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An Examination of Eisenia fetida Coelomic Fluid for Antimycobacterial Activity

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By

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Abstract

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Antibiotic resistance is a growing problem with real world consequences to human health and no known solution. The genus *Mycobacterium* contains several bacteria that can cause serious illnesses including tuberculosis and Johne's disease. Some of these pathogens also have resistance to several antibiotics. This project sought to find antimycobacterial activity related to the phagocytic coelomocytes or soluble substances in the coelomic fluid of the earthworm *Eisenia fetida*. The phagocytic activity was determined by observing the adherence of phagocytes to *Mycobacterium smegmatis* (strain mc^2155) bacteria. The antimycobacterial ability of the coelomic fluid was assessed by comparison of viable plate counts after treatment with coelomic fluid extracts. The effect of disrupting the microbial flora of the earthworm on these antimycobacterial affects was also investigated. No antimycobacterial activity

was observed in coelomic cells or extracts from earthworms with intact or disrupted microbial flora. However, a trend of increasing coelomic cell concentration after treatment of the worm with antibiotics was seen. There are some aspects of the experiment that could be refined to examine the coelomic fluid for antimycobacterial activity or antibacterial activity against other problem bacteria more precisely. However, the more fruitful area of research would appear to be examining the discrepancy in coelomic cell concentration between antibiotic treated and untreated earthworms.

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1. Introduction

The growing threat of bacterial resistance to antibiotics is a significant concern, as it makes treatment of diseases more challenging, more costly, or even impossible (1). Researchers have searched for new antibacterial drugs in increasingly diverse organisms and environments, including terrestrial invertebrates (2). Invertebrates lack the specificity provided by the antibody-based immunity of vertebrates (3). Instead, they have a non-specific immune system with humoral and cellular aspects (4,5). Investigation into the extracts of numerous organisms including terrestrial invertebrates has led to the discovery of several antimycobacterial compounds (6,7).

Earthworm coelomic fluid contains more than 40 proteins with cytolytic, proteolytic, antimicrobial, hemolytic, hemagglutinating, tumorolytic, and mitogenic activities (8-11). The coelomic fluid of the earthworm *Eisenia fetida andrei* has antimicrobial activity against *Aeromonas hydrophila* and *Bacillus megaterium*, both known earthworm pathogens (10,11).

Another aspect of earthworm innate immunity is the phagocytic activity of coelomocytes. Coelomocytes have two general populations: amoebocytes and eleocytes. The amoebocytes carry out phagocytosis and produce cytokines and eleocytes collect contaminants and other stray matter in the coelomic fluid (12). Amoebocytes are classified as either hyaline or granular based on their appearance (12). The first step of phagocytosis, cell adhesion, begins with the

association with surface receptors of the cell to the particle to be phagocytized (13). If the particle or bacterium is small enough to ingest, phagocytosis proceeds. However, if the particle is too large, such as an invading nematode, amebocytes encapsulate the particle and the brown body that is formed moves through the coelom toward the posterior body segments, which are detached with the particle (11). After the immune cells of the worm phagocytize bacteria, the bacteria clump together and then disperse. These bacteria are not found in the nephridial walls, indicating the phagocytes are able to efficiently breakdown and remove bacteria (14). The phagocytic activity of earthworm coelomocytes can be assessed by microscopy. The amount of coelomic cells adhered to bacteria out of the total coelomic cell population can be used as a measure of phagocytic activity. It has been observed that the coelomic cells of *Dendrobaena veneta* show an increased adherence of known earthworm pathogens (*Bacillus megaterium* and *Aeromonas hydrophila*) over non-pathogenic species (15). This phagocytic ability can be improved by humoral components and opsonins (12). An example of this is seen in *L. terrestris*, where phagocytosis of yeast was improved after opsonization with coelomic fluid (16). It is possible that this activity may result from a compound produced by flora of *E. fetida*, which may be able to enhance phagocytosis in its coelomocytes.

The association of bacteria and phagocytic cells is improved in the presence of opsonins. In mammals, opsonic activity is associated with complement and antibodies found in the serum (17). Beneficial bacteria may

also contribute; one study of mouse monocyte-macrophage cells *in vitro* demonstrated increased phagocytic activity in a dose-dependent manner using cell-free lactic acid bacteria extract (18). This indicates that secretions from the cells were promoting phagocytosis. Despite differences between mouse and earthworm immune systems, it is possible that this occurs as a result of secretions by earthworm bacterial flora as well. One of the bacteria in the earthworm *E. fetida* is *V. eiseniae*. This bacterium present in the nephridia of the worm still has no known function. It is possible that this bacterium or other flora may have antimicrobial properties.

Earthworms interact with numerous soil organisms including species of *Mycobacterium*. The genus *Mycobacterium* has had a significant impact on human and animal health. The most high profile disease associated with this genus is tuberculosis, caused by *Mycobacterium tuberculosis*. In 2013, tuberculosis had an incidence rate of 9 million worldwide (19). *Mycobacterium leprae,* the causative agent of leprosy, has had and still has an impact on the human population. In 2012, there was a prevalence of 189,018 cases and an incidence of 232,857 new cases was reported globally (20). *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a veterinary pathogen; it causes Johne's disease, a wasting disease of cattle. This disease if left untreated, causes malabsorption of nutrients in the cow intestine, which can eventually lead to death in infected cattle (21).

In humans, MAP has been suspected to be responsible for Crohn's disease, a disease with symptoms similar to those of in Johne's disease (22). The *Mycobacterium avium* complex is a collection of bacteria that cause significant human disease. It is causes an opportunistic infection that produces a vast array of symptoms including weight loss, diarrhea, fever, pneumonia, and hepatitis. It is a disease common to immune compromised individuals, in particular those in the HIV/AIDS population (23).

Antibiotic resistance in the genus *Mycobacterium* and in *M. tuberculosis* in particular is a concern. *M. tuberculosis* is able to withstand many broad spectrum antibiotics, which is problematic for treatment (24). Treatment of this pathogen includes a multi-drug regimen that can take as long as 6 to 12 months to complete and requires fairly strict patient compliance. The incomplete or improper administration of this chemotherapy treatment has led to the development of multi-drug resistant strains of the bacterium (25). In addition to mutations that enable resistance to antimicrobial agents, mycobacteria are naturally resistant to many common antibiotics. This is due to the intrinsic impermeability of their cell wall that stems from the mycolic acid (26-29). However, mycolic acids of the cell wall don't explain the entirety of the resistance seen in *Mycobacterium* (26). Synergistic effects have been investigated with respect to resistance; research has determined that genes not involved in cell wall assembly may work with those involved in cell wall assembly to enable resistance to certain broad-spectrum antibiotics (30-32).

With MAP infections in cattle, treatment with isoniazid alone or in combination with rifampin for the duration of an animal's life stops the progression of Johne's disease but doesn't cure the animal. When treatment is stopped, the bacterium again resumes growth and the animal progresses to a disease state (33). Because treatment is not curative, it is rarely undertaken; however, it may be considered for animals with exceptional genetic value (34). MAP is a difficult pathogen to remove from the livestock environment as infected cattle shed large numbers of the bacterium in their feces. MAP can persist in black earth for 11 months and manure for a year (35,36). If cattle graze on grass or eat soil contaminated by feces they have the potential to be infected.

Another cattle pathogen is *Mycobacterium bovis*, the causative agent of bovine tuberculosis (36). In the United States, the prevalence of this bacterium is much lower than that of MAP, as it has been eradicated in all but two states due to a surveillance program that started in 1917 (37,38). This organism has also been able to cause tuberculosis in humans, though this is a less common occurrence compared to tuberculosis due to *M. tuberculosis* (39).

Due to the damaging impact associated with mycobacterial diseases and the development of antibiotic resistant strains, new antibiotics and treatment regimens should be developed. One can look to organisms that have to deal with mycobacteria in their everyday environment. Since earthworms are residents of soil and manure, where MAP is known to persist, specific attention has been paid to earthworms (36). When *Lumbricus terrestris* earthworms were placed in a

highly MAP-contaminated environment and subsequently tested for the presence of the bacterium, few of them had culturable bacteria (40). The antimycobacterial activity of the earthworm *Dendrobaena veneta* stemmed from compounds produced by a bacterium that is a close relative of *Raoultella ornithinolytica,* (99% sequence similarity). *R. ornitholytica* is a suspected symbiont present in the midgut of the earthworm. The authors found the bacterium in the intestinal wall of *D. veneta* worms from multiple different soil sources indicating the organism is not merely an acquired soil organism. The metabolites made by the bacterium were postulated to interfere with cell wall synthesis as evidenced by the rounded or filamentous forms of the mycobacterial cells observed via scanning and transmission electron microscopy (7). Investigations of soil bacteria for antibiotic compounds have yielded results in the past. The soil organism *Streptomyces griseus* produces a compound used to derive the anti-tuberculosis drug streptomycin (41). Thus, the earthworm and its bacterial flora may provide a source of antimycobacterial substances.

Commensal and symbiotic bacterial association with the earthworm has been investigated in several instances, though much of the mechanism of establishment has yet to be elucidated. In one study, it was found that there was an increase in unrelated bacterial diversity when *E. fetida* was exposed to *E. coli* 0157:H7. The new bacteria then decreased following the elimination of *E. coli* on day 2 (42). It was proposed that one of the established gut bacteria, a *Bacillus* species, had an antagonistic relationship with the *E. coli*, likely secreting a

specific antimicrobial substance. In other words, bacteria antagonistic to *E. coli* including the *Bacillus* species exist as gut bacteria normally, but at a lower level. When a pathogenic challenge is detected, these bacteria increase to a detectable level and inhibit the invading bacteria (42).

In the earthworm *E. fetida* there is a symbiont, *Verminephrobacter eiseniae* that colonizes the nephridia of the worm. If this bacterium has a role in the earthworm it is unknown, but it is retained and vertically transmitted to their offspring cocoons (43,44). Following hatching of the juvenile worm from the cocoon, the worm is unable to acquire the symbiont or other bacteria (44). Thus, when one is assessing the presence or absence of *V. eiseniae* in an adult worm, one can either look to the nephridia of the worm or look to the cocoons produced by the worm.

The purpose of this study was to determine whether or not *V. eiseniae*, produces antimycobacterial compounds or stimulates phagocytosis. Unfortunately many of the *Mycobacterium* species discussed have slow generation times. It can take up to four months before visible MAP colonies can be seen on solid media; others take even longer (40). Therefore, in order to study the effects of substances on any mycobacteria, a common strain of *M. smegmatis*, mc^2155, is used for the study of *M. tuberculosis* and MAP due to its genetic similarity and faster generation time (45).

The purposes of this study were 1) to determine whether or not there are antimycobacterial compounds present in the coelomic fluid of *Eisenia fetida*

earthworms and whether there is a vertically transmitted bacterium, which affects that ability and 2) to determine whether the phagocytic cells present in the coelomic fluid interact with mycobacteria at a higher level when there is vertically-transmitted bacterium present in the worm.

The hypothesis for this study was: The coelomic cells and fluid of the *earthworm E.* fetida have antimycobacterial properties, which may or may not be influenced by the presence *of V. eiseniae*. If true, the treatment of *M. smegmatis* with *E. fetida* coelomic fluid should show a decrease in bacterial concentration. Also, phagocytic activity should be apparent based on adherence of phagocytes to *M. smegmatis*. If symbiotic bacteria have an affect on phagocytic coelomic cells, the amount of cells interacting with *M. smegmatis* should be positively or negatively with the loss of the symbiont.

2. Literature Review

Overview

The overall goal of this project was to assess the earthworm *Eisenia fetida* for antimycobacterial activity. In order to understand why this project was undertaken, information on the specifics surrounding the earthworm, the genus *Mycobacterium*, and antibiotic discovery need to be elucidated. For *E. fetida*, anatomy, immune function, and symbiotic bacteria of the earthworm will be discussed. For the mycobacteria, common pathogenic species and antibiotic resistance mechanisms will be analyzed. Natural sources of antibiotics will also be examined. These areas will provide a basis for understanding how new antimycobacterial compounds might be discovered in *E. fetida*.

Mycobacterium and Antibiotic Resistance

The genus *Mycobacterium* is largely composed of saprophytic soil microorganisms that are often associated with sphagnum moss, surface and drinking water, and soil with organic materials (36). This genus, composed of >50 species, is related to *Streptomyces* and *Actinomyces*. There are several successful pathogens in *Mycobacterium* including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium avium* subspecies *paratuberculosis* the causative agents of tuberculosis, leprosy, and Johne's disease, respectively (46). The saprophytic organisms in this genus are slower growing compared to many other bacteria. However, most pathogenic mycobacteria take weeks to months to form colonies, and one has yet to be grown in culture, therefore since

the saprophytes tend to only take 7 days to form colonies on a plate they are desirable for their use in research (47,48). *Mycobacterium tuberculosis* can take 2 to 4 weeks and *Mycobacterium avium* subspecies *paratuberculosis* colony formation takes 3 months or more (49,50).

