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CHARACTERIZATION OF AMOEBAE INTERACTIONS WITH FOUR NON-*PNEUMOPHILA LEGIONELLA* SPECIES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Microbiology

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by
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Abstract

Legionella is the causative agent Legionnaires' Disease and the number one cause of bacterial water-borne outbreaks in the United States. 85% of Legionnaires' Disease cases are attributed to one species, *L. pneumophila.* Other virulent *Legionella* species exist, yet we have limited knowledge of them. Four non-*pneumophila* species: *L. clemsonensis, L. gormanii, L. anisa,* and the uncharacterized strain D4482, were chosen to assess host interactions with two species of amoebae, *Acanthamoeba polyphaga* and *A. castellanii.* Interactions were assessed with both planktonic, amoebae grown and biofilm *Legionella*. For planktonic assays, *L. pneumophila* invaded significantly higher into *A. castellanii* than into *A. polyphaga*. Invasion of *L. pneumophila* was also higher than the four non-*pneumophila* species in both *Acanthamoebae* species. Amoebae grown *L. clemsonensis* showed an increased invasion ability compared to broth grown in *A. castellanii.* Both *A. polyphaga* and *A. castellanii* grazed equally from all *Legionella* biofilms started from planktonic culture. When amoebae grown bacteria were used to establish biofilms, *L. pneumophila, L. gormanii,* and *L. anisa* were grazed at lower amounts by *Acanthamoebae* than planktonically grown biofilms. Our results suggest that the *Acanthamoebae* host shows no preference for the *Legionella* species it consumes and that growth within an amoebae affects the host interaction. Characterization of host-pathogen interactions can aid in creating improved understanding of the microbial ecology and in turn predictive risk assessment for *Legionella.*

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Introduction

LEGIONELLA

Legionella is the number one bacterial cause of water-borne outbreaks in the United States, causing a severe form of pneumonia known as Legionnaire's Disease (LD). The genus *Legionella* contains Gram-negative bacteria that ubiquitously exist in most freshwater aquatic environments. These bacteria survive over a wide range of growth conditions, from 5-63°C and in pHs from 5-9.2 (1). *Legionella* also causes a milder flu like illness, Pontiac Fever, which usually goes undiagnosed as it resolves on its own in 1-3 days. Several cases of *Legionella* infection have also been reported after surgical procedures. Due to improperly cleaned surgical tools along with a lack of pre-surgical antibiotics, multiple cases of endocarditis have occurred. Both *L. pneumophila* and *L. dumoffii* were implicated in the infections (2). *L. pneumophila* Philadelphia 1, the type strain for the *Legionella* genus, was isolated from the American Legion outbreak in 1976, where 182 people were infected, and 29 of those patients died. This outbreak led to the discovery and identification of the bacteria (3).

Legionella bacteria are found in a variety of freshwater aquatic environments. Natural aquatic environments such as ponds and hot springs are home to various *Legionella* species, but rarely are cases of LD ever linked to isolates from natural environments. These locations are merely a reservoir for water containing *Legionella* before it is taken up into man-made water system such as hot tubs, water towers, ice-

makers and cooling towers. These man-made environments are where a vast majority of LD cases originate (4). Man-made water systems are where efforts to control *Legionella* are focused, since they are the disease causing environments.

BIOFILMS

In the environment, many species of bacteria exist as biofilms which provide stability and safety along with easy access to nutrients (5). Biofilms naturally go through multiple stages of initial attachment, biofilm maturation, and dispersal (1). The cyclic di-GMP secondary messenger stimulates formation of biofilms (6). Many bacteria found in water systems contain this messenger, so there will be many biofilms in the systems. Biofilm dispersal is initiated when bacteria require more nutrients or sense certain environmental signals. The dispersal stage can be induced by multiple factors including biochemical signals and nutritional deficits. Parts of a mature biofilm can be regularly detached by normal flow effects within the environment (1).

Legionella colonize existing biofilms containing species including *Escherichia coli, Acinetobacter baumanii,* and *Flavobacterium breve* (1). These species are commonly found in the same aquatic environments as *Legionella*, so can be used for *Legionella* biofilm initiation (1). Certain bacterial interactions have been shown to promote adhesion of new species to a biofilm. Monospecies biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumonia* have been shown to inhibit colonization of biofilms by *Legionella* (1). The makeup of biofilms play a role in if *Legionella* can persist in a system.

Bacterial attachment to biofilms is influenced by the material used to construct a water system. *Legionella* show higher attachment to biofilms formed on PVC and wood as compared to copper (7). The presence of shock absorbers and rubber stoppers also created niches for biofilm growth, increasing the contamination by *Legionella* (8)

The *Legionella* species itself may influence colonization of other *Legionella* species. *L. pneumophila* produces a biosurfactant. Biosurfactants often have antimicrobial properties, and the biosurfactant produced by *L. pneumophila* has been shown to have inhibitory effects on the growth of other *Legionella* species (9, 10). The production of the biosurfactant could not be linked to any another part of the *Legionella* life cycle, so likely it is used as a means to reduce species competition. Man-made water systems are typically low-nutrient environments. *Legionella* use the biofilms in these systems as a means of persistence and survival until they can find a host. Biofilms are often implicated as a source of pathogens causing various human infections and also as a source of disease (11, 12).

