Antimicrobial Properties of a Venom Alkaloid in a new Species of Panamanian Ant

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Evolution and Ecology in the undergraduate colleges of The Ohio State University

by

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April 2019

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Antimicrobial Properties of a Venom Alkaloid in a new Species of Panamanian Ant

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Abstract

Alkaloids are employed throughout multiple kingdoms, with one of the most well-known applications being defense. However, social organisms like ants have evolved a broader array of usages for these compounds, such as communication, resource monopolization, and as hygienic agents against microbial pathogens. *Megalomyrmex*, a small neo-tropical ant genus, produce a diversity of alkaloids for a variety of applications to aid them in defense and parasitism, yet the use of these substances as antimicrobials have not yet been documented in this group. For this study, we tested for the presence of *trans*-2-butyl-5-heptylpyrrolidine, a venom-derived alkaloid found in multiple ant genera and in or on the workers and brood of *Megalomyrmex* c.f. *wallacei*. We assessed the antimicrobial properties of this alkaloid using six diverse bacterial species and determined the minimum inhibitory concentration of the alkaloid for each using broth microdilution in a 96-well microtiter plate. This alkaloid inhibits the bacterial growth of six species (three Gram-positive and three Gram-negative strains). We also determined that *trans*-2-butyl-5-heptylpyrrolidine was the only alkaloid found in the venom of this ant species, and it can be detected on brood, suggesting possible transmission between workers and offspring. These findings suggests that *Megalomyrmex* c.f. *wallacei* may transfer the alkaloid-containing venom onto the brood to combat microbial pathogens, as has been shown in other ant species.

Introduction

A variety of organisms that span kingdoms (e.g., fungi, animals, plantae, bacteria) sequester or biosynthesize alkaloidal compounds (Aniszewski, 2015). Loosely defined as the class of naturally occurring heterocyclic organic compounds containing nitrogenous bases, alkaloids are used by organisms for a diversity of biological functions (Aniszewski, 2015). Alkaloids are often categorized into three groups, which include true alkaloids and protoalkaloids derived from cellular amino acids, as well as pseudoalkaloids, or those not derived from amino acids (Aniszewski, 2015). These three main types contain various alkaloidal chemical groups, including quinolines, piperidines, pyrrolizidines, steroids and many others, that are present in the alkaloid compounds produced by many organisms (Aniszewski, 2015). Alkaloids are produced throughout multiple families of plants as one of the main groups of defensive secondary metabolites that are used for protection against herbivorous organisms (Bennett and Wallsgrove, 1994) and microbial pathogens (Joosten and Van Veen, 2011). Endophytic fungi, found within plants, also produce a multitude of alkaloids that are toxic to various bacteria, fungi and insects (Zhang et al., 2012) (Riedell et al., 2017). While alkaloids produced by plants and fungi are toxic to most herbivores, a few insect groups, mainly lepidopterans and beetles, have evolved methods to sequester these alkaloids (Hartmann and Ober, 2007) and use them for individual defense (Hartmann, 1999) (Boppré, 1986), and can even allocate them to their offspring for protection (Dussourd et al., 1988). Certain predators of alkaloid-producing insects will also sequester these substances, with one of the most well-known examples being the conspicuously colored frogs of the family Dendrobatidae (Saporito et al., 2012). These frogs ingest alkaloid rich arthropods and appropriate the compounds for their own defense against predators and pathogens (Saporito et al., 2012). Other predators, such as *Pitohui* birds, will also sequester toxic alkaloids from arthropods for defensive usage (Dumbacher et al., 2004). Despite alkaloids being exploited as defensive compounds, a few groups of organisms have evolved much wider applications of alkaloids.

Ant-produced alkaloids have received much research attention for their broad range of functions, with the characterization of compounds in *Solenopsis* and *Monomorium* (Jones et al., 1982). *Solenopsis* alkaloids have been demonstrated to be important for inter-colony interactions such as communicatory pheromones between workers (Vander Meer et al., 2010) and reproductive signals (Eliyahu et al., 2011), whereas *Monomorium* ant alkaloids play a role in interspecific competition by repelling other ant species to allow the monopolization of food resources (Andersen et al., 1991). The neo-tropical genus *Megalomyrmex* consists of 44 species (Boudinot et al., 2013) and produces four classes of alkaloids (i.e., pyrrolidine, pyrrolizidine, piperidine, pyrrroline) (Adams and Jones, 2010) (Adams et al., 2013, 2015) (Jones et al., 1991a, 1991b). *Megalomyrmex mondabora, M. mondaboroides* and *M. silvestrii* are parasitic thief ants and produce nine different alkaloids from three different structural classes, releasing their alkaloids when stinging their fungus-farming ant host and as an aerosol when gaster-flagging (Adams et al., 2000, 2015). In addition, some species are known as guest ant parasites of the fungus-farming ants (e.g., *M. adamsae, M. symmetochus*) (Adams et al., 2012, 2013). *M. adamsae* use their alkaloidal venom against host queens while infiltrating the nest (Adams et al., 2012) whereas *Megalomyrmex symmetochus* use venom to suppress host aggression (i.e., appeasement substance) while also defending the farmer associates from a more lethal ant parasite (Adams et al., 2013). Remarkably, when *Megalomyrmex* sting the invading raider ant, their venom not only serves as a lethal toxin but as a propaganda substance (e.g., behavior disrupter), causing the raider ants to turn on one another and kill their own nestmates (Adams et al., 2013). However, most *Megalomyrmex* species are not social parasites but free-living predators, living in nests on the forest floor (Longino, 2010). Several free-living *Megalomyrmex* produce a wide array of pyrrolidine alkaloids (Jones et al., 1991a) which are likely used as repellents during competitive interactions when scavenging (Jones et al., 1991b) (Adams unpublished). Ants who nest in the soil have evolved many alkaloid-based chemical defenses to counteract the threats presented by soil-borne microbial pathogens, as many of