These organisms are nonmotile, nonsporulating, weakly Gram-positive, acid-fast bacilli, which appear as straight or slightly curved rods. They are 1 to 4 μm in length and 0.3 to 0.6 μm wide (51). These bacteria have mycolic acids that are critical to the cell wall (52). The waxy phenotype of the cell wall conferred by these acids results in: acid-fast staining, hypdrophobicity, and resistance to drying, acidity/alkalinity, and many antibiotics. Mycolic acids have immunostimulatory properties for the host immune system (53). These also allow for rapid diagnosis of mycobacteria by way of the acid-fast stain or Ziehl-Neelsen that identifies bacteria possessing mycolic acids (acid-fast) versus those that don't (non-acid-fast) (based off a translation in Kazda et. al.) (36,54,55). Using this technique one can easily detect acid-fast mycobacteria in sputum samples.

Much of the antibiotic resistance of *Mycobacterium* is an intrinsic resistance based primarily on the impermeability of the cell wall (26,27). No horizontal transfer via plasmids or transposons has been observed: thus, these resistances most likely arise by chromosomal mutations that occur as a result of selective pressure from antibiotic use (56). However, it has been found that cell wall composition doesn't entirely explain the observed antibiotic resistance (26). Thus, synergistic effects related to genes not involved in cell wall assembly that

enable resistance to some broad-spectrum antibiotics have been investigated (30-32). These include modification of drug targets such as ribosomal RNA, chemical modification of the antibiotic such as the acetylation of amine groups in aminoglycosides, enzymatic degradation of drugs such as β-lactamase activity, molecular mimicry of drug targets such as the production of MfpA as a decoy sequence for fluoroquinolones, and the use of efflux pumps to remove antibiotics from the bacterium (57-63).

Mycobacterium tuberculosis

The most well known member of this genus, *Mycobacterium tuberculosis*, causes the lung disease tuberculosis (TB). The symptoms of pulmonary TB include chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, and hemoptysis (64). Other less common bacteria in this genus including *Mycobacterium africanum, Mycobacterium canetti, Mycobaterium bovis, Mycobacterium caprae, Mycobacterium myroti,* and *Mycobacterium pinnipedii* can also cause tuberculosis (36). In 2013 there was an incidence of 9 million tuberculosis cases worldwide, that same year 1.5 million people died from the disease (19).

In more than 90% of cases of this disease, the bacterium is contained as an asymptomatic latent infection (65). Transmission of this bacterium is typically through contaminated aerosol from an infected person; however, it can also be shed in feces, urine, and other excretions and secretions (36). Without treatment, approximately 5-10% of TB infected individuals will develop active TB

(66). A latent infection is associated with a lower risk of reinfection after another exposure (67). Conversely, if progression to active infection occurs and is treated, there is an increased risk of a second episode of TB on reexposure (68,69). Diagnosis of the latent form of this disease is typically done by a tuberculin skin test or an interferon-gamma release assay (65). The tuberculin skin test or Mantoux tuberculin test is performed by injecting 0.1mL of tuberculin purified protein to the forearm; a further swelling of the injection site indicates an inflammatory action of T helper lymphocytes. This means the individual is likely infected with the bacterium, however a false positive can result if they have been infected with mycobacteria including *M. tuberculosis* previously; thus, a confirmation may be undertaken to confirm the diagnosis (70). Active TB diagnosis is typically performed via sputum microscopy and culture in a liquid medium with associated drug susceptibility testing (65). However, a new molecular test, Xpert MTB/RIF, detects *M. tuberculosis* complex within 2 hours using real time PCR to amplify a specific mutated sequence of the *M. tuberculosis rpoB* gene that indicates rifampin resistance. This method is a more sensitive method than smear microscopy (71-73).

One of the key aspects of *M. tuberculosis* pathogenicity is the invasion of macrophages of immunocompetent individuals (74). This bacterium is able to use several strategies to evade destruction by macrophages. These include disruption of: phagosome-lysosome fusion, recruitment of hydrolytic lysosomal enzymes, reactive oxygen and nitrogen species production, antigen presentation,

and MHC class II expression and trafficking, autophagy, and apoptosis (74). Entry into the macrophages requires recognition of the bacterium by several opsonic and non-opsonic receptors (75). The non-opsonic method uses pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) (76). In the case of opsonic uptake of *M. tuberculosis,* there is recognition of coated bacteria with complement factors, antibodies, and/or surfactants (77). However, most agree that ingestion of the bacterium occurs by non-opsonic means in the early stages of infection (77-80). Non-opsonic entry into the macrophage has been found to be associated with higher intracellular survival than opsonic entry; this suggests specific receptors need to be used to disrupt phagosome-lysosome fusion (78).

Immune $CD4^+$ T lymphocytes protect against TB, however some research indicates the participation of $CD8^+$ T lymphocytes (81-84). The risk of developing active TB increases soon after infection with HIV, due to a lower population of functioning T lymphocytes (85). In 2013 1.1 million were HIV positive of a total incidence of 9 million (19). Once CD4 cell counts reach less than 200 per mm^3, the presentation of TB becomes atypical. Subtle infiltrates, pleural effusions, hilar lymphadenopathy, and other forms of extrapulmonary TB appear in up to 50% of patients (65). When CD4 counts reach less than 75 per mm^3, pulmonary evidence may be lacking, and disseminated TB manifests as a nonspecific, chronic febrile illness with bacterial distribution to other organs. In these instances bacteremia is more frequent. This results in high early mortality. The

HIV positive status is also associated with multiple *M. tuberculosis* strain involvement (86).

Treatment of TB is not simple. For latent TB, the regimen is isoniazid alone for 9 months in a healthy individual or longer for HIV-infected individuals (87,88). For active TB, a four-drug regimen of isoniazid, rifampin, pyrazinamide, and ethambutol is effective in 90% of cases in which no drug resistance is detected (89). The 6-month treatment program requires an initial 2 months of treatment with all four antimycobacterial drugs followed by 4 months of isoniazid and rifampin. If risk factors for relapse (including cavitation, extensive disease, immunosuppression, or a positive sputum culture at 8 weeks) are present, the therapy can be extended up to 9 months (65). Barriers to the efficacy of this regimen include: inconsistent drug quality, treatment interruptions due to side effects, toxic effects, pharmacokinetic interactions (such as with antiretrovirals for HIV), and compliance issues due to the length of treatment (65,90). In an attempt to reduce the therapy to a more manageable 4 months, some trials have added or substituted fluoroquinolones or added higher doses of rifamycins (65).

A global report on TB by the WHO in 2014 estimated that the proportion of multi-drug resistant TB (MDR-TB) was between 14 and 28%, of these, 9% were extensively drug resistant TB (XDR-TB) (19). Eighty-four countries that have reported extensively resistant strains that have resistance to fluoroquinolones and the injectable drugs: kanamycin, amikacin, and capreomycin (89,91,92). Improper use of anti-TB drugs including using monotherapy and adding a single

drug alongside currently failing drugs has favored the cultivation of these resistant mutants via selective pressure (65). Unfortunately, it is estimated that only 10% of cases of MDR-TB are currently diagnosed worldwide, and only half of these receive appropriate treatment (89,91,92).

MDR-TB is typically treated using either standardized treatment or individualized treatment once the empirically determined resistance pattern of the bacterium becomes known (65). These regimens typically require combination drugs from five groups of first-line and second-line drugs, which need to be used for 20 months or 30 months for those who have had MDR-TB previously (65,93). Another treatment, the Bangladesh regimen, is a 3-stage regimen that involves a shorter treatment time of 21 months with fewer adverse effects and more positive outcomes including some showing no identifiable bacteria at 3 months. This treatment is a standardized treatment based on the resistance pattern observed in a sampling of the population (94). This allows for less extensive hospitalization and less time waiting on individual culture tests. XDR-TB is a greater challenge to diagnose and treat; it has been associated with an extremely high death rate among HIV-infected individuals (95,96).

Some new drugs have shown some activity against MDR and XDR-TB and are being investigated further; several of these drugs have new mechanisms of action against *M. tuberculosis.* These include new fluoroquinolones that inhibit topoisomerase IV and DNA gyrase, new nitroimidazoles that inhibit mycolic acid synthesis in replicating TB and generate highly reactive nitrogen radicals in non-

replicating TB, bedaquiline that inhibits ATP synthase, new oxazolidinones that inhibit translation by blocking binding at the A site of peptidyl transferase center of the mycobacterial ribosome, and SQ109 that inhibits the MmpL3 transporter, necessary for the integration of trehalose mycolate,into the cell wall (65). One three-drug treatment including moxifloxacin, pyrazinamide, and PA-824 showed 14-day bactericidal effect similar to that of the standard four-drug therapy (97).

The *Mycobacterium bovis* Calmette-Guérin (BCG) attenuated vaccine is commonly used in areas where TB is endemic (65). The prevention efficacy of this vaccine is approximately 50% (98). The vaccine is not appropriate for use in immunosuppressed individuals as it has the potential to cause a fatal disseminated infection (65). The restrictions of this vaccine have led others to investigate other options; over 30 vaccines are in development (99). Another TB vaccine uses *Mycobacterium microti*, which has the same level of efficacy (100). Unfortunately, the use of these vaccines undermines the efficacy of tuberculin skin testing (66).

Mycobacterium leprae

Leprosy, caused by *Mycobacterium leprae*, was first described in 460 BC by Hippocrates, though archaeological evidence from Egypt suggests indicates it has been around since 4000 BC (48). Leprosy, also called Hansen's disease, has an array of symptoms including skin lesions; growths on the skin; thick, stiff, or dry skin; severe pain; numbness on affected areas of skin, muscle weakness or paralysis, eye problems including eventual blindness, enlarged nerves, and

ulcers on the soles of feet (101). Like many others in the genus *Mycobacterium*, *M. leprae* has a long incubation period and it can take from 5 to 20 years to appear (20). This disease is not just one of historical record; in 2012 there was a prevalence of 189,018 cases, with 232,857 new cases globally (20).

Even though this disease has been around since early-recorded history, the causative agent, *M. leprae*, wasn't determined to be the cause until 1875 (102). This proved to be a difficult pathogen to grow outside of the infected host; it wasn't done until 1960 when it was successfully grown in mouse footpads. It has yet to be cultured *in vitro* (103). A more recent discovery is that another species, *Mycobacterium lepromatosis*, also causes leprosy (104). In the 1960s, a multidrug regimen including dapsone, rifampicin, and clofazimine was developed after resistance to dapsone alone occurred (20). Fortunately, at this point no resistance to this multidrug treatment has been reported (20).

It is generally understood that transmission of this disease is airborne and by direct skin contact by an infected individual (36). This bacterium has also been isolated from feral nine-banded armadillos, which indicates that infected individuals aren't the only source of this pathogen. It has also been postulated that polluted drinking water may be a source (36). Some support for this was seen in Indonesia, where PCR testing was performed for *M. leprae* DNA. These tests indicated a higher prevalence of leprosy amongst individuals that used contaminated water for bathing and washing clothes or dishes (105).

Mycobacterium bovis

Mycobacterium bovis is a cattle pathogen, the causative agent of bovine tuberculosis (36). This is currently of lower concern than other cattle pathogens as it has been eradicated in all but two states due to an eradication program that started in 1917 (37,38). This organism has also caused tuberculosis in humans, but is a much less common occurrence compared to that caused by *M. tuberculosis* (39). However, disseminated infections in individuals with immunodeficiencies can result from vaccination with BCG. As such, it has been seen that areas with large populations of AIDS patients are showing these complications to the vaccine (106). Unfortunately, treatment of adverse reactions and disseminated BCG infection is still controversial, as the multiple strains with different resistance patterns are used, and there is no standardized treatment at present (107-109). Since multiple strains of BCG are used in the creation of this vaccine, the treatment of each strain needs to be done according to the strain's susceptibility pattern to different antituberculosis drugs (110).

Mycobacterium avium subspecies *paratuberculosis*

There are several diseases associated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP); some are veterinary diseases, while others are found in human populations. A German veterinarian Heinrich Johne first described MAP as the causative agent of Johne's disease in 1895 (111). Several other *Mycobacterium avium* subspecies are also associated with disease. These include *M.a. hominisuis,* which causes avian mycobacteriosis in humans, pigs,

cattle, and other animals, *M.a. silvaticum*, which causes disease in wild and domestic birds, and *M.a. avium,* the causative agent of avian tuberculosis in birds, domestic pigs, cattle, and other mammals (36). The chronic inflammation of Johne's disease results in malabsorption of nutrients in the intestine, which can lead to death in the cow (8).