HOSTS

Biofilms provide a mechanism for persistence of *Legionella,* where they employ necrotrophic growth to gain needed nutrients, and as long as there are at least 100 dead bacteria/*Legionella,* the *Legionella* can survive (13). But *Legionella* are believed to be unable to replicate as part of a biofilm. *Legionella* replicate by parasitizing various types of protozoa. These protozoan hosts serve as the replication niche for *Legionella.* Many

are commonly found in the same water systems and environments as *Legionella* (14). The degree of replication differs between bacteria and protozoan species though. In amoebae – a preferred host – *Legionella* resist fusion with the lysosome and form a *Legionella* containing vacuole (LCV). Within the vacuole, the *Legionella* recruit and use host nutrients until exhausted, at which point the host is lysed and the bacteria are released (15). Replication in this manner can result in released *Legionella* within the water column. If the water systems then aerosolizes the water, this creates an exposure risk for humans upon inhalation of the bacteria contaminated water aerosols. In some cases, *Legionella* will interact with a host and be ingested, as they are unable to avoid the initial phagocytosis response of the host, so the LCV is fused with the lysosome and the bacteria are degraded. In other cases, the *Legionella* will be ingested by a host and be able to lyse the amoeba, but little to no replication of the bacteria will have occurred. For example, the ciliate host *Tetrahymena tropicalis* ingests *Legionella,* where is stays in food vacuoles until being released without having replicated (16).

Many different genera of protozoa can act as hosts to *Legionella,* including *Naegleria, Vermamoeba, Tetrahymena,* and *Cylcodium,* but of the most commonly studied genera *Legionella*-host interactions is *Acanthamoeba. Acanthamoebae* species are commonly considered soil-dwelling amoebae that also frequently inhabit aquatic systems. They are often found in man-made water systems and are part of the free living amoebae (FLA) group of protozoa (17). *Acanthamoebae* are between 15 and 35 μm in size and naturally graze on various species of bacteria as a food source.

Interactions of *Acanthamoebae* species with *Legionella* end in the lysis of *Acanthamoebae* species and the amplification of the *Legionella* (18). When *Legionella* infect the lungs they invade and replicate within alveolar macrophages just as they would in an amoebae. Alveolar macrophages are similar to amoebae so they are able to act as hosts for *Legionella* (19).

Legionella infectivity is typically assessed based on the ability of the bacteria to invade and replicate within a particular host cell (20). Only transmissive (stationary phase) bacteria are capable of causing infection. These bacteria are characterized by the presence of flagella unlike the exponential phase bacteria which are non-flagellated and susceptible to degradation if engulfed by phagocytic cells (21). Bacteria that are either planktonic and aerosolized or contained in an amoebae can be inhaled into human lungs. These free swimming stationary phase bacteria are also capable of invading new host cells where they continue to proliferate in the water column (21). This proliferation will depend on which host the *Legionella* interacts with. The study of planktonic phase *Legionella* has clearly delineated transmissive, infectious *Legionella* from exponential, replicative bacteria but biofilm bacteria have been more difficult to fit into this pattern.

INTERACTION

Biofilms serve as a common food source for these free-living amoebae. Since both the amoebae and the bacteria reside with man-made water systems, it is natural to assume that contact is made at the biofilm surface. *Acanthamoebae* will graze the

biofilm and ingest the bacteria. The bacteria will then replicate and lyse the host. *Legionella* released from a host can then aggregate on the surface of existing biofilms. Planktonic culture *Legionella* will colonize a biofilm surface, but they tend not to aggregate together. During biofilm formation, the amoebae grown *Legionella* release more polysaccharides than the culture grown, so aggregated growth would be advantageous (22). If the *Legionella* exist on the surface of biofilms, they will be at a high risk of amoebae grazing. While most bacteria acquired through grazing become food for the amoebae, *Legionella* avoid the normal phagocytosis and use this as a replication opportunity (23). However not all amoebae take up all *Legionella*. *Willaertia magna* resists the cytotoxic effects of internalized *Legionella* and inhibits its replication (24).

Many potential amoebae hosts for *Legionella* exist in man-made water systems, but most have not been characterized for any amplification potential of various *Legionella* species. *Legionella* are currently known to replicate within at least 23 different kinds of protozoan hosts, including 20 amoebae, two ciliates, and one slime mold (15). The combination of over 60 *Legionella* species and 25 different hosts presents the possibility of significant variability in the host-parasite interaction. Not all species of *Legionella* will likely be able to replicate in all 25 hosts, as evidenced by published works (24).

LEGIONELLA RISK

During known or suspected outbreaks of LD, the CDC and its affiliates work to identify the source of the outbreak as well as the strain of *Legionella* responsible for the infection. Samples are collected from both the patients and the aquatic and soil environments surrounding the area in which the outbreak took place. The DNA from these isolates is then sequenced and stored to keep a record, not only of which *Legionella* species are causing disease, but which *Legionella* species are in the environment and in what habitat they are persisting.