these alkaloids contain antibacterial and antifungal properties (Vander Meer, 2012) (Vander Meer and Morel, 1995) (Jouvenaz et al., 1972).

Although the production of antimicrobial alkaloids is known in multiple organisms, this subject has only been studied in limited capacity in ants. Piperidine alkaloids—the main alkaloidal components of *Solenopsis, Solenopsis* venom (MacConnell et al., 1971)—are known to inhibit the growth of several Gram-positive and Gram-negative bacteria (Jouvenaz et al. (Jouvenaz et al., 1972). *Solenopsis invicta* queens utilize these antimicrobial properties by spreading venom onto their eggs, inhibiting the growth of pathogenic fungi and bacteria, all while applying pheromones to attract workers to tend to the offspring (Vander Meer and Morel, 1995). *Solenopsis invicta* workers also produce these piperidine alkaloids and have additionally been suggested to disperse their venom onto offspring through gaster-flagging within the brood chambers (Obin and Vander Meer, 1985). Free-living *Megalomyrmex* species produce some of the same alkaloids found in *Solenopsis* (e.g., 2,5-dialkylpyrrolidine) (Jones et al., 1982, 1991a), as well as alkaloids unique to *Megalomyrmex* (Jones et al., 1991a), yet the antimicrobial application of these substances in *Megalomyrmex* remains largely unknown, despite the prospect of discovering versatile natural products that can have biomedical and agricultural uses (Escoubas and Blum, 1990).

trans-2-Butyl-5-heptylpyrrolidine is a naturally occurring alkaloid that has been previously found in *Monomorium smithii* (Westermann et al., 2015), *Solenopsis fugax* (Blum et al., 1980) and *Megalomyrmex goeldii* (Jones et al. 1991a). With this alkaloid present across genera, and one genus (*Solenopsis*) being well known for using alkaloids as antimicrobials (Vander Meer, 2012) (Vander Meer and Morel, 1995) (Jouvenaz et al., 1972), we sought to investigate the antimicrobial properties of this alkaloid. For this study, we tested for the presence of *trans*-2-butyl-5-heptylpyrrolidine in *Megalomyrmex* c.f. *wallacei,* a potentially new *Megalomyrmex* species. We analyzed the effects of this venom alkaloid against six species of bacteria by assessing the minimum inhibitory concentration (MIC), or the lowest concentration of a

substance that inhibits bacterial growth after 24 hours (Andrews, 2001). In addition, we conducted a chemical analysis to confirm the presence of this alkaloid on the brood, providing evidence to suggest that the workers or the queen disperse venom alkaloids to offspring for protection against pathogens. This study has many broad applications, one being applied to biomedical research, as naturally derived chemicals from insects are often at the forefront of antibiotic, antiviral and even anticancer applications (Dossey, 2010) However, our focus is to understand the toxicity of ant venom and the evolution of novel strategies to protect ant colonies from pathogens to improve their fitness and survival.

Methods

Ant Colonies and Care

In 2018, two colonies of *Megalomyrmex* c.f. *wallacei* were collected near El Llano, Panamá (El Llano forest, 9°16'46.40"N 78°57'41.40"W, 365 m) (RMMA180623 and CRC180623) to obtain venom samples for chemical analysis. In the lab, colonies were placed in a darkened wooden cabinet in a controlled insect-rearing room at an average temperature of 23 °C. Ant enclosures consisted of multiple containers lined with Plaster of Paris ™, as well as tubes and petri dishes lined with moistened cotton, which provided ample choice for the ants to nest. Containers lined with Plaster of Paris ™ were watered three times a week, and cotton from the tubes and dishes were rehydrated and changed as necessary. A diet of Bhatkar Agar, consisting of water, honey, eggs, agar, Wesson salts ™, Vanderzants vitamin mix ™, and live flightless *Drosophila hydei* fruit flies were provided three times a week. The fruit flies were actively hunted, stung and killed by the workers and the artificial diet was readily consumed (Mularo pers. obs.).