Johne's disease is a significant problem in agriculture with an estimated cost between $200 and $250 million based on lost dairy production (112). Another estimate puts the costs at closer to $1.5 billion (113). These costs are a result of direct and indirect losses. Direct losses include mortality of infected animals and the resulting decreased slaughter value, decreased milk production in quantity and quality resulting from mastitis, decreased pregnancy rate and postpartum complications, poor food conversion in clinically or sub clinically infected animals, decreased productive age in infected animals, and increased predisposition to other chronic diseases. Indirect losses include unrealized future income by breeding animals being prematurely culled, increased expenses from idle production, increased expenses for herd replacement, expenses for diagnostic testing for MAP and associated veterinary care for ill animals, expenses for a control program, lost genetic value of desirable animal traits, expenses associated with trade restrictions, and lost reputation of a farm with infected animals (114).

Due to the high losses associated with this disease, a means of successful treating of cattle would be desired. Treatment with isoniazid alone or

in combination with rifampin for the duration of an animal's life can stop the progression of MAP but doesn't cure the animal. When treatment is stopped, the bacterium again progresses to the disease state (33). Because treatment is not curative, it is rarely undertaken, although it may be considered for animals with exceptional genetic value for the breeding life of the animal (34). MAP is also a difficult pathogen to remove from the environment of the cattle. Infected cattle can shed the bacterium in their feces; the bacterium can persist in black earth for 11 months and manure for a year (35,36,115). As such, MAP can be found in soil, manure, and surface water as a result of cattle fecal contamination (116-118). Some MAP bacteria have been found in free-living amoebae in fields, which indicates they may persist due to their ability to infect the amoebae (119). Due to the ubiquity of the bacterium in the farm environment, it is easy for uninfected cattle to acquire the pathogen simply from grazing and drinking. Without a proper curative treatment, the cost of an MAP infection in a herd of cattle can be devastating to the financial success of the farm.

Organisms belonging to the *Mycobacterium avium* complex (MAC) cause opportunistic infections with a vast array of symptoms including weight loss, diarrhea, fever, pneumonia, and hepatitis. The MAC cause a disease common to immune compromised individuals, particularly those in the HIV/AIDS population (23). It can cause chronic lung disease in immunocompetent individuals and disseminated disease in those with AIDS (120). This disease has a similar resistance pattern to other mycobacteria. A multidrug regimen including

macrolides has been demonstrated to be the most effective treatment (121,122). Unfortunately, the newer regimen has been found to have increased adverse effects including drug toxicities exacerbated by interactions with other drugs (123). Also, despite an improved treatment success, it is still not deemed a satisfactory regimen (120). MAC has also been found to be a rising problem in those with functioning immune systems (124).

A human disease with a suspected mycobacterial origin is Crohn's disease (CD), a chronic inflammation of the bowel in humans. It can affect those with a normal immune system; it is also suspected that MAP causes this disease (22). Some of the support for MAP as an etiologic agent of CD includes:

1. Examination of diseased intestinal tissue from Crohn's patients and Johne's diseased intestinal tissue show similarities. This led researchers to conclude they may be caused by the same bacterium (125). Both of these diseases share symptoms such as weight loss and chronic diarrhea, they also both produce tissue with granulomas and inflammatory action in the small bowel and the colon (126). Unfortunately, detection of MAP in human patients with Crohn's was unsuccessful in these studies.
2. Tests for the MAP-specific protein tyrosine phosphatase A have shown a significant increase in CD patients in comparison to the control group (127).
3. Several studies have detected MAP at a higher frequency in the blood and tissue of CD patients than in those of unaffected individuals (128-131).

4. MAP isolated from a CD patient was inoculated into a young goat, which subsequently acquired Johne's disease, this indicates that the transference of the inflammatory symptoms after infection (132).

The etiology of the disease remains unknown but it is typically understood to involve genetic, environmental, and immune factors coupled with increased inflammatory action (133).

Proponents of a bacteria-associated disease have postulated that pathogenesis occurs when a genetically susceptible host ingests the bacterium. The organism enters the intestinal wall via disrupted epithelium. MAP then stimulates an immune response perpetuated by immune reactivity to the organism. The bacterium is then ingested by intestinal macrophages, the bacteria have been observed in a different form devoid of a cell wall (spheroplast) (134). Once inside the bacterium can exist in a latent form for years, with the potential to produce several inflammatory mediators that have been observed in CD affected individuals (135). These mediators also stimulate naïve T cells to differentiate into T-helper 1 cells, which stimulate macrophages to secrete further inflammatory mediators. These cytokines activate natural killer cells, which produce the tissue damage common to CD (136). Another observation that supports an association between MAP and CD is that peripheral blood mononuclear cells (PBMCs) isolated from CD patients could be induced to produce T cells and secrete inflammatory cytokines such as TNF-α and IL-10 when incubated with MAP (137).

The *NOD2/CARD15* gene is one associated with the development of CD (135,138). Individuals with mutations in this gene have a malfunctioning innate response to bacterial infection; thus, it may be associated with a poor clearing of intracellular MAP. This is a proposed connection for CD and MAP causation (139). Some supporting evidence of this is shown in PBMCs from CD patients who have *NOD2* mutations, thus they lack proper recognition of MAP (140). Some support for this idea is given in the identification of *CARD15* single nucleotide polymorphisms; this genetic type in cattle has shown a higher susceptibility to MAP infection (141).

As was stated previously, infected cattle can shed MAP containing feces, which can contaminate soil and surface water (116-118). Contaminated manure from cattle can also contaminate crops and drinking water, which can contribute to human exposure (142,143). One study has shown that MAP bacteria are able to form biofilms in water systems for several weeks even under high flow conditions (144). In addition to these forms of contamination, dairy products including pasteurized milk and milk powder for infant formula can also harbor MAP, which may expose individuals to MAP bacteria (145-147). Though there hasn't been conclusive evidence that the consumption of MAP in these has lead to the development of CD (148). There has been evidence of a systemic infection with the detection of MAP in breast milk of CD patients whereas lactating control individuals didn't have the bacterium (149). Despite the presence of MAP in breast milk, no evidence of vertical transmission to offspring

has been observed (150). It has been postulated that MAP can exist in systemic and local forms (130). It has also been postulated that soil may serve as a source of mycobacterial infections including *M. avium* infection (151-154).

As it stands there has been difficulty in detecting MAP in the blood and tissues of immunocompetent CD patients via direct observation (155). However, there has been some success in culturing the bacterium from the intestines of several Crohn's disease patients (132). Some research supports the idea of a morphologic change to a smaller spore morphotype that survives heat and other stressors, which may allow for survival in the host and the environment (156). This may also explain the difficulties in culturing of the organism, this challenge in culturing continues to contribute to the debate as to whether MAP is a pathogen causing damage to the host or is just a transient bacterium associated with the environment (126). Some research indicates bacterium has a modified cell wall when present in humans, thus it no longer has an acid-fast character (157). It is challenging to isolate MAP DNA from soil, as humic acids and other organic material often affect its extraction. Instead either denaturing gradient gel electrophoresis or T-RFLP alongside cloning and sequencing is often performed (158-160). Due to challenges culturing the bacterium, PCR for IS*900*, an insertion sequence specific to MAP is commonly used for detection of the bacterium (116,117,161,162).

The evidence linking the relationship between MAP and CD is conflicting, for example, as some phenotypes of CD are not present in Johne's including

fibrosis, fistulae, fissures, and pseudopolyps (163). It has also been shown that farmers consistently exposed to MAP-infected cattle have shown no higher prevalence of CD (164). Another somewhat confusing observation is that MAP presence is decreased in immunosuppressed individuals. One explanation is that MAP doesn't cause CD, but is able to thrive in the immune dysfunction and inflammatory environment caused by CD (126). Based on current evidence it is no defined etiology of CD by MAP is possible.

Mycobacterium smegmatis as a Model Organism

Mycobacterium smegmatis is a common environmental bacterium found in soil, dust, water, animal tissue, and human genital secretions (165). It was first isolated from syphilitic chancres in 1884 by Sigmund Lustgarten (166). It was later determined that *M. smegmatis* is not associated with disease when it was found in normal human genital secretions (smegma) (translation from article by He and De Buck) (167,168). This bacterium has only rarely has acted as a pathogen; these are typically disseminated infections in soft tissues, usually associated with immunosuppression (165,169-171). As with other mycobacteria, *M. smegmatis* is resistant to some antibiotics including isoniazid and rifampin, however it is susceptible to ethambutol, imipenem, doxycycline, sulfamethoxazole, amikacin, and ciprofloxacin (171). It also has cell wall similar to its pathogenic relatives such as *M. tuberculosis*, MAP, *M. leprae* (168). The similarities don't end at its cell wall; the genome of *M. smegmatis* has homology to those relatives, which makes it ideal for studying those pathogens (45).

Due to these similarities, this organism is commonly used to study pathogenic mycobacteria (172). *M. smegmatis* is a faster-growing bacterium (3 to 4 hr. doubling time) with a fully sequenced genome, making it a useful model organism The mc^2155 strain is commonly used (168,173).

Natural Sources of Antimicrobial Compounds

Antimicrobial resistance in bacterial populations is a significant problem. The treatment of disease becomes more challenging, more costly, or impossible in these instances (1). Many currently available antibiotics have been derived from natural sources including terrestrial plants, microorganisms, vertebrates, and marine invertebrates (174). In one survey, approximately 66% of 148 were either natural products or derived from a natural product scaffold (175).

One of the most famous antibiotics is penicillin, a compound that was discovered by Alexander Fleming in 1928. He observed that the fungus *Penicillium notatum* secreted a substance that inhibited the growth of *Staphylococcus aureus* (176). Therapeutic use of the drug wasn't fully realized until the first successful treatment of a patient with streptococcal septicemia in 1942 (177). Penicillin (which acts on enzymes referred to as penicillin-binding proteins (PBPs)) contains a beta-lactam ring that interrupts the formation of peptidoglycan in bacterial cell walls (178).

An antibiotic more germane to the subject at hand is streptomycin, the first drug used to remedy tuberculosis (179). Selman Waksman, a soil microbiologist, isolated this antibiotic. It was found to be the most potent antimycobacterial

substance of 10 other candidates (180,181). This compound was isolated from the bacterium *Streptomyces griseus* by one of Waksman's collaborators Albert Schatz (182). This drug acts as a protein synthesis inhibitor and works by binding the 16S rRNA of the 30S subunit of the bacterial ribosome (183). However, bacteria gain resistance to this antibiotic via mutations of the ribosomal proteins (184).

As mentioned above, some terrestrial invertebrates have been examined for antimicrobial activity. Limited specific attention has been focused on these invertebrates in regards to antimycobacterial activity. Most studies are indirect and linked to the fact that MAP is able to persist in soil and feces, which earthworms can inhabit (36). A few studies have examined the interaction between mycobacteria and earthworms. In one study, *Lumbricus terrestris* earthworms were placed in a highly MAP contaminated environment; these worms were then tested for the presence of the bacterium and a low proportion of them had culturable bacteria (40). However, this study did not elucidate the mechanism the observed failure of MAP to colonize the worm. Another study found that antimycobacterial activity of the midgut of the earthworm *Dendrobaena veneta* stemmed from a close relative (99% sequence similarity) of *Raoultella ornitholytica*. It has been postulated that this suspected symbiont produced metabolites that disrupted cell wall synthesis. This was evidenced in rounded or filamentous forms of mycobacterial cells when observed using scanning and

transmission electron microscopy. *R. ornithinolytica* is consistently found in the intestinal wall of worms from several different soil sources (7).

Eisenia fetida and its Symbiont *Verminephrobacter eiseniae*

Several pathogens are associated with soil: opportunistic or emerging pathogens present in the soil microbiota such as *Aspergillus fumigatus* spores that may be inhaled causing disease and soil-based diseases caused by pathogens that exist in the soil naturally or enteric pathogens that enter via human or animal excrement such as *Clostridium tetani*, *Bacillus anthracis*, and *Clostridium perfringens* (185). Current human waste disposal practices may allow the accumulation of large concentrations of enteric pathogens into soil that can contribute to some of these exposures (186). Several outbreaks have stemmed from water exposed to contaminated soil (187-190). Other common outbreaks of disease are related to fecal-contaminated soil in produce (190-192). MAP has been detected in soil as well as *M. leprae* indicating soil may be a reservoir for *M. leprae* (154,193,194). Contamination of this soil also occurs by fecal contamination by animals and survival for up to a year or longer due to association with amoebae or other protozoa (119). Soil bacteria also interact with various invertebrates including earthworms (195-199). In the case of earthworms, most of these bacteria go through the digestive tract and are excreted (200).

The earthworm *Eisenia fetida,* from the family *Lumbricidae,* typically inhabits areas rich in organic material such as manure, horticultural land, and

forests with large amounts of leaf liter (201). These annelids are commonly used in vermicomposting and ecotoxicological studies. They are also used for comparative biochemistry, physiology, and immunology studies (202). The worm is epigeic, meaning it exists in the top layer of its habitat. The top layer of soil is rich in decaying organic matter; this layer also contains a high variability in microbiota (202). As earthworms digest organic matter, their digestive system disintegrates, grinds, and degrades this material. These activities can be affected by the activity and concentration of beneficial and pathogenic microbes; these can include beneficial and pathogenic fungi, actinomycetes, and bacteria (203). Microorganisms in the digestive system are the most important to earthworms with regard to the degradation of organic matter (204). In addition, organisms in earthworm intestines including *Bacillus*, *Pseudomonas*, *Klebsiella*, *Azotobacter*, *Serratia*, *Aeromonas*, and *Enterobacter* species are plant growth promoters, free-living nitrogen fixers, and phosphate solubilizers that are deposited by earthworms in their passage through the soil (204-207). The results of one study on intestinal microbial diversity revealed that the gut of *E. fetida* housed 21 taxa of bacteria (208).