Survival and persistence of *Legionella* in man-made water systems such as heating and cooling towers is one of the reasons for the number of LD outbreaks. Over 160,000 such systems exist in the United States alone (26). Each system serves as an environment for *Legionella* to survive and replicate, and with the continued installation of more and more man-made systems, there is an increasing exposure risk to humans. Humans are however a dead end host. *Legionella* is transmitted through aerosols from the environment into human lungs, but is not usually considered passable between humans. There has only been one reported case of LD spread between humans, and it occurred in a non-ventilated, small space and involved a patient with severe LD having close contact to another person (27) Without these specific conditions, *Legionella* would not likely be transmitted between humans.

Most cases of LD in the United States are caused by a single species, *L. pneumophila*, therefore, most studies on *Legionella* are conducted with various strains of this species. However more than 60 identified species of *Legionella* have been found in the environment, and at least half of these are known to have the ability to cause disease in humans (28). *L. pneumophila* is mentioned in over 5400 articles, while *L. anisa* and *L. gormanii,* two *Legionella* species implicated in fewer cases of LD, are mentioned in less than 100 research papers each. *L. anisa* and *L. gormanii* are both virulent, though both are more commonly found as environmental samples. *L. anisa* has been implicated in cases of LD, but can be difficult to grow using standard culture techniques (29). Many illnesses caused by *L. anisa* result in the milder form of the pneumonia, Pontiac Fever, making it possible that this species of *Legionella* is responsible for many undiagnosed cases of Pontiac Fever (30). *L. gormanii* has also been implicated in cases of LD, both alone and as a co-infection with *L. pneumophila* (31). While *L. gormanii* has not often been found as the causative agent of LD cases, but it could be co-infecting with *L. pneumophila* on a more regular basis.

The species that is implicated in the second highest number of LD cases, specifically in the southern hemisphere, is *L. longbeachae* (32). In Australia and New Zealand, from 30-80% of the cases of LD are caused by *L. longbeachae* (33). One possible reason for the increase of *L. longbeachae* LD cases in Australia and New Zealand is the make-up of their potting soil. Australians tend to use pine waste products for soil, such as sawdust and hammer mill bark, which could supply a more suitable

home for *L. longbeachae* (34). The cases of LD caused by *L. longbeachae* also show a spike in spring, when gardens are planted and people have more interaction with potting soil. Avid gardeners are often seen as the patients in the cases of *L. longbeachae* LD pneumonia (35). A majority of these infections were linked to hanging plants, in which dripping and aerosolized water containing *L. longbeachae* was able to infect humans (33).

The number of *L. longbeachae* LD cases in Australia exhibit that soil dwelling species of *Legionella* also readily infect humans. In the case of *L. gormanii,* another soil dwelling species, soil aerosolization could potentially be an avenue for infection. Multiple species of *Legionella* have been isolated from soil, and have been present in sampled air during soil manipulation (36). While in Australia many cases of LD in specific areas are attributed to *L. longbeachae,* worldwide the majority of cases are still caused by *L. pneumophila.* So *L. pneumophila* is used for the majority of the *Legionella* research. Using one, or even two, species as the basis of knowledge for an entire genus creates gaps in the understanding of *Legionella*-host interactions. This is turn makes it difficult to assess the true amount of LD cases caused by non-*pneumophila* species

The environment in which the different species are found may affect the prevalence of LD in the human population. *L. pneumophila* is found primarily in aquatic environments, while *L. longbeachae* more commonly resides in soil. The genome of *L. longbeachae* shows adaptations unique for a soil environment (37). Other species of

Legionella may have the same preferences, but a majority of *Legionella* species have yet to have their genomes sequenced. *Legionella* species adapted to soil environments would have different interactions with amoebae dependent on which amoebae species are present in the soil. Aquatic environments have different temperature and pH conditions than soil environments, so *Legionella* would be required to infect in varying conditions. Expression of a capsule and diverse metabolic processes by bacteria make the soil environment more favorable, as evidenced by *L. longbeachae* (37). Certain environments could be more favorable to specific bacteria and amoebae, therefore a host-bacteria interaction would be more likely to occur. Understanding the interactions of *Legionella* and its preferred amoebae host could also help improve preventative risk assessment in the efforts to prevent LD.

The amount of biofilm and the number of protozoan hosts in a system correlate positively with each other (38). With significant numbers of hosts in the environment having sufficient biofilms to graze on, interactions between *Legionella* and appropriate host cells are likely. This positive correlation leads to more interaction and therefore more risk, which requires a higher need to detect that risk. While *Legionella* can survive for weeks as a member of a biofilm, they survive much longer when amoebae are also present (39). *Legionella* numbers in water system biofilms have been shown to increase significantly in the presence of *A. castellanii* (40). This creates a significant health risk as there are various types of protozoa living in water systems, and *Legionella* have so many potential hosts (41).

The number of LD cases has increased in the US 217% between 2000 and 2010 (42). An increase in the number of man-made water systems, along with an increase in the human population and a change in climate has led to an increase in water systems and usage of them, which in turn increases the chance of *Legionella* interacting with humans. Most man-made water systems in the United States contain detectable levels of *Legionella,* suggesting that even if this level is currently below the acceptable risk level, it could develop into a problem. If *Legionella* is in the water system, the potential that the bacteria could replicate within, and be released from, its host into the water column exists. This would exponentially increase the risk of human inhalation and infection. This water and any organisms within it has the potential be aerosolized, leading to the potential for infection. Between 2009-2012, there were a reported 51 outbreak caused by *Legionella,* with 302 patients and 30 fatalities (43). The current increase of outbreaks and cases demonstrates a need for an improved risk assessment tool focused on LD in man-made water systems.