Chemical Analysis

One worker and three larvae from each of the two colonies were collected in ca. 20-80 µl of methanol for chemical analysis. Gas chromatography-mass spectrometry (GC-MS) was performed on each sample, set to EI mode, using a Shimadzu QP-2010 GC-MS or a Shimadzu

QP-2020 GC-MS with an RTX-5, 30 m x 0.25 millimeter i.d. column. This device was set for analysis from 60°C to 250°C at 10°/min. GC-MS was carried out in the EI mode using a Shimadzu QP-2010 GC-MS or a Shimadzu QP-2020 GC-MS equipped with an RTX-5, 30 m x 0.25 mm i.d. column. The instrument was programmed from 60 °C to 250 °C at 10 °/min.

Bacterial Cultures

We tested *trans*-2-butyl-5-heptylpyrrolidine against six different species of bacteria. Three of the bacteria species (*Bacillus subtilis, Corynebacterium ammoniagenes, Staphylococcus saprophyticus*) were Gram-positive bacteria and three (*Ralstonia picketti, Aquaspirillium serpens, Escherichia coli*) were Gram-negative bacteria. All bacteria cultures were streaked on either Mueller-Hinton or Tryptic Soy agar and incubated between 26 and 37 °C. Before an assay was performed, the test strain was inoculated in Mueller-Hinton broth at 37° C for approximately 24 hours until there was visible growth in the liquid media.

Artificial Preparation of Alkaloid

trans-2-Butyl-5-heptylpyrrolidine was prepared according to the method of Jones et al., 1980, by the reductive amination of 5,8-pentadecadione to provide a *cis/trans* mixture of 2-butyl-5-heptylpyrrolidine. The synthetic alkaloid was diluted in molecular grade ethanol to produce a stock solution concentration of 20 μg/μL, a concentration confirmed to have inhibited the growth of bacteria by disk-diffusion assays. A two-fold serial dilution was performed on this stock solution to yield eight diluted concentrations. These eight solutions were stored in glass vials and placed in a -80 ° C freezer for short-term storage.

Minimum Inhibitory Concentration: MIC Assay

In order to assess the minimum inhibitory concentration of *trans*-2-butyl-5-heptylpyrrolidine, procedures modified from Wiegand et al., 2008 and Cockerill et al., 2012 were used. Each species of bacteria tested against the alkaloid was inoculated overnight in Mueller-Hinton broth at 37°C. Once inoculated, the MIC of the alkaloid was determined using a broth

microdilution procedure, as modified by Wiegand et al. (2008). The bacterial cultures were adjusted to the 0.5 McFarland Standard, or approximately $1x10^8$ cfu mL^{-1} (OD600 nm 0.08-0.10) and diluted by a factor of 100, as recommended in Wiegand et al. (2008). Each MIC assay was carried out on a 96-well microtiter plate, where 190 µL of Mueller-Hinton broth and 5 µL of inoculum were placed into each well (apart from the negative growth control in column 11 and blank sterility control in column 12). For columns 1-8, 5 µL of each alkaloid treatment were placed into each well, with each column of wells representing a different alkaloid concentration. Smaller amounts of the potential antibiotic alkaloid were applied than was recommended by Wiegand et al. (2008) and Cockerill et al., 2012 due to the minute amount of alkaloid that was produced during artificial synthesis. 5 µL of tetracycline (1 µg/ µL) was placed in each well of column nine as a positive, broad-spectrum antibiotic control, and 5 µL of molecular grade ethanol was placed in each well of column 10 as a positive solvent control.

Once the respective 96-well plate was prepared, it was placed into an ELx808i™ Absorbance Microplate Reader. Plates were run at 37°C for 24 hours, with a plate shake and an optical density (OD600) read every 5 minutes for the 24-hour period (Quigley, 2008). This protocol allowed for 288 reads for each well over 24 hours, for a total of 2,304 OD reads per treatment. After the trial, the growth in optical density for each well was calculated by taking the range in OD measurements and subtracting the OD range of the blank broth measurements (column 12). A line plot was generated in JMP Pro 14 ™ by taking the mean growth in OD from all eight trials and plotting the growth (y-axis) against the alkaloid treatment (x-axis), which allowed for the visualization of the change in growth between alkaloid treatments for each of the bacteria. In addition, bar graphs were generated in JMP Pro 14 ™ to compare growth in OD between the MIC concentrations and the positive ethanol control, as well as the positive tetracycline control.