Symbiotic bacteria have many roles in the lives of their hosts; these include development, nutrition, reproduction, speciation, antimicrobial defense, and immunity (209-219). Obligate symbionts show very specific interactions with the host's cells in numerous invertebrates. They are transmitted vertically from the mother to eggs or embryos. The bacteria then go on to colonize the progeny

during their development (209). Mutualistic bacteria have been able to develop such that they don't harm their host, but confer beneficial attributes to the host; this symbiosis is sometimes necessary for bacterial survival (220). In one well-studied bacterial symbiosis, *Vibrio fischeri* allows the squid *Euprymna scolopes* to mask its position from predators by using *V. fischeri* bioluminescence to hide its shadow (221). Another example of a highly beneficial relationship is that of reef-building corals and photosynthetic algae. In this instance the algae perform photosynthesis, producing glucose, glycerol, and amino acids. In some instances the growth and formation of reefs is only possible with algae present in the coral (222).

Numerous bacterial symbionts of various earthworm species have been identified. Often these bacteria are identified via isolation on selective media and genetic identification (223-228). Identification is often performed indirectly by sequencing the contents of the worm, as some are not cultivatable at present (228). Suspected symbiotic microbes present in the earthworm gut can be identified by their genome; however, they need to be distinguished from soil organisms that transit through the digestive tract (208). These microbes have been found to be highly beneficial to the earthworm health. In one report, young hatched from sterilized *E. fetida* cocoons were unable to reach sexual maturity when raised in sterilized soil unless protozoa were added to their food (229).

In *E. fetida*, the first described obligate symbiont doesn't lie in the intestine but in the nephridia of the worm. The nephridia are paired organs with three

loops; these are present in every segment of the earthworm, there are 200 in total. These take in fluid that comes from the coelom, a cavity surrounding the intestine of the worm (230). The ciliated mouth of the nephridia, called the nephrostome, takes in this coelomic fluid from the coelom and blood from the vessels. After passage through the different loops of the nephridium, nitrogenous waste is excreted through the nephridiopore. The nephridia also have an osmoregulatory function (230). The symbiont present in the ampulla (the second loop) of the nephridia was first identified in 1926 by microscopic observation (translation in Davidson et. al.) (44,231). These bacteria were first genetically identified as belonging to the genus *Acidovorax* (232). A closer examination via 16S rRNa analysis of this bacterium identified it as being in a newly identified genus and species *Verminephrobacter eiseniae* (233). The function of this bacterium in the worm remains unknown; one proposed purpose has been proteolysis to allow better absorption and amino acids (translation from Lund et. al.) (234,235). This may increase nitrogen excretion by earthworms, which releases nitrogen into the soil (232).

While the function of the bacterium has been thus far ambiguous, the colonization of the symbiont has been well investigated. These bacteria are transferred from adult worms to their offspring in egg capsules during mating. It was also demonstrated that it wasn't acquired from the environment (44). The bacteria are secreted through the clitellum after exiting the bladder, the third loop of the nephridia; these symbionts then enter the albumin of the egg capsule (43).

A model for colonization of the earthworm embryo in the cocoon has also been elucidated. The work of Davidson and her collaborators has shown that colonization of the embryonic nephridia occurs when the nephridial canal matures in a segment, this releases an attractant to draw in the *V. eiseniae* bacteria (236). Another study looked into some *V. eiseniae*'s components necessary for motility and colonization of embryonic nephridia. It was found that t flagellar motility and type IV pili were necessary for proper colonization. The twitching motility afforded by *pilB* and *pilC* is necessary for migration from the albumin of the cocoon through the colonization pore and duct, the nephridiopore, and the bladder. The flagellar motility conferred by *flgK* and *flgL* genes is necessary for the final migration from the bladder to the ampulla where the bacterium binds (237).

A further investigation of the nephridia and the symbiont has shown there are additional bacteria involved in the process; these include novel *Microbacteriaceae* and *Flexibacteriaceae* members and *Hebraspirillum* species. The *V. eiseniae* bacterium, *Microbacteriaceae,* and *Flexibacteriaceae* bacteria were present in both the cocoon and the adult nephridia; however, the *Hebraspirillum* bacteria were at a lower than detectable level in the cocoon, and may have been acquired from bedding or were in a lower than detectable level in the cocoon. It was hypothesized that the presence of these bacteria other than *Hebraspirillium* protects the earthworm embryos during development (43). This was supported by an observation of suppressed growth of *Herbaspirillum*

bacteria later in development, as it has a pathogenic role in the earthworm. Another study has investigated *Verminephrobacter* species in multiple earthworms. Nineteen of 23 lumbricid earthworms had one of these symbiont species present. Sequencing of 16S rRNA and *rpoB* genes revealed that the genetic similarity of symbionts corresponded to genetic similarity of the earthworm host species, indicating coevolution (235). Despite ambiguity as to the function of the symbiont, it is clear that it is beneficial for the bacterium, the host, or both, as *E. fetida* and other related earthworms conserve the bacterium.

Earthworm Immunity

In order to evaluate the antimycobacterial activities of earthworms, knowledge of their immune system is necessary. Invertebrates, including earthworms, lack the antibody-based specific immunity of vertebrates (3). Instead, they have an innate immunity with humoral responses mediated by pattern recognition receptors and phagocytic responses including brown body formation. These are associated with the coelomic fluid in the worm (4,45). In addition to these, earthworms have skin consisting of an epidermis and a thin cuticle containing mucopolysaccharides that act as an antimicrobial barrier (238,239). The epidermis is composed of a layer of epithelial cells, basal cells, and secretory cells. These basal cells help in the process of wound healing and graft rejection, and often exert phagocytic activity (240,241).

The coelom is a cavity filled with coelomic fluid. This fluid contains cells called coelomocytes; the transport of these cells and the fluid in the coelom is

done using channels between segments of the worm. Each segment possesses nephridia and a pore allowing draining of fluid into the environment (12). Since this pore to the environment exists, the coelom is not aseptic and always has bacteria, protozoans, and fungi, however the worm has ways to keep these populations in check (242). One study reports that the coelomic fluid of earthworms contains 6 x 10^5 bacteria/mL, with the number of suspected phagocytic cells being ten times higher in concentration (243). The combination of these phagocytic coelomic cells and humoral compounds keeps microbial populations at tolerable levels (12).

The investigation into the compounds present in the coelomic fluid of earthworms has led to the isolation of more than 40 proteins, that show cytolytic, proteolytic, antimicrobial, hemolytic, hemagglutinating, tumorolytic, and mitogenic activities (8-11). The majority of hemolytic proteins in *Eisenia fetida* species show either bactericidal and/or bacteriostatic activity against numerous pathogenic soil bacteria including *Aeromonas hydrophila* and *Bacillus megaterium*, which are suspected earthworm pathogens (10,244-246). Some of the proteins with antibacterial activity in the coelomic fluid are called *Eisenia fetida andrei* factors or EFAFs, two specific glycoproteins secreted by chloragocytes and eleocytes (244,247,248). These show antibacterial activity against both Gram-positive and Gram-negative bacteria (10,249,250). These have a particularly strong activity against bacteria pathogenic to the earthworm (245,246,251). These EFAFs have now been characterized more specifically

and named fetidins, one of these proteins with a corresponding DNA sequence 40 kDa length was cloned and found that its amino acid sequence has an N-glycosylation site and a peroxidase component (252,253). Another hemolytic protein type with a length of 41 kDa that was identified independently was termed a lysenin (254). Three discovered lysenin-related proteins were found to have high sequence homology with fetidins (255). These proteins bind sphingomyelin in the target cell membrane and create 3 nm pores (256,257). However, lysenin acts on bacteria differently, since their membranes lack sphingomyelin (255).

Another of these substances is a bacteriolytic lysozyme, which breaks down 1,4-β-D linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in peptidoglycan of bacterial cell walls. This helps to protect the worm against bacterial infections, particularly those caused by Gram-positive bacteria. Lysozyme effects are seen in extracts from both coelomocytes and coelomic fluid extracts (258). Earthworm lysozyme has both lysozyme and isopeptidase activities and is upregulated after a challenge with either Gram-positive or Gram-negative bacteria (12).

Coelomic cytolytic factor (CCF) is a protein with known cytolytic activity against invading bacteria; it is also a pattern recognition molecule (259). Pattern-recognition molecules bind microbial pathogen-associated molecular patterns (PAMPs). The PAMP recognized by CCF is the O-antigen of LPS of Gram-negative bacteria and muramyl dipeptide and muramic acid of peptidoglycan of Gram-positive bacteria as well as β-1,3-glucanase and N,N'-diacetylchitobiose of

yeast. (260-262). The mRNA for CCF is also upregulated upon microbial stimulation (263). This molecule activates a prophenoloxidase cascade, causing cytotoxic and antimicrobial compounds to form (264). CCF has two lectin-like domains; one has homology with the polysaccharide and glucanase motifs of β-1,3-glucanases and is located in the central part of the CCF. The C-terminal tryptophan-rich domain interacts with N,N'-diacetylchitobiose, muramyl dipeptide, and muramic acid (262). Despite homology with β-1,3-glucanases, this CCF doesn't show the same enzymatic ability (265-267). This compound is a contributor to cell-mediated cytotoxic reactions and can be secreted by phagocytic coelomocytes once stimulated by lipopolysaccharide (268). It has also been demonstrated that CCF agglutinates Gram-positive and Gram-negative bacteria (260). CCF and Tumor necrosis factor (TNF) also show some similarity. However, anti-TNF antibodies don't inhibit CCF indicating that different mechanisms of action for lysis must exist between the two molecules (12).

Once bacteria have been disabled, they can be removed from the coelom in several ways including: excretion through the nephridia, engulfment by cells in the nephrostome or ampulla of the nephridia, phagocytosis by specific coelomocytes and phagocytic cells (nonfunctional cells can then be excreted), and encapsulation large foreign bodies including agglutinated bacteria or parasites (11,269-271). In encapsulation, the process begins with recognition of foreign material; this is then surrounded by coelomocytes, which form a capsule also called a brown body around the material. When capsule then reaches a size

of 1-2 mm wide, external cells lose adhesion with the epithelium such that the capsule migrates toward the posterior segments to be eliminated (272-275). In *E. fetida*, most typical brown bodies have wastes, agglutinated bacteria, gregarines, or nematodes (11).

The coelomocytes present in the coelomic fluid are typically identified via morphological and cytochemical differences (276,277). There are three main types of coelomocytes: eleocytes and hyaline or granular amoebocytes. Eleocytes or chloragogen cells have nutritive and accessory functions (278). These include the collection of contaminants and other stray particulate in the coelom and the also release some antimicrobial substances (12). Eleocytes also have an identifiable collection of vesicles in their cytoplasm called chloragosomes (279). Amoebocytes have immune functions including phagocytosis and cytokine production (12). Granular amoebocytes tend to have increased phagocytic activity over hyaline amoebocytes (276). The materials these phagocytes engulf include inert particles, bacterial cell wall pieces, and foreign cells (269). Phagocytosis by coelomocytes can be modified by humoral components as well as opsonins, which coat the particle to enhance the process (280). In one experiment, the phagocytic activity of coelomocytes was improved in the presence of mammalian opsonins, IgG, and C3b (281). It has been observed that the coelomic cells of *Dendrobaena veneta* show an increased phagocytosis of known earthworm pathogens (*Bacillus megaterium* and *Aeromonas hydrophila*) over non-pathogenic species, indicating specific recognition by the

phagocytes (15). There is also evidence that EFAFs and CCF may have roles in opsonization (259,282).

A Brief Restatement of Hypothesis and Methods

This study sought to determine whether or not the symbiotic bacteria of *Eisenia fetida* have a role in immune-mediated defense against *Mycobacterium smegmatis*. Thus, the hypothesis for this study is: the coelomic cells and fluid of the *earthworm E. fetida* have antimycobacterial properties, which may be influenced by the presence *of V. eiseniae* or other symbionts. In order to examine the immune activity of the coelomic fluid of this worm against *M. smegmatis* strain mc^2155, the viability of mc^2155 after treatment was studied. Phagocytic interactions between coelomic cells and mc^2155 bacteria were observed with microscopy. The coelomic fluid and cells of both symbiont-positive and symbiont-negative earthworms were assessed.

3. Materials and Methods

Worm Populations

An earthworm colony (originally purchased from Carolina Biological) has been maintained for several years at Minnesota State University, Mankato. For this study, worms were routinely maintained on either a sphagnum moss or cellulose bedding and fed a commercial worm food (Magic Worm Food, Magic Products, WI). Bedding was replaced as needed and feeding occurred weekly.