Water systems are disinfected on a regular basis, but *Legionella* continue to persist in the same towers, so identifying which water systems pose a threat is important for knowing where to focus intensive cleaning efforts. A recent outbreak of LD in New York infected 128 people, killing 12, all of whom were immunocompromised adults (44). The suspected source of the outbreak stemmed from was identified and disinfected per the New York regulations at the time. Within two months, another outbreak started in New York that was tracked again to the same water tower. This led

to more stringent testing regulations required for all water systems in New York. This is an example of the common persistence of *Legionella* in water systems despite the fairly regular cleaning and removal of biofilms.

When *Legionella* detach from the biofilm, they can end up inside the water column. *Legionella* take advantage of biofilm dispersal as a means to move within the water system. Protozoa can take up planktonic bacteria from the water column or graze bacteria from a biofilm. LD can be caused by the inhalation of *Legionella* that have been detached from biofilms in man-made water systems or from inhaling infected amoebae. Since *Legionella* grow as biofilms in water systems, understanding the interaction between biofilm and host is imperative in understanding the virulence of the genus. *Legionella* in a biofilm do not express flagella, so would - by the typical characterizations of the genus - be considered avirulent (45), but biofilms have been shown to be more virulent within a host than planktonic bacteria. Replication of *Legionella* within murine macrophages is significantly higher with biofilm bacteria as opposed to planktonically grown *Legionella* (46). This increased virulence seen from biofilm bacteria after host invasion could allow for more favorable interactions of the *Legionella* with more host species.

After passage through a macrophage, bacteria have shown an increase in virulence and lethality (46, 47). If *Legionella* show increased virulence after passage through a host, this would increase the risk caused by water systems. All *Legionella* in

water systems have the ability to cause disease in humans given the right exposure and susceptibility. Therefore, regular disinfection of water systems is done to attempt to remove the bacteria. But current methods remove *Legionella* from only the water column and the tops layers of biofilms. This leaves the persisting biofilm to continue replication and interaction with hosts, maintaining the initial risk of the water system (48). The persistence of biofilms containing *Legionella* along with the increased host interactions and virulence of the *Legionella* in those biofilms confirms the need for a way to better determine risk and treat contaminated water systems.

Recent work in our lab tested eight water towers for the presence of *Legionella.* We have shown that some species of *Legionella* was present in all samples. Within the samples collected, the amount of *Legionella* varies. *L. pneumophila* was found in all samples taken from eight water towers at the Savannah River site, but the amount of *L. pneumophila* – measured by qPCR – ranged from 0.003 – 80.2% of the sample. These samples spread over the course of a year and multiple samples were taken from each site during various seasons. Despite the small sample size, these findings demonstrate that *Legionella* is truly ubiquitous in man-made water systems and that its presence persists over time. Other studies show that *Legionella* is detected in >40% of samples taken, with over 90% in hospital samples (49, 50). The persistence of *Legionella* in water systems is a large problem. Even after disinfecting, *Legionella* are often found in water systems, and this could in part be due to the combination of bacterial persistence in biofilms, along with the host interactions that allow for further replication.

The recent LD outbreaks in New York led to the installment of new regulations for water systems in the city. These regulations state that each water tower will be tested twice a year for the presence of any *Legionella* species. If any level of the bacteria is detected, intense cleaning protocols must be followed to remove the bacteria (51). The decision to implement this strategy is problematic in a few ways. There approximately 15000 water towers in New York, so the testing alone will be immensely expensive. Also, it is very likely that every test will find some number of *Legionella.* Washing the systems will do little to prevent the problem.

Cleaning using the current methodologies does not entirely remove *Legionella* from water systems. It clears the *Legionella* out of the water column and removes the top layers of the biofilms that are consistently present. The biofilms are never fully removed though so they continue to regrow and the *Legionella* returns. Removal of the amoebae from water systems could be an alternative solution to this problem. The use of "probiotic amoebae" could allow for improved control of *Legionella* numbers within systems. If an amoebae, for example *W. magna,* could inhibit *Legionella* growth*,* and it could outcompete *Legionella* permissive species like *Acanthamoebae,* there could be a reduction in the amount of *Legionella* in a system. A French company is currently trying this probiotic amoebae protocol, with some success (24). After characterization of host interactions between amoebae and *Legionella*, there will be certain interactions that reduce the number of *Legionella* found in the system (24). In this case, these amoebae can be added to water towers. They will then graze on the biofilms and reduce the

presence of *Legionella* instead of amplifying it as many amoebae do. Identification of bacterial and amoebae species in a specific water system, knowledge of how those particular species interact, could provide means to assess the risk of individual water towers. When *Legionella* is highly amplified in a host and consistently ends up in the water column, there would be a higher risk for human infection. Conversely, if a host reduced *Legionella,* the risk of that specific water system would be lower.