Statistical Analysis

Minimum inhibitory concentration was determined by assessing the lowest concentration of the alkaloid that inhibited bacterial growth over the 24-hour trial (Andrews, 2001). We considered there to be significant growth when the optical density grew by a factor of at least 0.08 (McFarland Standard), and the MIC was determined as the lowest alkaloid concentration that produced no significant growth in bacteria. Before determination of the MIC, A Kruskal-Wallis test was implemented in JMP Pro 14 ™ to assess the variation in growth for each bacterial species. If the variation was due to alkaloid treatments, an MIC value was determined for each trial, and a set of descriptive statistics were calculated to determine MIC data patterns for each strain, including the MIC_{50} and MIC_{90}. This is a calculation of the alkaloid concentration that inhibited 50 and 90 percent of the bacterial isolates, respectively (Kowalski et al., 2005). In addition, the median, mode and range of MIC values were calculated for each strain as recommended by Kowalski et al. 2005. Growth for each alkaloid concentration was also averaged for all eight trials, and the MIC of the average growth was determined for each strain. A Wilcoxon Signed Rank test was generated in JMP Pro 14 ™ and used to compare the growth values at MIC_{50} with the positive ethanol control for each strain of bacteria and with the positive tetracycline control.

Results

Chemical Composition

The GC-MS results indicated the presence of only one alkaloid, *trans*-2-butyl-5-heptylpyrroline in *Megalomyrmex* c.f. *wallacei* workers (n=2 nests). Of the five sampled brood, one sample had no detectable presence of the alkaloid, two contained trace amounts, and three had a significant detectable amount of *trans*-2-butyl-5-heptylpyrrolidine.

MIC Assay and Bacterial Trends

The line plot of bacterial growth showed a consistent inhibition of growth for alkaloid concentrations greater than the MIC (e.g. 20, 10 and 5 μg/μL in all bacteria), followed by a sharp increase in growth for concentrations less than the MIC (e.g. 0.3125 and 0.15625 μg/μL in all

bacteria) (**Figure 1).** The minimum inhibitory concentrations, including the average, MIC_{50}, MIC_{90}, Median, Mode and Range for each bacterial species are displayed in **Table 1**. All of the bacterial species had an average MIC between 1.25 and 2.5 μg/μL, except for *Staphylococcus saprophyticus,* whose average MIC value was 0.625 μg/μL. Two species (*Corynebacterium ammoniagenes, Staphylococcus saprophyticus*) did not yield MIC_{90} values, as seven out of eight trials had an MIC of 1.25 μg/μL, and one out of eight trials had an MIC of 0.625 μg/μL. The MIC_{50} values showed a greater amount of bacterial inhibition when compared to the ethanol control, yet a smaller amount of inhibition when compared to the tetracycline control **(Figure 2, Figure 3)**. The results of the Wilcoxon Signed Rank Test for the MIC_{50} compared to ethanol is shown in **Table 2,** in which all MIC_{50} values for each strain of bacteria had a Z probability value less than 0.01, showing a significant difference between ethanol and MIC bacterial growth. In addition to this, the results of the Wilcoxon Signed Rank Test for the MIC_{50} compared to tetracycline is shown in **Table 3**, where only two bacteria (*Corynebacterium ammoniagenes, Aquaspirillum serpens*) has significantly different growth between treatments.

<u>Discussion</u>

trans-2-Butyl-5-heptylpyrrolidine is an alkaloid produced in multiple species of Solenopsidini ants (Westermann et al., 2015) (Jones et al. 1991b) (Blum et al., 1980). This study has confirmed that this alkaloid is present in *Megalomyrmex* c.f. *wallacei* and is the exclusive alkaloid in the venom of this potential new ant species. Larvae contained variable amounts of the alkaloid, suggesting that workers may apply this venom alkaloid to the brood. However, without behavioral experiments, we can neither confirm nor deny the purposeful application of venom on brood, like that described in Obin and Vander Meer (1985), where *Solenopsis invicta* workers were observed gaster-flagging and releasing venom as an aerosol in brood chambers. While it is possible that there is broad gaster-flagging application of venom on brood, we have so far not observed this behavior. It is plausible that the workers may apply the

alkaloid to the soil walls of the nest in response to pathogens or for use as a prophylactic (Vander Meer, 2012), but this remains to be tested. These ants may also apply alkaloids for purposes other than hygiene, as some species of *Megalomyrmex*, particularly social parasites, have been observed to dispense venom alkaloids in a defensive response to other ant species (Adams et al., 2000, 2012, 2013, 2015), which may lead to hygienic disinfection of the nest in the process. Due to the free-living habits of *Megalomyrmex* c.f. *wallacei*, the probability of encountering other ant species within the nest is lower than that of parasitic species, which, combined with the fact that chemical samples were taken from laboratory colonies, makes accidental application of venom to the brood unlikely.