Elimination of the Symbiont

Elimination of symbionts required sequential treatments with antibiotics. Control worms (symbiont (+)) were treated in the same manner without antibiotics. For each treatment 1:100 dilution of an antibiotic mix (10,000 units/mL of penicillin, 10 mg/mL of streptomycin, and 25 μg/mL of amphotericin B (Sigma; #A5995)) was prepared. A maximum of 11 worms were exposed in 10mL of antibiotics in 4 oz. glass jars per treatment period. The control worms were treated with deionized water. Twenty-four seven-month-old *Eisenia fetida* earthworms were cleaned to remove any bedding or slime on their body prior to placement in antibiotics or water. These worms remained in jars with 20 mL of deionized water, a 2 cm^2 piece of paper towel and 3-5 mg of Magic Worm Food until the next treatment. The antibiotic treatment was repeated 24, 48, and 72 hours after the initial treatment. The antibiotic-treated and control worms were then placed

in an 8 oz. plastic container along with moistened bedding. The container had a perforated lid for airflow. These were kept at room temperature in the dark.

Development of a symbiont free colony

The treated worms were given food once a week and any cocoons produced by the worms were counted and placed in jars. Any hatched worms were also counted and placed in 8 oz. plastic containers. These worms were kept separate by symbiont status and generation and were fed and checked for adult worms weekly. Once adult worms were discovered they were placed in a larger 12 oz. plastic container. This allowed for breeding of symbiont free populations. The generation after the antibiotic treatment was used in the experiments as symbiont (-) worms. All containers were kept in the dark at room temperature.

Confirmation of Symbiont Status

Cocoons from symbiont positive and negative worms were observed under a dissecting microscope. Tested cocoons from each population had embryos that hadn't yet formed elongated worms and appeared to either have 4 eggs or be at a 4-cell stage. The cocoons were washed and their contents were squeezed out into a microfuge tube using forceps. The cocoon contents were suspended in 50 μL of 4% paraformaldehyde (50mM phosphate buffer, pH 7.4, 0.15M NaCl). The tube was then incubated at room temperature for 2 hours. The microfuge tube was centrifuged at 8 for 10 minutes (Mini-Centrifuge, Bel-Art Products, NJ), the supernatant was poured off and the pellet resuspended in 1mL of phosphate buffered saline (PBS); it was centrifuged at speed 8 for another 10 minutes. The

supernatant was poured off and the PBS wash step was repeated. Then the pellet was suspended in 50 μL of warmed hybridization buffer [(0.9 M NaCl, 0.02 M Tris-HCl pH 8, 30% formamide, 0.01% SDS) (Sigma-Aldrich; F9037), distilled water, and 10% SDS with 10 μL of *Verminephrobacter*-specific probe, LSB 145 (S-G-Acidov-0145-a-A-18)] were added and the tube was incubated at 47°C for 2 hours. The tube was then centrifuged for 10 minutes at speed 8 and the supernatant was removed. The pellet was suspended in 1mL of warmed wash buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 8, 0.005M EDTA, distilled water, and 0.01% SDS) then incubated at 47°C for 20 minutes. The tube was then centrifuged at speed 10 for 2 minutes to quickly pellet the cells, the supernatant was poured off and the remaining pellet was suspended via pipetting. The suspension was then pipetted on a slide, and a coverslip was sealed in place with clear nail polish added along the edges. This slide was then observed using a fluorescence microscope with TRITC and DAPI filters for the probe and the worm tissue fluorescence, respectively. The symbiont fluoresced orange and the symbiont-negative cocoon was confirmed as free of any fluorescence. The symbiont positive cocoon was confirmed as having orange fluorescing bacteria.

Coelomic Cell Harvest

For each experiment four worms from control and treatment populations were selected for coelomic cell harvest. These worms were cleaned in deionized water and massaged to induce defecation. The worms were then transferred to a sterile Petri dish containing 5mL of phosphate buffered saline (PBS). Each worm

was then exposed to electric shock for 10 seconds using electrodes attached to a 9-volt battery. The worms were then left in the dish for 10 minutes to allow for coelomic fluid extrusion. The worms were then stripped of any mucus fluid, containing cells, that covers their body and they were returned to their bedding to recover for future procedures. The PBS and mucus fluid was then transferred to a sterile tube. Five milliliters of PBS were then added to the plate to collect any additional mucus fluid remaining and added to the tube. This tube was then centrifuged for 10 minutes at 1000 rpm (200 x g). The supernatant fluid was passed through a 0.45μm filter into a sterile tube; this was used for the filtered extract treatment. The pellet was resuspended with 2.5mL of PBS and 20μL of the fluid were added to a hemacytometer. This suspension was examined under the microscope and all coelomic cells were counted in five 0.1 mm^3 grids. The number of cells per 0.1 mm^3 was used to calculate coelomic cell concentration. The remaining suspension was used for coelomic cell association trial.

mc^2155 Maintenance and Dilution

A frozen stock culture of *Mycobacterium smegmatis* strain mc^2155 provided by Dr. Timothy Secott was used to inoculate 9mL of tryptic soy broth (TSB)(BBL). This culture was grown at 37°C for 3 days and was used as the source of weekly cultures used for the experiment. Several days prior to an experimental trial, a loopful of the TSB culture was added to 9 mL TSB containing 5 μL of Tween 80 and incubated for 3 days at 37°C. The culture was measured for turbidity using a Spec 20 spectrophotometer (Bausch and Lomb) set at 600nm. The culture was

then diluted using 1% peptone blanks to optical densities of $1x10^{-4}$, $5x10^{-5}$, $2.5x10^{-5}$, and $1.25x10^{-5}$.

Coelomic Fluid Extract Treatment of mc^2155

In microfuge tubes, 200 μL of PBS were added to either 200μL of the symbiont positive or negative extracts for extract controls. Also, 200μL of the different *M. smegmatis* dilutions and 200 μL of PBS were added to tubes for mycobacterial controls. Aliquots of 200μL of symbiont-positive or -negative extracts were added to the *M. smegmatis* dilutions for the experimental trials. After a 30-minute incubation at room temperature, 100μL of each solution was spread-plated on of tryptic soy agar (TSA). These plates were then incubated at 37°C for 3 days. The plates were then counted for *M. smegmatis* colonies and any colony merging or contaminants were noted.

Coelomic Cell Association Treatment

Using a concentration of mc^2155 determined by spectrophotometer (between 0.140 and 0.980 across the trials), the culture was diluted in three 1:10 dilutions in 1% peptone. A volume of 200μL of each dilution and the undiluted bacteria was mixed separately with 200μL of coelomic cells harvested from symbiont (+) and symbiont (-) worms. The coelomic cells alone and the mycobacteria alone were added to 200μL of PBS as control treatments. These tubes were then allowed to incubate at room temperature for 30 minutes. After incubation, the tubes were centrifuged at speed 5 for 10 minutes (Bel-Art Mini centrifuge) and the supernatant was poured out. The pellet was then suspended using the

remaining liquid and approximately 100μL were smeared onto each slide and allowed to dry. The smears were then Gram stained. These slides were then observed at 1000x magnification and 300 total coelomic cells were counted as either adhered with or not adhered with mycobacteria. If any slides had too high of a mycobacterial concentration causing nearly all coelomic cells to appear as having adherent mycobacteria they were not used for the count or the study. This was also true if a slide had less than 300 coelomic cells. The coelomic cells without mycobacteria were used as a control to ensure there were no contaminant bacteria giving false positives for the experimental bacteria.

Statistical Analysis

To determine differences between the symbiont (+) and (-) populations as well as between the untreated mycobacteria, statistical analyses were performed in SPSS. For the coelomic association trials, a univariate analysis was performed to determine differences in percent association at the different dilutions. Individual differences were determined by a Tukey HSD or by Games Howell in the case of nonhomogeneous variances determined by a Levene's Test. Univariate analyses were also performed on the variables associated with the cell-free coelomic extract treatments of mc^2155. These were also tested for homogeneity of variances with the Levene's test and individual significant differences assessed by the Tukey HSD test or Games-Howell test for nonhomogeneous data. To determine differences among the survival

percentages for symbiont (+), symbiont (-), and mycobacterium alone populations, univariate analysis was performed.

4. Results

The antimycobacterial activity of soluble compounds and coelomic cells in the coelomic fluid of symbiont (+) and (-) *E. fetida* earthworms were assessed in this project.

Adherence

The number of cells with mycobacteria adhered to their surface was divided by the total cells (300) counted to obtain the percent association; three concentrations of mc^{2}155 bacteria were used. As the concentration decreased, so did adherence (Fig. 1). Differences exist between the percent associations corresponding to the varying dilutions when p=0.01 was used as the level for significance, but not by symbiont status (+) or (-) at each dilution (p= 0.663). All individual probabilities are displayed in Appendix A.

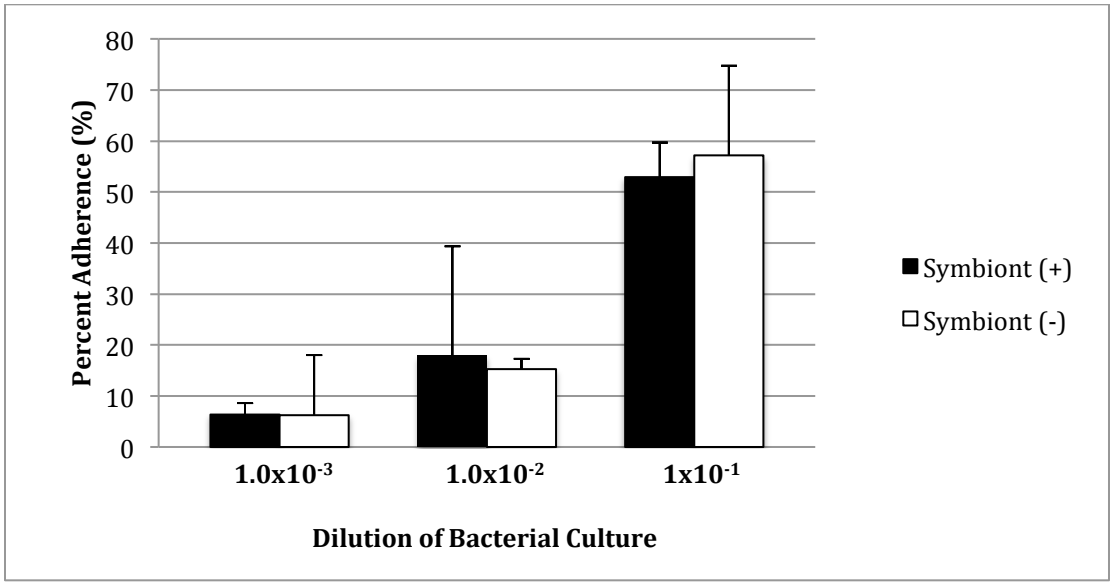

Figure 1-Percent Association by Dilution-Significant differences exist between the different dilutions, but not by symbiont status.

Antimycobacterial Activity

To determine if the coelomic fluid had antimycobacterial activity, the fluid was mixed with different concentrations of mc^2155 cells, incubated, and aliquots of the mixes spread-plated for viable bacterial counts. Using a 72-hour culture, dilutions with PBS were made to $1x10^{-4}$, $5x10^{-5}$, $2.5x10^{-5}$, and $1.25x10^{-5}$ OD. Two dilutions were used per trial.

Table 1-The Effect of Coelomic Fluid Treatment on *Mycobacterium smegmatis* mc^{2}155 Concentration Viability

Trial Number	Concentration of mc^{2}155 (OD_{600})	Symbiont (+) Average CFU/mL mc^{2}155	Symbiont (-) Average CFU/mL mc^{2}155	Mycobacterium Alone Average CFU/mL mc^{2}155
1	$1.00x10^{-4}$	TMTC	TMTC	$1.90x10^{7}$
	$5.00x10^{-5}$	$2.60x10^{7}$	$4.14x10^{7}$	$4.46x10^{7}$
2	$1.00x10^{-4}$	$1.73x10^{7}$	$1.34x10^{7}$	$1.17x10^{7}$
	$5.00x10^{-5}$	$2.14x10^{7}$	$2.24x10^{7}$	$2.08x10^{7}$
3	$1.00x10^{-4}$	$8.50x10^{6}$	$1.05x10^{7}$	$1.42x10^{7}$
	$5.00x10^{-5}$	$2.10x10^{7}$	$2.40x10^{7}$	$1.88x10^{7}$
4	$1.00x10^{-4}$	$1.46x10^{7}$	$1.93x10^{7}$	TMTC
	$5.00x10^{-5}$	$2.76x10^{7}$	$3.02x10^{7}$	$2.86x10^{7}$
5	$5.00x10^{-5}$	$3.48x10^{7}$	$3.18x10^{7}$	$2.98x10^{7}$
	$2.50x10^{-5}$	$8.16x10^{7}$	$6.08x10^{7}$	$4.69x10^{7}$
6	$2.50x10^{-5}$	$2.60x10^{7}$	$3.80x10^{7}$	$1.52\ x10^{7}$
	$1.25x10^{-5}$	$3.92x10^{7}$	$3.84x10^{7}$	$7.28\ x10^{7}$
7	$2.50x10^{-5}$	$3.60x10^{7}$	$3.28x10^{7}$	TMTC
	$1.25x10^{-5}$	$4.16x10^{7}$	$3.12x10^{7}$	TMTC

No treatment type was significantly different than the others. TMTC: Too Many Colonies to Count (> 300).

