics ampicillin and chloramphenicol were used as positive controls and substantially inhibited *S. aureus* growth (Fig. 12). Methanol was used as a negative control to be sure that the process of spotting methanol onto the agar and allowing it to dry was not creating some sort of inhibitory effect on the *Staphylococcus.* Whole cell extracts of Bubbles, Hargis, and Small all inhibited *S. aureus* (Fig. 12A). In a second whole cell extract spot inhibition assay, Jackson also inhibited *S. aureus* (Fig. 12B). Hargis showed the largest zone of inhibition, inhibiting the *Staphylococcus* in a large area around the applied droplet (Fig. 12A). The antimicrobial metabolite produced by Hargis seems to have diffused through the agar into the surrounding area.

Figure 12: Whole cell extract spot inhibition assay against *S. aureus*.

Whole cell extract of Bubble, Hargis, Jackson, and Small plated against *S. aureus* in a spot inhibition assay. Jackson did not inhibit *S. aureus* in the first assay (Fig. 12A), but did show inhibition in subsequent assays (Fig. 12B). Ampicillin and chloramphenicol were used as positive controls. Methanol was used as a negative control.

TLC Separation. Whole cell extracts of Bubbles, Hargis, Jackson, and Small were individually spotted onto a TLC plate and run in a chloroform/methanol mobile phase. R_f measurements were recorded for each separated spot. Figures 15 and 16 show the final R_f measurements from TLC plates separating whole cell extract from Bubbles, Hargis, Jackson, and Small.

I tried two different methods of whole cell metabolite extraction of Bubbles and Hargis, one using methanol as described in the methods section and one using ethyl acetate and water (Small World

Initiative, 2012). The ethyl acetate method was supposed to yield an ethyl acetate layer containing extracted organic metabolites and a water layer containing cell debris. Unfortunately, with some bacteria, the ethyl acetate and water did not separate, producing an emulsion which contained both cell debris and extracted metabolites. Since cell debris made TLC separation more difficult, I chose to use the methanol extraction for the rest of my research. The first TLC plate of Hargis compares separation results using the methanol or ethyl acetate extraction methods (Fig. 13).

Bubbles did not separate very well with either type of extraction, although fresh extracts separated better than old extracts (Fig. 14 and 15A). Many of the TLC spots were indistinct and difficult to separate. Some spots were only visible under short wave UV light and some were only visible under long wave UV light. Figures 13 through 15 show all TLC spots, regardless of viewing wavelength.

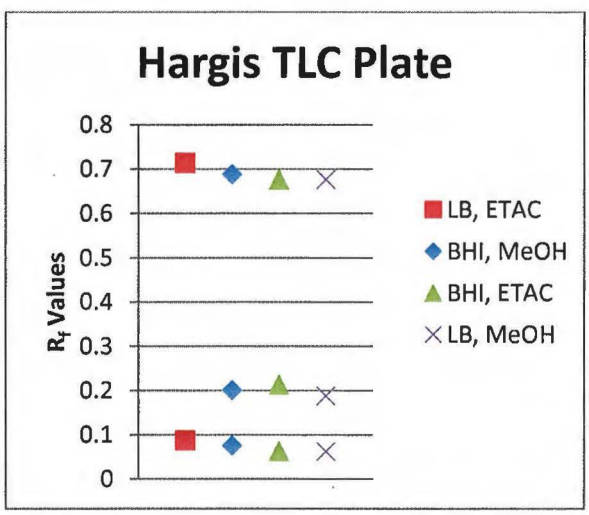


Figure 13: TLC Separation of whole cell Hargis ethyl acetate or methanol extract following growth in LB or BHI broth

Hargis did not grow well in BHI broth and was grown in LB broth prior to plating.

Ethyl acetate (ETAC) and methanol (MeOH) were compared as extraction solvents.

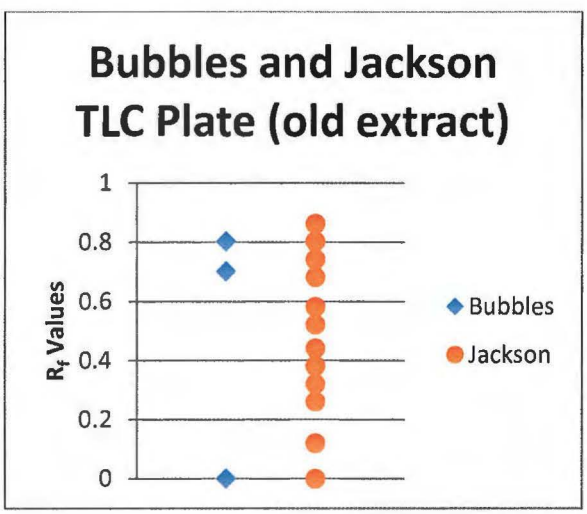


Figure 14: Old extract reduces separation in TLC.

Old whole cell metabolite extract does not separate well on TLC plates. The extract becomes viscous and sticky. Fresh extract yields much better separation (Fig. 15).