Predictive risk assessments are currently used for a myriad of situations. Businesses use a risk assessment when looking into their finances and making decisions for a company like making investments and outcompeting business rivals. This practice includes using past and current information to forecast the outcome of future events, including predicting financial trends and making plans. Risk assessment is used for chemistry and the specific hazards of chemicals being used. Depending on the chemicals involved, a risk factor is calculated for each reaction. One problem with this calculation is that these assessments include only the two chemicals being mixed. There is no inclusion of any substrates the chemicals are in, catalysts, or environmental factors such as temperature or pressure (52). Risk assessments are commonly used for testing microbiological risks for food safety. Bacteria are grown in a variety of conditions and a mathematical equation is developed to estimate the number of bacteria that would be present under such conditions. Pathogens such as *Listeria* and *Salmonella* are some common organisms that this risk assessment looks for. This equation can then be used in future food processing conditions along with a handling assessment to discover the

risk factor of certain foods (53). An improved *Legionella* risk assessment will be more useful in detecting unsafe water systems.

Current American Society for Heating, Refrigerating, and Air Conditioning Engineers (ASHRAE) guidelines require that any building with a water system report the set-up of the system, including the flow of water. They must also have plans in place for starting and disinfecting the system before turning it on. Furthermore, any testing for *Legionella* must be done in an accredited lab (54). While these guidelines are very comprehensive in regards to checking for *Legionella* in a system, there is no mention of other bacteria or hosts which would play a role in persistence and replication that the *Legionella* may interact with. Since certain species of bacteria can either promote or inhibit biofilm colonization of *Legionella,* this would be an important factor to consider. Adding this information to a *Legionella* predictive risk assessment can make it much more useful, as the amplification of *Legionella* in water systems along with *Legionella* persisting as part of a biofilm are of major concern in regards to *Legionella* persistence and potential infection. To add this kind of information to the assessment requires a better understanding of the interactions of the bacteria and host.

Based on the lack of effective cleaning procedures for man-made water systems, an incomplete predictive risk assessment, and a concentration on one of over 60 species of *Legionella*, there is a continual problem of LD. If *Legionella* host interactions were better characterized, a more precise predictive risk could be implemented. We

therefore investigated the interaction of five *Legionella* species (one *pneumophila* and four non-*pneumophila*) with two species of *Acanthamoebae.* These experiments will expand the understanding of varying host interactions, and begin to fill the gap of knowledge regarding non-*pneumophila* species. We postulate that the non-*pneumophila* species will interact with the *Acanthamoebae* species differently than *L. pneumophila*. To characterize the host interactions, *Legionella* were grown planktonically or as a biofilm and then the bacteria were exposed to *Acanthamoebae* species. The same assays were repeated using amoebae grown *Legionella* to check for increased virulence after passage through a host.

Each year, we have seen a steadily increasing number of outbreaks and cases of LD (55) and the CDC reports 80% of LD cases likely go undiagnosed annually (56). The current diagnostic test for *Legionella* requires the use of a urinary antigen test which strictly test for *Legionella* serogroup 1. But the gold standard for *Legionella* still requires growth on and isolation from Buffered Charcoal Yeast Extract (BCYE). Growth of *Legionella* on BCYE takes a minimum of three days, so can be slow in clinical cases when a diagnosis is needed. When using either diagnostic test, non-*pneumophila* species of *Legionella* are often missed. While some of these cases are likely missed because the patient either improves or passes away, there is also a chance that the bacteria is never cultured on the proper growth media, so the cause is never determined to be *Legionella.* Some LD cases are undiagnosed because they are caused by non-serogroup 1 *Legionella* species, and so they are not detected using the regular tests. An improved

diagnostic test could lead to fewer undiagnosed cases of LD, and better annotate which *Legionella* species are actually causing disease (57). This focus on one species, *L. pneumophila,* leaves significant gaps in the knowledge base of *Legionella* as a whole*. L. pneumophila*, while prevalent, does not necessarily have the same characteristics as the rest of the genus, but researchers have generalized the genus based on *L. pneumophila* information. We hypothesize that the four experimental non-*pneumophila* strains will exhibit different host interactions than *L. pneumophila* with *Acanthamoebae* species. Our use of less characterized non- *pneumophila* strains will enhance the understanding of the genus by uncovering host-pathogen interactions between various amoebae hosts and *Legionella* species.

Materials and Methods

Growth and Cultivation of *Legionella* species

Legionella species used in this study were *Legionella pneumophila* Philadelphia 1 (ATCC 33152), *L. clemsonensis* (CDC D5610)*, L. anisa* (CDC 4252)*, L. gormanii* (CDC NAV11-1-55c1)*,* and a novel strain, D4482. All strains except *L. pneumophila* Philadelphia 1 were acquired from Dr. Claressa Lucas at the Centers for Disease Control (CDC). Isolates were collected during routine sampling of known cases of LD. *L. pneumophila* Philadelphia 1 is the type strain for the genus and was used as a control for all experiments (Table 1). *L. clemsonensis* was collected from a bronchial wash from a pneumonia patient in Ohio. *L. anisa,* also a clinical isolate, was collected from a lung

sample of a patient in Minnesota. *Legionella* strain D4482 and *L. gormanii* were both acquired from environmental samples. *L. gormanii* was isolated from a water cistern on a Navajo reservation, while D4482 was collected from an unknown environmental location. All species were grown on Buffered Charcoal Yeast Extract agar (BCYE, recipe in Appendix I) at 35°C and 5% CO_2 for 3 days prior to use in experiments, except *Legionella* strain D4482 which required 5 days of growth before use. Biofilm cultures were grown in ACES Buffered Yeast Extract (AYE, recipe in appendix I) and incubated at 35°C with 5% CO_2.