Overall, when mc^{2}155 bacteria were treated with the fluid, no difference in surviving bacteria occurred by source of the coelomic fluid (symbiont (+) or (-))

nor did the coelomic fluid-treated bacterial concentrations differ from the untreated mycobacteria (Table 1).

ANOVA of the different trials indicated a significant difference (p= 0.001) among the trials of surviving bacteria. Post hoc tests indicated differences between trials 2 and 5 (p= 0.004), 2 and 6 (p= 0.033), 3 and 5 (p= 0.002), and 3 and 6 (p= 0.019). These correspond to differences due to the concentration of bacteria added.

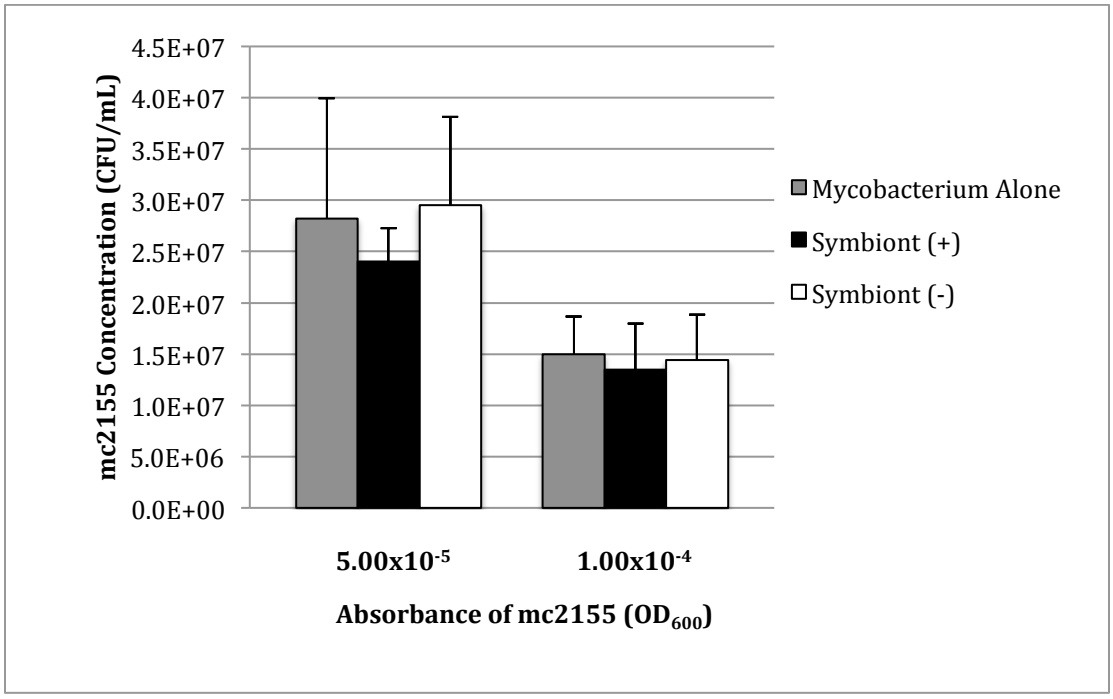

Figure 2a- The Effect of Coelomic Extract Treatment on Higher Concentrations of *Mycobacterium smegmatis* mc^{2}155- The diluted concentration of mc^{2}155 in trials 1-4 are compared amongst the different treatments for the lower concentration dilutions. Trial 5 was excluded from the figure due to results falling between the high and low concentration groups.

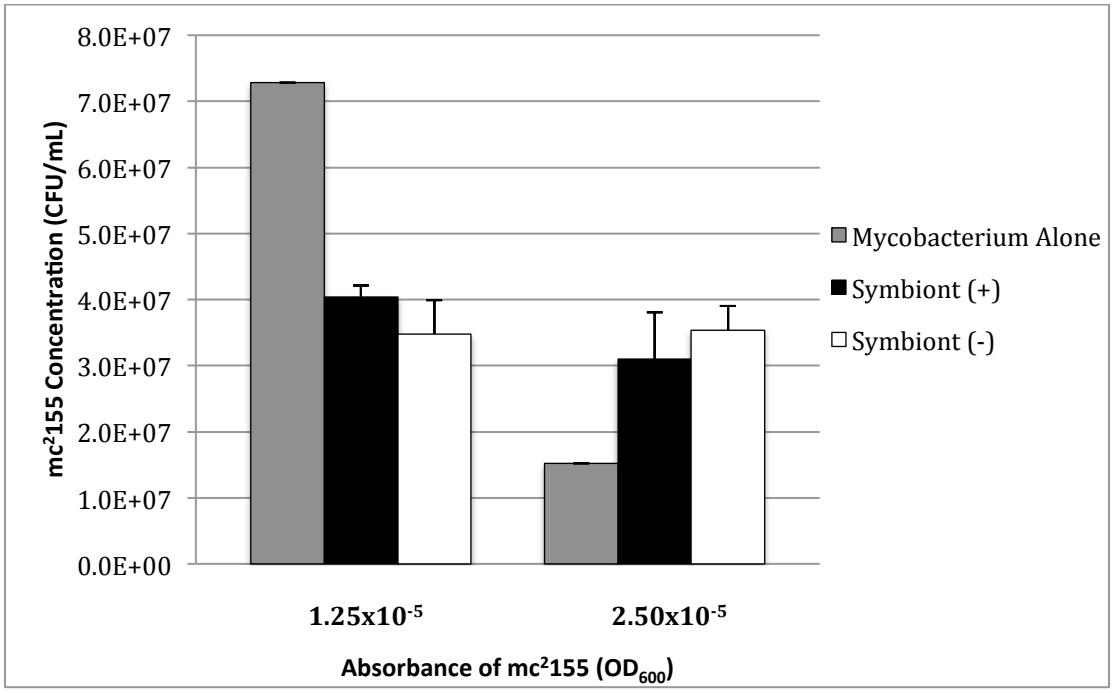

Figure 2b- The Effect of Coelomic Extract Treatment on Lower Concentration *Mycobacterium smegmatis* mc^{2}155-The diluted concentration of mc^{2}155 in trials 6 and 7 is compared amongst the different treatments for the lower concentration dilutions. As bacterial concentration in OD increased, viable concentration decreased.

ANOVA revealed a significant difference between the surviving mycobacteria by the different dilutions ($p < 0.001$). This difference was found using the Welch test, as there was a lack of homogeneity of variance ($p = 0.006$). The individual differences were found via a Games-Howell test also due to the lack of homogeneity of variance. The only significant difference was between the $1x10^{-4}$ and $5x10^{-5}$ dilutions ($p<0.0001$).

Mycobacterium smegmatis mc^{2}155 produced a higher bacterial concentration at lower absorbance dilutions than at lower concentration dilutions (Table 1, Figs. 2a & 2b). However, despite significant increases in initial concentration, no significant differences existed between the two coelomic fluid

treated bacteria. The only significant differences found were between some of the different initial concentrations within each coelomic fluid treatment (Appendices B-D) and some at the differences between the coelomic fluid treatments (Appendices E-G).

Table 2-Average Pooled Percent Survival by Treatment- Differences in percent survival occurred by dilution but not by treatment (symbiont (+) or (-)).

Initial Concentration of mc^2155 (OD_{600})	Symbiont (+) Coelomic Fluid Mean Percent Survival ± SD	Symbiont (-) Coelomic Fluid Mean Percent Survival ± SD
$1x10^{-4}$	89.98 ± 30.12	96.21± 29.96
$5x10^{-5}$	91.72 ± 19.68	105.05 ± 26.41
$2.5x10^{-5}$	186.01± 115.18	170.47± 57.87
$1.25x10^{-5}$*	55.49 ± 2.33	47.80 ± 6.99

***-**Only one set of untreated controls yielded countable plates

The percent survival statistic was calculated using the average concentration of untreated mycobacteria as 100%. The bacterial concentration from each dilution was used to calculate the percentage (Table 2). Significant differences occurred by dilution ($p=0.004$) but not by population ($p=0.963$). No interaction was observed between dilutions and treatment populations ($p=0.940$). All individual differences are noted in Appendices H-J.

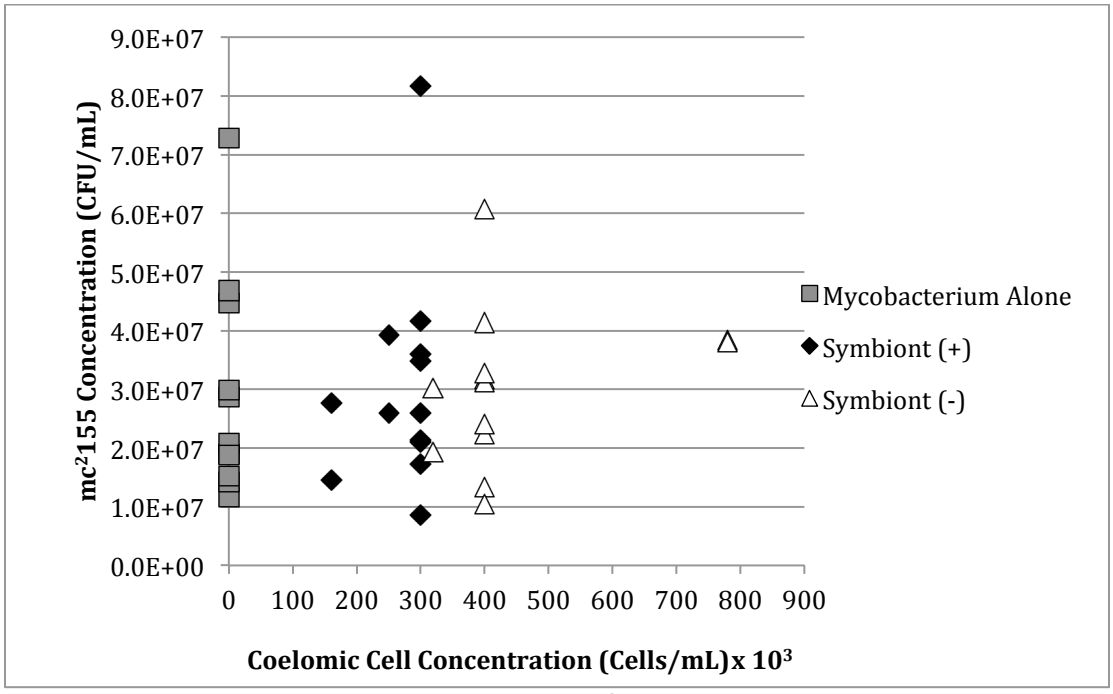

Figure 3-*Mycobacterium smegmatis* mc^{2}155 Concentration After Treatment with Coelomic Cell Extract by Coelomic Cell Concentration and Treatment Group- The symbiont (+) and (-) treatments seem to stratify with a bit of overlap with the symbiont (-) garnering more coelomic cells. However, there is no significant difference between the coelomic cell concentrations or treatment types.

Coelomic cell concentration varied from trial to trial, however coelomic cell concentration didn't correspond to an increase or decrease in mc^{2}155 concentration by cluster (Fig. 3). A correlation of 0.165 existed (r^2=0.027) between coelomic cell concentration and surviving mycobacteria, but this was not found to be significant (p= 0.421).