Despite the transmission of the venom alkaloid remaining speculative, *trans*-2-butyl-5-heptylpyrrolidine has strong antibacterial properties, with the ability to inhibit bacterial growth at low concentrations (2.5-0.625 μg/μL). *Escherichia coli* and *Aquaspirillum serpens* required a higher dose of the alkaloid to effectively inhibit growth (2.5 μg/μL), while others (*Staphylococcus saprophyticus*) were more sensitive to the alkaloid. The MIC_{50} values for all species were significantly greater than the positive ethanol control, meaning bacterial growth was strongly inhibited by the alkaloid and this inhibition was not just due to the solvent. In contrast, there were only two bacterial strains where the MIC growth was significantly greater than the tetracycline control (*Aquaspirillum serpens, Corynebacterium ammoniagenes*). This suggests that this alkaloid is not as potent as the control at 1 μg/μL, likely due to the effectiveness of tetracycline as an antibiotic, as evident by low MIC values (Ryan and Adley, 2013) (Bryan et al., 2004) (Santamaria et al., 1985). When compared to other evaluated alkaloids in the pyrrolidine class, this alkaloid of has a lower potency level. MIC analysis of the pyrrolidine alkaloids bgugaine and irniine found in the plant *Arisarum vulgare* yielded very low concentrations of bacterial inhibition (6.25-50 μg/mL) (Melhaoui et al., 1993). Another study of artificially generated benzene pyrrolidine derivatives suggests that two of the three tested were also more

potent than our compound (32-256 μg/mL) (Arslan et al., 2006). It is also worth noting that one pyrrolidine derivative (1,3-Dipyrrolidinobenzene) in the study done by Arslan et al. (2006) did not inhibit the growth of any bacteria, showing variability in pyrrolidine antimicrobial effects. While most pyrrolidines were stronger antimicrobial agents, it cannot be argued that *trans*-2-butyl-5-heptylpyrrolidine was consistently successful at inhibiting all six of our species of bacteria.

Since this is the only venom alkaloid found in *M.* c.f. *wallacei*, it likely plays an important role in the natural history of these ants. Free-living *Megalomyrmex* ants live in a range of habitats, from shallow subterranean nests (e.g., *Megalomyrmex foreli*) (Peeters and Adams, 2016) to small, temporary shelters found between dead leaves in deep forest litter (e.g., *Megalomyrmex wallacei*) (Boudinot et al., 2013). *Megalomyrmex* c.f. *wallacei* was collected by following baited foragers back to their nest, deep within the leaf litter. These nesting preferences inevitably have rich microbial biodiversity, and many may be entomopathogens. Thus, having venom that inhibits harmful soil microbial growth at low concentrations could be beneficial. This is documented in other neo-tropical ant species, such as *Crematogaster pygmaea,* a species also nests in a humid soil environment and produces antimicrobial venom (Quinet et al., 2012). Two bacterial species we tested have been known to colonize soil communities (e.g., *Bacillus subtilis* and *Ralstonia picketti*). *Bacillus subtilis* is most commonly found in the vegetative state in soil communities (Earl et al., 2008) and was susceptible to *trans*-2-butyl-5-heptylpyrrolidine at relatively low concentrations. *Ralstonia picketti*, while being a potential pathogen toward humans, can also be isolated from soil (Stelzmueller et al., 2006). This species is notable for its ability to process and degrade organic chemicals, making this a potential bioremediation agent in contaminated ecosystems (Ryan et al., 2007). Regardless of its ability to resist organic chemicals, *R. picketti* was still susceptible to *trans*-2-butyl-5-heptylpyrrolidine at 1.25 μg/μL. *Escherichia coli*, although commonly associated with animal intestinal systems, is known to also persist in the soil under ideal environmental conditions (Van Elsas et al., 2011) and was

inhibited by *trans*-2-butyl-5-heptylpyrrolidine. The similarities between other ants and the potency of the pyrrolidine alkaloid—especially against various soil microbes mentioned—suggest that the venom of *Megalomyrmex* c.f. *wallacei* may be used for colony hygienic activities against soil-borne bacteria.