Growth and Cultivation of Amoebae Species

Acanthamoeba polyphaga and *A. castellanii* served as amoebae host cells (Table 1). *Acanthamoebae* species were grown and maintained at 35°C with 5% CO_2. *A. polyphaga* was grown in Tryptic Soy Broth (TSB) while *A. castellanii* was grown in Peptone Yeast Glucose broth (PYG, recipe in Appendix I). All amoebae were cultivated in 25 cm^2 cell culture flasks with vented caps to allow oxygen into the system. Amoebae were continuously passed every 2-5 days throughout the course of the experimentation as the amoebae reached 80% confluency. Spent media was removed from the amoebae flasks and replaced with 2 mL of the appropriate media. The flasks were then tapped to dislodge the amoebae from the side of the flask for collection, passage, and use in experiments.

Table 1. List of bacteria and amoebae used for all experiments. *L. pneumophila* Philadelphia 1 was used as the control for all experiments. For each set of experiments, each *Legionella* species was interacted with each amoebae species.

Bacteria species	Amoebae species
L. pneumophila Philadelphia 1	*A. polyphaga*
L. clemsonensis	*A. castellanii*
Legionella strain D4482	
L. gormanii	
L. anisa	

Amoebae infections

Amoebae infections were conducted as previously described (58, 59). Briefly, after collection from flasks, amoebae were centrifuged (129 g, 12 minutes) and resuspended in the appropriate media. Amoebae were then counted using a hemocytometer and live/dead cell counts were determined by trypan blue assay. Amoebae were added to two six wells plates at a concentration of $1x10^5$ amoebae per well and media was added to a final volume of 3 mL per well. The plates were incubated overnight. For invasion and replication assays, amoebae monolayers were washed once with Phosphate Buffered Saline (PBS) to remove any non-attached amoebae. *Legionella* strains were resuspended from a BCYE plate to an OD of 0.12 in sterile PBS. This OD is roughly equal to a bacterial concentration of $1x10^8$ bacteria/mL. Actual concentrations of all bacterial suspensions were determined by dilution plating on BCYE for each experiment. *L. pneumophila* Philadelphia 1 served as a control in all experiments. Approximately $1x10^8$ *Legionella* (control or experimental) were added to two of the wells on the six well plate. Each well plate was centrifuged (50 g, 10 minutes) to increase

contact of the bacteria with the amoebae and then incubated at 35°C with 5% CO_2 for 2 hours.

Planktonic Invasion and Replication Assays

After the six well plates were incubated for 2 hours, both plates were washed three times with sterile PBS to remove extracellular bacteria. Three mL of the appropriate media was added to each well of one of the plates and that plate was incubated for a further 46 hours, for a total of 48 hours. This plate was used to assess replication ability. To the first plate, 1.5 mL of PBS was added to each well and the amoebae were removed from the bottom of each well using a cell scraper. The amoebae were collected, centrifuged (129 g, 8 minutes), and resuspended in 500 µL of sterile PBS. Amoebae were lysed by passage through a 31G syringe 4-5 times to release the *Legionella*. *Legionella* CFU/mL were calculated by dilution plating on BCYE. At 48 hours the supernatant of each well of the second plate was collected. 2 mL of PBS was then added to each well and the amoebae were removed with a cell scraper and added to the corresponding supernatant. These suspensions were then centrifuged (3220 g, 12 minutes) and resuspended in 1 mL of PBS. Amoebae were lysed using the syringe and CFU/mL of *Legionella* were determined as described above.

Amoebae-biofilm interaction assays

As previously described in Raftery et al. (60), *Legionella* biofilms were set up on slides in glass petri dishes. *Legionella* strains were suspended in ACES buffered Yeast

Extract (AYE) to an OD_{600} of 0.600. Four mL of each bacterial suspension were added to two individual glass petri dishes containing sterile glass microscope slides. Twenty mL of 10% AYE was added to each dish. The slides were incubated at 35°C and 5% CO_2 for 24 hours to allow for biofilm establishment. At 24 hours, the 10% AYE media was exchanged for 20 mL of 100% AYE. The biofilms were then further incubated for four days to produce mature, well-established biofilms. On day five, biofilms were washed once with Moderate Hard Water (MHW), and then 20 mL of MHW was added to each petri dish along with $1x10^6$ amoebae. The MHW was used to prevent further growth of both the amoebae and the bacteria. Amoebae were collected from flasks as described above and resuspended in 3 mL of MHW prior to addition to the biofilm. Control biofilms received no amoebae as a way to calculate a baseline of biofilm surface area. Amoebae and biofilms were incubated for 48 hours at 35°C and 5% CO_2.

To assess grazing, supernatants were removed from each biofilm and added to separate 50 mL conical tubes. The biofilms were then gently washed twice with 10 mL of MHW, each time the wash being saved in the respective tube. 10 mL of MHW were then added to each of the biofilms which were then placed on ice for 20 minutes to release any remaining amoebae from the slide. After incubation, this MHW wash was then added to the rest of the collected supernatant. The collected MHW was centrifuged (3220 g, 12 minutes), and the amoebae were resuspended in 500 µL of MHW and number and viability were determined using a trypan blue assay and compared to the initial number of amoebae that had been added to the biofilm.