Variability in Bacterial Cultures and Coelomic Cell Concentration

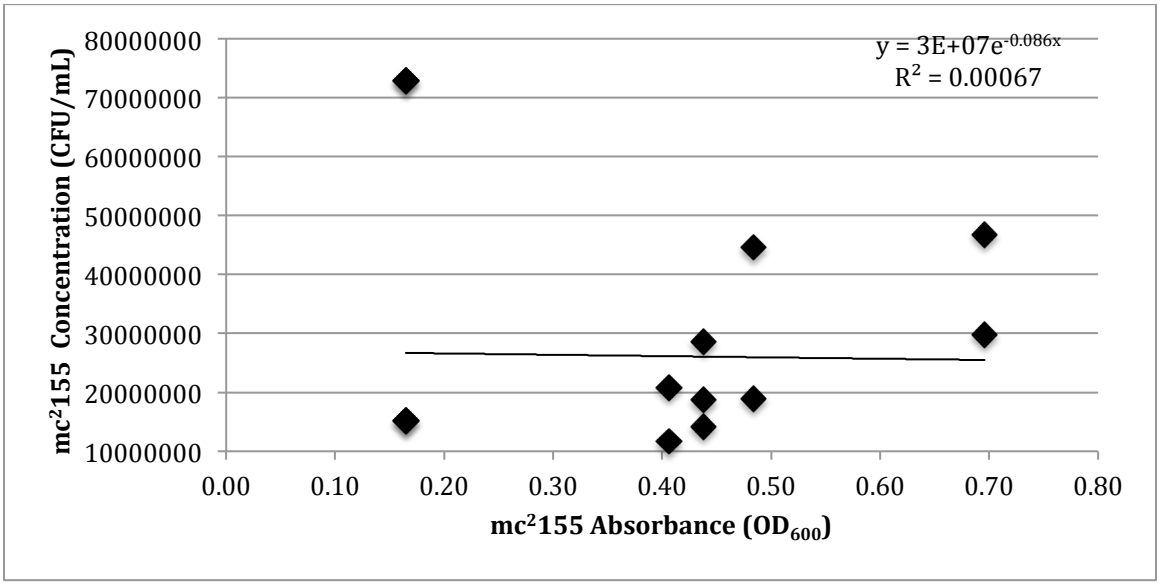

Figure 4- *Mycobacterium smegmatis* mc^{2}155 Concentration by Absorbance of Starting Culture-There were increasing plate counts with increasing absorbance, dropping off at 0.696. No significant differences were detected overall.

Quantified bacterial concentrations exhibited great variability in the 72-hour cultures of mc^{2}155 used in these experiments. Generally speaking as the absorbance of the culture increased there was a resulting increase in plate count. However, an examination of the starting culture absorbance values (Fig. 4) revealed no significant difference between plate counts (p= 0.066) at the different measured absorbance values for a 72-hour culture. Due to a lack of homogeneity of variances, a Welch test and a Games-Howell post-hoc test was also performed. This did reveal a difference (p= 0.037) in bacterial concentration between the starting absorbance values of 0.165 and 0.484.

The viable counts increased when the bacteria were diluted (Fig. 5). No significant differences in concentrations were found between the different

dilutions (p=0.055). This was found using a Kruskal-Wallis test, which was necessary due to a lack of normality of the 1.25 x 10^{-5} dilution.

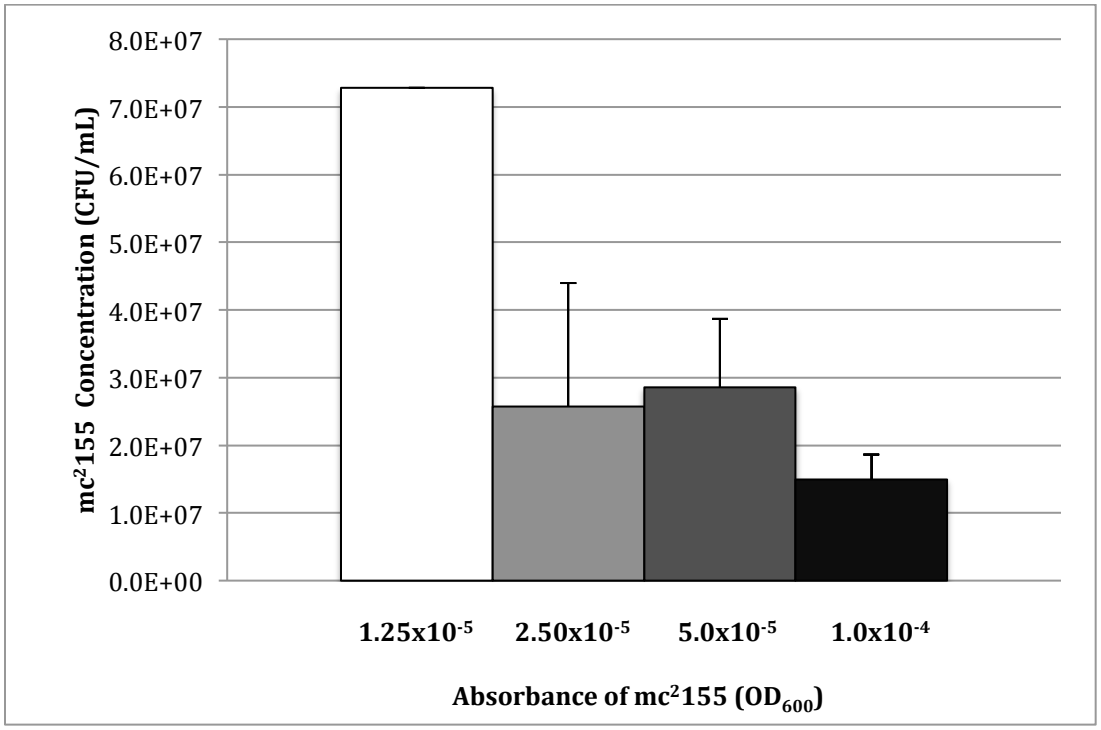

Figure 5-Average Colony Counts of Untreated *Mycobacterium smegmatis* mc^{2}155 by Dilution-As the absorbance increased bacterial concentration decreased, however no significant differences were found. Only one sample of the 1.25x10^{-5} dilution was viable so no standard deviation was calculated.

An examination of the mycobacterial concentration in relation to coelomic cell concentration revealed a clustering of coelomic cell concentration by population (Fig. 3). A comparison of the coelomic cell concentrations (Figures 3 & 6) between the symbiont (+) and (-) shows a significant difference (p= 0.001). This difference shows an increased mean coelomic cell concentration in the symbiont (-) population of 4.46 x 10^5 coelomic cells/mL over the symbiont (+) population of 2.70 x 10^5 coelomic cells/mL.

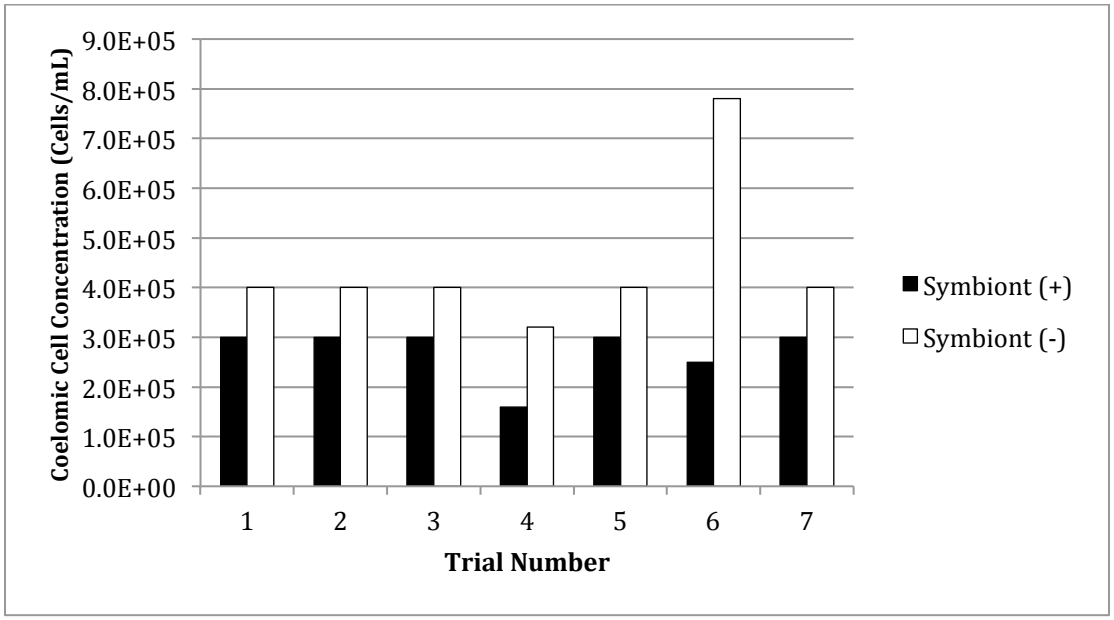

Figure 6- Variable Coelomic Cell Concentration by Trial and Worm Population-Coelomic cell counts are the compilation from four earthworms' ceolomic exudates.

5. Discussion

Adherence of Coelomic Cells with Mycobacteria

This project sought to elucidate any possible antimycobacterial activity present in the earthworm *Eisenia fetida* and whether a previous disruption of normal microbiota would affect this activity. The first system used coelomic cells extracted from symbiont (+) and (-) earthworms and three dilutions of *Mycobacterium smegmatis* mc^2155 bacteria. It was found that the percentage of coelomic cells with associated mycobacteria was not significantly different in the worm populations after disruption of the earthworm microflora. This indicates that the nephridial symbionts had no effect on the coelomic cell binding of mc^2155 cells.

Furthermore, as the bacterial concentration in the mixtures decreased, the percentage of coelomic cells with adhered bacteria also decreased, however this decrease in percentage adhered was not proportional to the 10-fold decrease in concentration. The high association observed may have been due to the higher number of bacteria remaining after the wash step. The remaining unbound bacteria may have associated with coelomic cells once they were placed on slides. The lower two concentrations of bacteria used differed in percent adherence by 50%. Thus, it appears as though either a low proportion of coelomic cells can bind *M. smegmatis* or the binding requires an increased incubation time or different conditions for adherence. If phagocytes specifically

recognized mycobacteria, similar binding proportions would be seen across at least two of the dilutions. The higher concentrations of bacteria were not useful for determining coelomic cell association, due to the sheer numbers of bacteria on the slide.

There may be other considerations to take into effect with the experimental design including the use of PBS in the experiment. There was passive adherence of the bacteria to the coelomic cells in this solution, but without an energy source the coelomic cells may be unable to bind tightly to the bacteria. It is possible that a longer incubation time may be necessary for proper recognition and adherence of the mycobacteria and a nutrient source may be necessary for that longer incubation. However, an incubation time longer than 3-4 hours may allow *M. smegmatis* to replicate, affecting bacterial concentration in solution (173). Thus, the bacteria may have to be inactivated or a slower growing species like MAP may have to be used.

Assessment of Coelomic Fluid for Antimycobacterial Activity

The assessment of antimycobacterial activity by reductions in viable plate counts was plagued with issues related to inconsistent growth by the bacteria. However, cell-free coelomic-cell extracts from symbiont (+) and (-) worms resulted in no significant difference in the concentrations of surviving bacteria. Two compounding problems arose with this method. There is a certain amount of variation in coelomic cell concentrations from trial to trial, as well as differences in bacterial plate counts. If there was variability related to the coelomic cell

concentration prior to filtration of the fluid, it was not significant. Also even though the coelomic cell concentration varied from trial to trial, no resulting significant difference in plate count was observed.

Compared to common model bacteria such as *E. coli* that are easier to manipulate, *Mycobacterium smegmatis* culture presents difficulties. Firstly, this bacterium grows slower than some common model bacteria, taking 2-3 days to grow colonies or a turbid culture. However, *M. smegmatis* is preferable to other strains of mycobacteria that take weeks to months to grow. Also, this bacterium grows in clumps; in broth culture it tends to form a flaky mat on the surface of the liquid and on agar plates the bacteria form spreading colonies. To aid dispersal of bacteria, the surfactant Tween 80 was added to the culture medium. However, inconsistencies in the dilutions still occurred. However, more dilute starting concentrations yielded more countable plates. A related issue is also the fluctuations in the absorbance of the starting cultures for each trial. The mid-log phase for *M. smegmatis* (at 600nm) is 0.5 OD and most of the cultures fell near that absorbance (283). However, if the bacteria aren't fully in solution, the OD may be inaccurate and the dilution may be too high or low as a result.

Despite the variability of bacterial cultures, no evidence of mycobactericidal activity in the cell free coelomic fluid was found. Since the concentration of coelomic cells used to make the extracts also varied with each trial, they added to possible inconsistency in results. While coelomic cell concentration differed by trial overall, the symbiont (-) population yielded a

significantly higher coelomic cell concentration than the symbiont (+) population. How these differences in coelomic yield relate to the loss of bacterial symbionts remains unclear. Coelomic cells function in the innate immune defenses of the earthworm (12). It may be a compensatory increase in immune cells to act on bacteria that have taken up residence in areas normally occupied by symbiotic bacterial flora. Thus, the increase in coelomic cell production may be a result of a persistent inflammatory reaction in response to disrupted bacterial populations in the worm. Conversely, the increase in coelomic cell concentration may indicate the microflora of the worm may be essential to the regulation of innate response. Another possibility is that the symbiont's presence during development helps to acclimatize the cells' immune response.

This project has assessed the coelomic fluid and coelomic cells of *E. fetida* earthworms for antimycobacterial activity. By the parameters of this project, there has been no obvious activity against *M. smegmatis*. An assessment of *Lumbricus terrestris* earthworms in MAP-infested soil suggested resistance to mycobacteria, as few worms in that environment had detectable MAP in their gut (40). If that kind of antimycobacterial activity also exists in *E. fetida*, it likely isn't occurring via phagocytic recognition or antimycobacterial substances in the coelomic fluid. MAP may be recognized differently by *L. terrestris* than *M. smegmatis* by *E. fetida*. A survey of tissue samples and coelomic cells of the both earthworm guts for mycobacteria in both of these instances might be useful to elucidate the method of elimination.