Future studies in this line of research will confirm through behavioral assays whether the transfer of venom is inadvertent, or there is clear application of the ant venom to protect brood. Another important step is to determine the potency of the alkaloid compared to the rest of the venom, as other components, such as proteins (dos Santos Pinto et al., 2012), may have stronger inhibition of bacterial growth. It is also worth exploring the mechanism in which this alkaloid inhibits the growth of bacteria. Several classes of alkaloids (e.g. indolizidines, isoquinolines, quinolines, agelasines, squalamines) are shown to inhibit bacterial growth by inhibiting nucleic acid synthesis, reducing oxygen consumption, inhibiting specific bacterial enzymes, or compromising cell membrane structural integrity (Cushnie et al., 2014). On the other hand, pyrrolidines are suggested to play a role in compromising cell membrane integrity (Wang et al., 2015), yet this question still needs to be addressed for this specific alkaloid. With new questions arising, this study confirmed that the alkaloid exclusive to the venom of *Megalomyrmex* c.f. *wallacei*, *trans*-2-butyl-5-heptylpyrrolidine, has the potential to be used as a hygienic disinfectant within the free-living ant colony. This substance acts as a powerful antimicrobial, inhibiting the growth of several types of bacteria, some of which are known soil dwellers. The antibiotic properties of venom likely contribute to the success of soil-dwelling ant species (Quinet et al. 2012) (Vander Meer 2012), and *Megalomyrmex* c.f. *wallacei* is a good example of a species potentially utilizing antimicrobial venom components to their advantage.

Bacteria Species	Average MIC	MIC_{50}	MIC_{90}	Median	Mode	Range
Aquaspirillum serpens (-)	2.5	2.5	2.5	2.5	2.5	2.5-2.5
Bacillus subtilis (+)	1.25	1.25	1.25	1.25	1.25	1.25-1.25
Corynebacterium ammoniagenes (+)	1.25	1.25	-	1.25	1.25	0.625-1.25
Escherichia coli (-)	2.5	2.5	2.5	2.5	2.5	2.5-2.5
Ralstonia picketti (-)	1.25	1.25	1.25	1.25	1.25	1.25-1.25
Staphylococcus saprophyticus (+)	0.625	0.625	-	0.625	0.625	0.625-1.25

Table 1: Descriptive statistics of minimum inhibitory concentration (MIC) values. Average MIC is the concentration that inhibited the average growth of all eight trials. MIC_{50} and MIC_{90} are the concentrations that inhibited 50% and 90% of the trials, respectively. Median is the middle MIC value for all trials in a strain, and the mode is the most frequent MIC value for all trials in a strain. OD range states the minimum and maximum values throughout all eight trials. MIC values are measured in μg/μL.

Bacterial Strain	Alkaloid MIC vs Ethanol S	Z	Prob Z
Aquaspirillum serpens	36	-3.31060	**0.0009**
Bacillus subtilis	100	3.31304	**0.0009**
Corynebacterium ammoniagenes	100	3.31060	**0.0009**
Escherichia coli	36	-3.31548	**0.0009**
Ralstonia picketti	100	3.313793	**0.0009**
Staphylococcus saprophyticus	100	3.31060	**0.0009**

Table 2: Wilcoxon Signed Rank test of each bacteria, comparing the alkaloid minimum inhibitory concentration (MIC) to the negative ethanol control. All bacteria had significant differences between the alkaloid MIC and control (indicated in bold).

Bacterial Strain	Alkaloid MIC vs Tetracycline S	Z	Prob Z
Aquaspirillum serpens	40	-2.89447	**0.0038**
Bacillus subtilis	53.5	-1.48567	0.1374
Corynebacterium ammoniagenes	43	-2.57871	**0.0099**
Escherichia coli	50.5	-1.79063	0.0734
Ralstonia picketti	63.5	-0.43000	0.6672
Staphylococcus saprophyticus	50	-1.84603	0.0649

Table 3: Wilcoxon Signed Rank test for each bacterial species, comparing the minimum inhibitory concentration (MIC) to the tetracycline treatment. Only two bacteria (*Corynebacterium ammoniagenes, Aquaspirillum serpens*) had significant differences between treatments (indicated in bold).