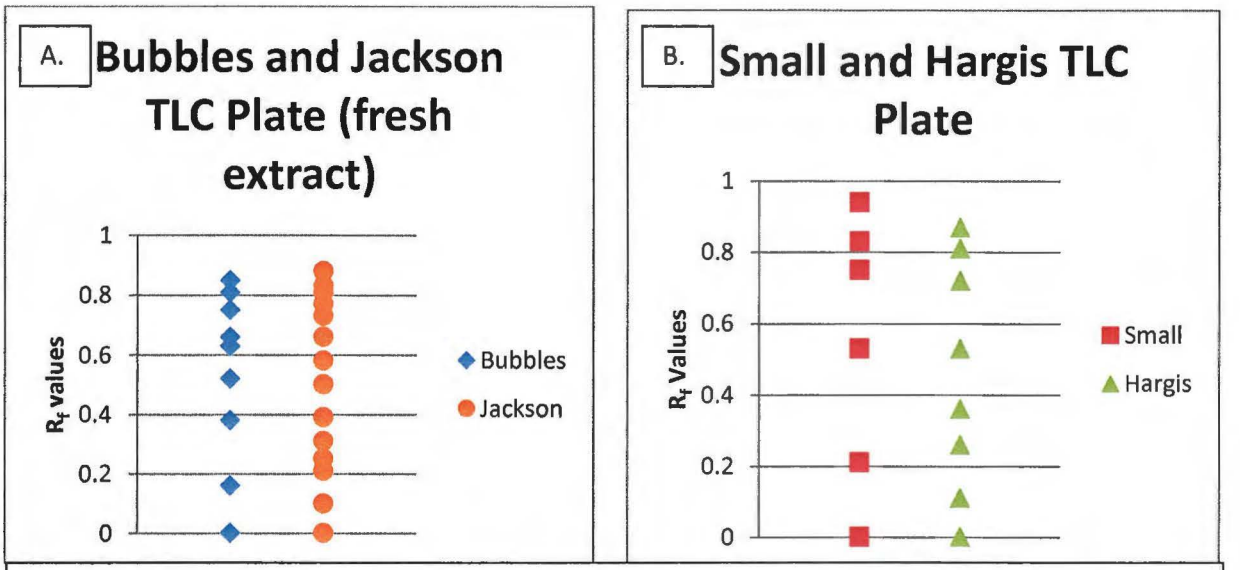


Figure 15: Final TLC plates separating whole cell extracts of Bubbles, Hargis, Jackson, and Small.

All four whole cell extracts were successfully separated by TLC and R_f values are reported here. These isolated spots were then cut out, resuspended in methanol and used in a spot inhibition assay too test inhibitory effectiveness against *S. epidermidis.*

TLC Spot Inhibition Assay. All spots from the TLC plates were cut out and resuspended in methanol. A spot inhibition assay was then performed on these resuspended metabolic compounds. Four compounds were found to inhibit the growth of *S. epidermidis*. One of these compounds was isolated from the Hargis whole cell extract and the other three were isolated from the Jackson whole cell extract. The compound isolated from Hargis inhibited growth on top of the area of the pipet droplet and, in addition, showed slightly more inhibition in a faint halo around the droplet area. All three compounds from Jackson inhibited growth only on top of the area that the pipet droplet covered (data not shown). The Hargis compound may have caused more inhibition because it is smaller in size, allowing the molecules to diffuse more freely through the agar and therefore inhibiting a larger area of *S. epidermidis* than the metabolites isolated from Jackson. The whole cell extract from Hargis also had the largest zone of inhibition in the whole cell extract spot inhibition assay.

Discussion and Conclusions

My research this summer has optimized methods for antimicrobial metabolite extraction and separation. This work contributes to the resolution of the antibiotic crisis by using old methods made new again to search for novel antibiotics that might be able combat the rise in antibiotic resistant bacterial infections. Overuse of antibiotics in humans and in food-producing animals has contributed to this increase in antibiotic resistant bacteria. Decreased discovery and production of new antibiotics by pharmaceutical companies has exacerbated the antibiotic crisis.

A variety of soil bacteria were isolated that inhibited the growth of *S. epidermidis* and *S. aureus*, including the four bacteria that I worked with on this project—Bubbles, Hargis, Jackson, and Small. The growth inhibition assay was used to identify antibiotic producing bacteria. Metabolites were extracted from these bacteria and their inhibitory ability against *Staphylococcus* was assessed to confirm that the antibiotic was in the extract. The whole cell extract was then separated using thin layer chromatography with a methanol-chloroform mobile phase and each isolated compound was put through another growth inhibition assay to see which individual compounds have antimicrobial activity against *Staphylococcus*.

Future plans for this project include further separation using 2D TLC to ensure that no spots are stacked on top of one another. Using an additional mobile phase with different affinities for the metabolites, we will be able to separate these potentially stacked metabolites. A hexane/chloroform mobile phase is a prospective option for these 2D TLC plates. Other future plans include using the R_f measurements from the TLC plates created in this research to run larger amounts of the whole cell metabolite extract through high performance liquid chromatography (HPLC) to get larger quantities of isolated antimicrobial metabolites. These large quantities of antimicrobial metabolites will be further

characterized using mass spectrometry and will be put on plant seeds to make sure that the compounds are not toxic to eukaryotic cells.

Compounds isolated and identified in this project can be assessed for viability as new antibiotics for therapeutic use in humans. The ultimate goal of this project is to find new antibiotic treatments, which work by different mechanisms, to combat multidrug resistant pathogens and to protect these new treatments from misuse. We must not follow the same paths that first lead us to the antibiotic crisis. Antibiotics should be used only when absolutely necessary in both humans and food-producing animals. We must use these drugs responsibly and ethically to maintain their viability for the generations of the future.

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