The null hypothesis that *E. fetida* coelomic cells and fluid exhibit no antimycobacterial properties was upheld in this instance. Disruption of symbiotic bacteria in this earthworm had no direct effect on phagocytic activity of the coelomic cells or bactericidal/bacteriostatic properties of the coelomic fluid. Under the conditions of these experiments, treatment of mycobacteria with coelomic fluid showed no apparent negative effect on mycobacterial growth compared to the untreated mycobacteria after treatment, as there was no significant reduction in viability in either of the treated populations. The data demonstrated that no antimycobacterial activity against *M. smegmatis* could be observed.

There are some variations on methodology that one could explore in future research. This research only used 4 worms for coelomic cell harvest; higher concentrations of coelomic cells may have yielded detectible activity. This would necessitate additional worms per harvest. If additional worms were used, a more concentrated fluid of any antimicrobial substances could possibly show more obvious results and gradations down to a minimum inhibitory/bactericidal concentration could be found.

Another area to investigate would be the potential injury suffered by the mycobacteria after coelomic fluid treatment. The bacteria may be damaged in some way that is not readily apparent by plate count. If working with more concentrated coelomic fluid than used in this study in combination with visualization, perhaps by using electron microscopy, one might be able to see

changes in the cell wall structure or atypical cell shape as a result of treatment. This was a method used previously to observe the damage of another earthworm derived antimycobacterial substance (7). Alternatively, injury may make the bacteria more susceptible to a second assault that could result in death. This research used only *M. smegmatis*. Since there has been noted antimicrobial activity in coelomic fluid previously, it is possible that these worms may have activity against another bacterium of interest such as certain troublesome Gram (-) bacteria (4,249,250,284). Another possibility is that an injury might progress to a fatal injury after a longer incubation period was used prior to plating.

The most intriguing data from this project are the coelomic cell concentration differences between symbiont (+) and (-) earthworms. A larger survey of these earthworm populations, either with more worms per harvest or more harvests overall, would be beneficial to see if this is a consistent trend in regards to symbiont status or if this was only in relation to the two separate breeding populations used in this study. Since the aim of the study didn't directly address coelomic cell concentrations, the data are unfortunately limited in this area. However, resident microflora do exert an antimycobacterial influence in another earthworm species (7). Thus, disruption of the microbiota of the worms used in the study may exhibit different susceptibilities to earthworm pathogens.

The increased concentration of coelomic cells in the disrupted worms indicates that there is a need for more immune cells than in untreated worms. The loss of *Verminephrobacter eiseniae* cannot be determined as the cause of

the increase in coelomic cells, as other bacteria were likely eliminated from the worms when they were treated with antibiotics. Only *V. eiseniae* was assayed as it is a well-described symbiont of the worm and the loss of this symbiont indicated a disrupted microbiota.

This experiment focused on the phagocytic coelomocytes or amoebocytes but also indirectly investigated the production of antimicrobial substances by eleocytes (12). It appears as though no phagocytic recognition occurred, based on the data collected in this experiment. However, it is possible that an antimycobacterial substance produced by eleocytes in the coelomic fluid may have been present, but at too low a level to show a noticeable effect on mc^2155 plate counts.

Conclusion

In closing, this project investigated the effects of disrupting the bacterial flora and symbionts from *E. fetida* on potential antimycobacterial defenses. No significant trends with regard to phagocytic activity or direct antimycobacterial activity were observed. While some aspects of this study could be modified to further examine these trends possibly using more concentrated extracts, this would appear to be an unproductive area to pursue. The most likely area of interest would be the effect of symbiont removal on the coelomic cell populations. A closer examination of the earthworm's physiological response to the antibiotic treatment may elucidate the source of the coelomic cell concentration discrepancy.

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Appendices

Appendix A-Probability Values of Average Mycobacterial Concentration Between All Dilutions for Both Symbiont (+) and (-) Treated Mycobacteria

Dilution and Treatment	p-value against $1x10^{-3}$ Symbiont (+)	p-value against $1x10^{-3}$ Symbiont (-)	p-value against $1x10^{-2}$ Symbiont (+)	p-value against $1x10^{-2}$ Symbiont (-)	p-value against $1x10^{-1}$ Symbiont (+)	p-value against $1x10^{-1}$ Symbiont (-)
$1x10^{-3}$ Symbiont (+)	-----	0.793	0.032	0.001	<0.0001	<0.0001
$1x10^{-3}$ Symbiont (-)	0.793	-----	0.012	0.002	<0.0001	<0.0001
$1x10^{-2}$ Symbiont (+)	0.032	0.012	-----	0.534	0.003	<0.0001
$1x10^{-2}$ Symbiont (-)	0.001	0.002	0.534	-----	.535	<0.0001
$1x10^{-1}$ Symbiont (+)	<0.0001	<0.0001	0.003	.535	-----	0.636
$1x10^{-1}$ Symbiont (-)	<0.0001	<0.0001	<0.0001	<0.0001	0.636	-----

Tukey HSD testing was used to determine p-values; in cases of inequality of variances the Games-Howell test was used.

Appendix B-Probability Values of Untreated Mycobacteria Average Mycobacterial Concentration Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Mycobacterium Alone	$5.00x10^{-5}$ Mycobacterium Alone	$2.50x10^{-5}$ Mycobacterium Alone	$1.25x10^{-5}$ Mycobacterium Alone
$1.00x10^{-4}$ Mycobacterium Alone	---	0.390	0.648	0.001
$5.00x10^{-5}$ Mycobacterium Alone	0.390	---	0.985	0.005
$2.50x10^{-5}$ Mycobacterium Alone	0.648	0.985	---	0.005
$1.25x10^{-5}$ Mycobacterium Alone	0.001	0.005	0.005	---

All p-values were found by Tukey HSD testing.

Appendix C-Probability Values of Symbiont (+) Coelomic Fluid Treated Average Mycobacterial Concentration Between Different Dilutions

Dilution and Treatment	$1x10^{-4}$ Symbiont (+)	$5x10^{-5}$ Symbiont (+)	$2.5x10^{-5}$ Symbiont (+)	$1.25x10^{-5}$ Symbiont (+)
$1.00x10^{-4}$ Symbiont (+)	---	0.057	0.403	0.011
$5.00x10^{-5}$ Symbiont (+)	0.057	---	0.657	0.014
$2.50x10^{-5}$ Symbiont (+)	0.403	0.657	---	0.967
$1.25x10^{-5}$ Symbiont (+)	0.011	0.014	0.967	---

Differences were found by Games-Howell (lack of heterogeneity of variances p=0.005)

Appendix D- Probability Values of Symbiont (-) Coelomic Fluid Treated Average Mycobacterial Concentration Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Symbiont (-)	$5.00x10^{-5}$ Symbiont (-)	$2.50x10^{-5}$ Symbiont (-)	$1.25x10^{-5}$ Symbiont (-)
$1.00x10^{-4}$ Symbiont (-)	---	0.156	0.014	0.132
$5.00x10^{-5}$ Symbiont (-)	0.156	---	0.223	0.917
$2.50x10^{-5}$ Symbiont (-)	0.014	0.223	---	0.700
$1.25x10^{-5}$ Symbiont (-)	0.132	0.917	0.700	---

All p-values were found by Tukey HSD testing.

Appendix E-Probability Values of Untreated Mycobacteria Against Coelomic Fluid Treated Average Mycobacterial Concentration Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Mycobacterium Alone	$5.00x10^{-5}$ Mycobacterium Alone	$2.50x10^{-5}$ Mycobacterium Alone
$1.00x10^{-4}$ Symbiont (+)	0.680	0.049	0.365
$5.00x10^{-5}$ Symbiont (+)	0.011	0.478	0.885
$2.50x10^{-5}$ Symbiont (+)	0.025	0.688	0.677
$1.25x10^{-5}$ Symbiont (+)	0.002	0.080	0.298
$1.00x10^{-4}$ Symbiont (-)	0.874	0.060	0.355
$5.00x10^{-5}$ Symbiont (-)	0.035	0.847	0.698
$2.50x10^{-5}$ Symbiont (-)	0.002	0.353	0.419
$1.25x10^{-5}$ Symbiont (-)	0.002	0.495	0.560

The untreated mycobacteria didn't have countable plates at the $1.25x10^{-5}$ dilution

Appendix F- Probability Values of Symbiont (+) Coelomic Fluid Treated Average Mycobacterial Concentration Against Symbiont (-) Coelomic Fluid Treated and Untreated Average Mycobacterial Concentration at Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Symbiont (+)	$5.00x10^{-5}$ Symbiont (+)	$2.50x10^{-5}$ Symbiont (+)	$1.25x10^{-5}$ Symbiont (+)
$1.00x10^{-4}$ Mycobacterium Alone	0.680	0.011	0.025	0.002
$5.00x10^{-5}$ Mycobacterium Alone	0.049	0.478	0.688	0.080
$2.50x10^{-5}$ Mycobacterium Alone	0.365	0.885	0.677	0.298
$1.00x10^{-4}$ Symbiont (-)	0.812	0.013	0.026	0.004
$5.00x10^{-5}$ Symbiont (-)	0.026	0.252	0.252	0.046
$2.50x10^{-5}$ Symbiont (-)	0.003	0.800	0.800	0.136
$1.25x10^{-5}$ Symbiont (-)	0.039	0.046	0.046	0.346

The untreated mycobacteria didn't have countable plates at the $1.25x10^{-5}$ dilution

Appendix G-Probability Values of Symbiont (-) Coelomic Fluid Treated Average Mycobacterial Concentration Against Symbiont (+) Coelomic Fluid Treated and Untreated Average Mycobacterial Concentration Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Symbiont (-)	$5.00x10^{-5}$ Symbiont (-)	$2.50x10^{-5}$ Symbiont (-)	$1.25x10^{-5}$ Symbiont (-)
$1.00x10^{-4}$ Mycobacterium Alone	0.874	0.035	0.002	0.002
$5.00x10^{-5}$ Mycobacterium Alone	0.060	0.847	0.353	0.495
$2.50x10^{-5}$ Mycobacterium Alone	0.355	0.698	0.419	0.560
$1.00x10^{-4}$ Symbiont (+)	0.812	0.026	0.003	0.039
$5.00x10^{-5}$ Symbiont (+)	0.013	0.252	0.004	0.150
$2.50x10^{-5}$ Symbiont (+)	0.026	0.800	0.409	0.538
$1.25x10^{-5}$ Symbiont (+)	0.004	0.046	0.136	0.346

The untreated mycobacteria didn't have countable plates at the $1.25x10^{-5}$ dilution

Appendix H-Probability Values of Mean Percent Survival of Symbiont (+) Treated Mycobacteria Between Different Dilutions

Dilution and Treatment	$1x10^{-4}$ Symbiont (+)	$5x10^{-5}$ Symbiont (+)	$2.5x10^{-5}$ Symbiont (+)	$1.25x10^{-5}$ Symbiont (+)
$1.00x10^{-4}$ Symbiont (+)	---	1.000	0.593	0.411
$5.00x10^{-5}$ Symbiont (+)	1.000	---	0.596	0.046
$2.50x10^{-5}$ Symbiont (+)	0.593	0.596	---	0.416
$1.25x10^{-5}$ Symbiont (+)	0.411	0.046	0.416	---

Differences were found by Games-Howell (lack of heterogeneity of variances p=0.005)

Appendix I-Probability Values of Mean Percent Survival of Symbiont (-) Treated Mycobacteria Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Symbiont (-)	$5.00x10^{-5}$ Symbiont (-)	$2.50x10^{-5}$ Symbiont (-)	$1.25x10^{-5}$ Symbiont (-)
$1.00x10^{-4}$ Symbiont (-)	---	0.985	0.115	0.479
$5.00x10^{-5}$ Symbiont (-)	0.985	---	0.122	0.282
$2.50x10^{-5}$ Symbiont (-)	0.115	0.122	---	0.019
$1.25x10^{-5}$ Symbiont (-)	0.479	0.282	0.019	---

All p-values were found by Tukey HSD testing.

Appendix J- Probability Values of Mean Percent Survival of Symbiont (+) and (-) Treated Mycobacteria Between Different Dilutions

Dilution and Treatment	$1.00x10^{-4}$ Symbiont (-)	$5.00x10^{-5}$ Symbiont (-)	$2.50x10^{-5}$ Symbiont (-)	$1.25x10^{-5}$ Symbiont (-)
$1.00x10^{-4}$ Symbiont (+)	0.812	0.514	0.122	0.128
$5.00x10^{-5}$ Symbiont (+)	0.832	0.394	0.135	0.008
$2.50x10^{-5}$ Symbiont (+)	0.308	0.347	0.851	0.173
$1.25x10^{-5}$ Symbiont (+)	0.142	0.013	0.075	0.346

All p-values were found using a t-test accounting for lack of homogeneity of variances (Levene's Test for Symbiont (+) p=0.005)