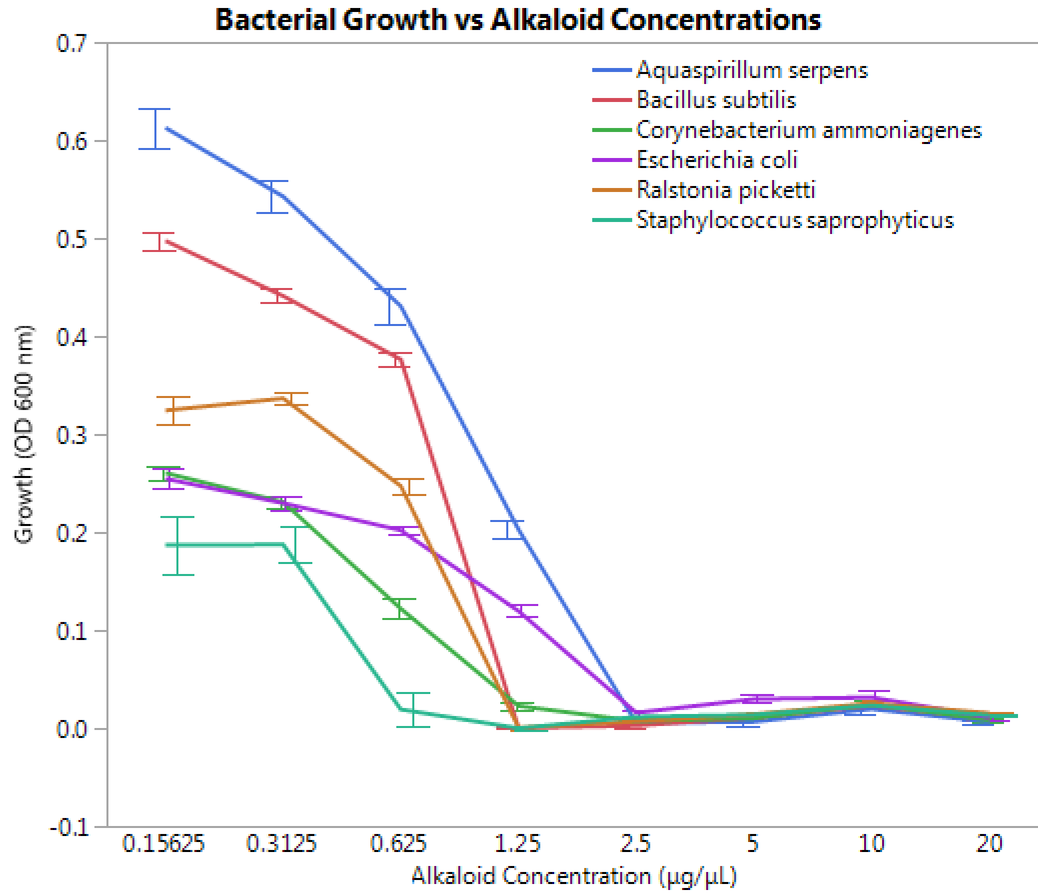

Figure 1: Change in optical density (OD) growth between bacteria species. Bacterial growth in each well was calculated by taking the range in OD and subtracting it from the blank well reading. Mean growth of all eight trials for each treatment was calculated and plotted with standard error bars.