After removal of amoebae, the slides were removed from the petri dishes and allowed to air dry. Slides were fixed in methanol for 10 minutes and again air dried. The slides were then stained with Giemsa stain (2.5% of Giemsa solution in Ultra-Pure Water, EMD) for 20 minutes. At that time, the stain was gently washed with water and the slides were dried and stored at 4°C until imaged. Images were taken on a Nikon Eclipse E600 at 400x magnification. The total surface area of the biofilms was then calculated using COMSTAT software (61). Three images were taken of each biofilm slide.

Amoebae reinfection assays

For reinfection assays, extracellular *Legionella* were collected at the 48 hour time point from an initial invasion assay (diagrammed below, Figure 1). These bacteria were then added into new 6 well plates containing amoebae monolayers. The plates were centrifuged (50 x g, 10 minutes) and invasion and replication assays were then conducted as described above. To assess the concentration of the *Legionella* transferred

to the new wells, 100 µL of the supernatant was removed from each of the initial 48 hour plate wells, diluted and spot plated to calculate CFU/mL.

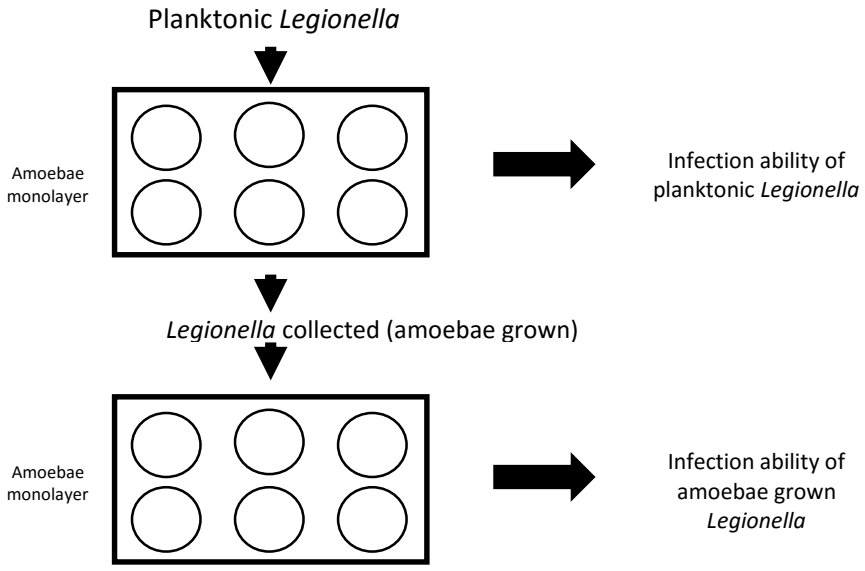

Figure 1. Amoebae reinfection protocol. Planktonic *Legionella* were added to an amoebae monolayer. At 48 hours, the *Legionella* were collected from the amoebae and reinfected into a second amoebae monolayer and invasion and replication were again calculated.

Amoebae grown biofilm assay

Biofilms were established according to the above protocol. Amoebae were then added and allowed to graze those biofilms. After 48 hours of grazing, the amoebae were collected from the biofilms. The amoebae were centrifuged (3220 x g, 12 minutes). The collected amoebae were then resuspended in 5 mL AYE. Amoebae were then lysed using a 31G syringe and the resulting suspension containing *Legionella* was split between two glass petri dishes with sterile glass slides inside. 20 mL 10% AYE was added to each dish. The biofilm was incubated for 5 days as described above. The same amoebae species in

which the bacteria grew was again added after biofilm maturation to determine grazing efficiency (Fig. 2).

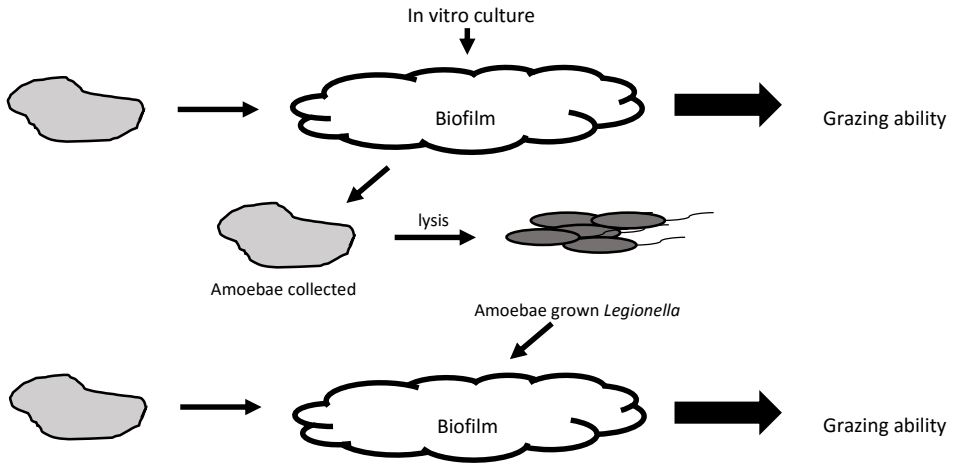

Figure 2. Amoebae grown biofilm protocol. Biofilms were grown from a planktonic culture and amoebae were allowed to graze. After grazing, amoebae were collected and the *Legionella* were isolated. These amoebae grown biofilms were then used to grow second generation biofilms which were then grazed by amoebae.