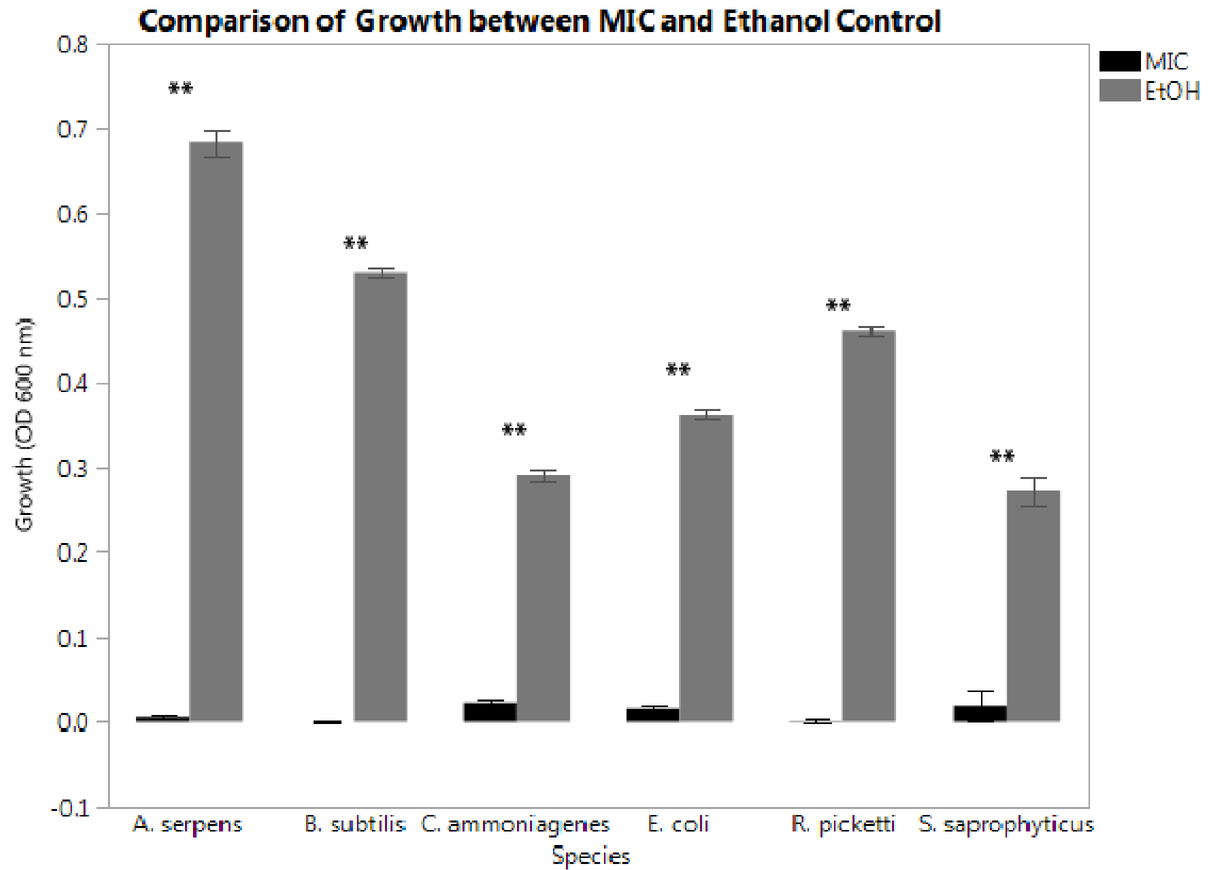

Figure 2: Optical density growth means at the alkaloid minimum inhibitory concentration (MIC_{50}) and for the positive ethanol control for each species of bacteria tested, with error bars created using 1 standard error from the mean. Significant differences of $p<0.01$ are indicated with **.

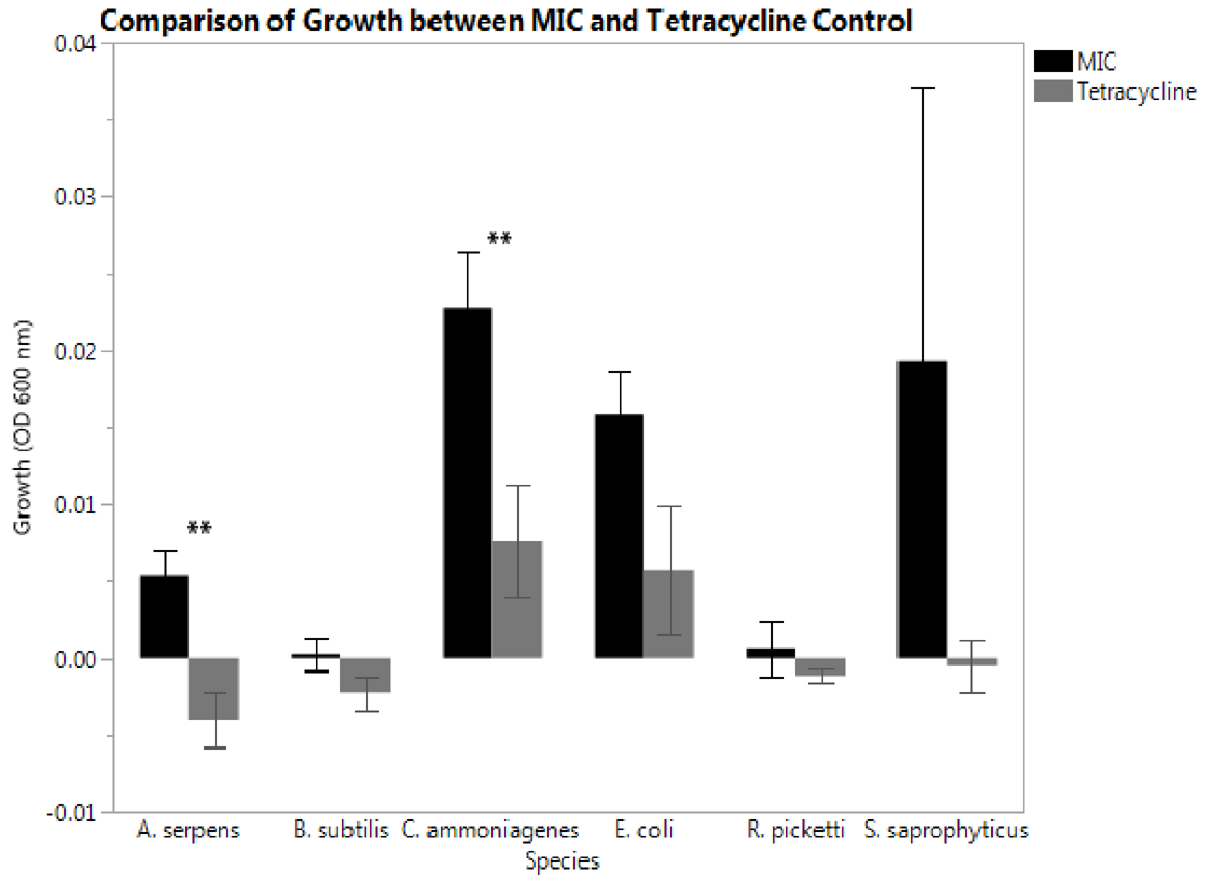

Figure 3: Optical density growth means at the alkaloid minimum inhibitory concentration (MIC_{50}) and the positive tetracycline control for each species of bacteria tested, with error bars created using 1 standard error from the mean. Significant differences of $p<0.01$ are indicated with **.

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