Quantitative analysis of biofilms

Each image taken from the microscope was analyzed using COMSTAT software as previously described (61). For each image, a threshold was adjusted manually to match the original image. This threshold provided a three-dimensional matrix to be quantified. The surface area of each image was then calculated using the software. Each biofilm slide had three images taken, and the average of the three images was calculated. Each control biofilm slide was then quantitatively compared to the biofilm-amoebae grazed slide.

Statistics

Students T-test was used when comparing two specific *Legionella* strains or two amoebae strains. Comparisons between all *Legionella* species were done using ANOVA performed on SAS studio. A p-value of 0.05 was used to determine significance for all experiments.

Results

Planktonic Invasion Assessment

Invasion into host species is imperative for the replication of *Legionella. L. pneumophila* invasion ability has been well characterized in both *Acanthamoebae* species used here (15, 62, 63), but a direct comparison of the invasion and replication potentials has never been completed. Percent invasion for each experiment was the number of *Legionella* from the added inoculum that successfully invaded the host. *L. pneumophila* Philadelphia 1 invaded *A. castellanii* at significantly higher percentages (13.4%) than into *A. polyphaga* (4.7%) ($p<0.05$). The invasion potential of the non-*pneumophila* species has not been well characterized, and both *L. clemsonensis* and *Legionella* strain D4482 are novel species with no studies having been completed on their ability to invade amoebae hosts. In both *A. castellanii* and *A. polyphaga,* all non--*pneumophila Legionella* species displayed similar invasion abilities ($p>0.05$) (Fig. 3). ANOVA analysis showed that across the board, the non-*pneumophila* species of *Legionella* showed similar invasion abilities into each host, with approximately 5% of the

initial bacterial suspension added to the amoebae actually invading into the host cell after 2 hours (p>0.05). Within *A. castellanii, L. pneumophila*'s invasion ability was higher than all of the non-*pneumophila* species in that host. *L. pneumophila* invasion was significantly higher than that of *Legionella* strain D4482 within *A. castellanii* (p<0.05). In *A. polyphaga* – a less commonly studied host for *Legionella* – *L. pneumophila* invasion rate was equivalent to other *Legionella* species (p>0.05). Comparison of non-*pneumophila Legionella* species between the two *Acanthamoebae* hosts also showed no significant differences in invasion ability. The lowest amount of invasion was seen by *Legionella* strain D4482 into *A. castellanii.* Less than 2% of the bacteria added to the amoebae invaded the host cell.

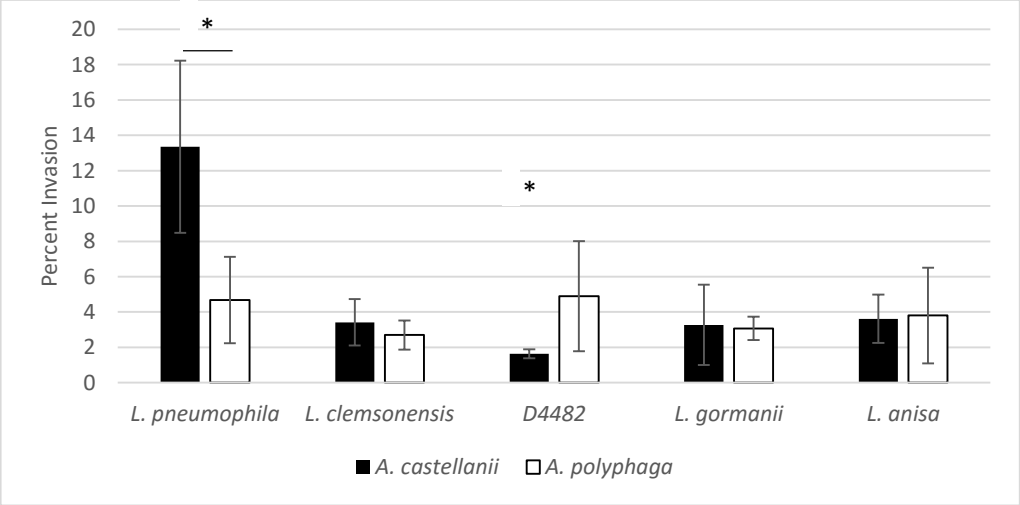

Figure 3. Comparison of invasion ability of various *Legionella* species into both *A. castellanii* and *A. polyphaga. L. pneumophila* showed significantly higher invasion ability into *A. castellanii* than into *A. polyphaga* (p<0.05). The non-*pneumophila* species all showed similar invasion ability into both amoebae hosts. Clinical isolates are patterned, environmental isolates are striped.

The two clinical isolates of *Legionella, L. clemsonensis* and *L. anisa,* showed reduced invasion compared to *L. pneumophila* Philadelphia 1 in both species of *Acanthamoebae.* Less than 3.5% of *L. clemsonensis* cells invaded the *A. castellanii* host and *L. anisa* invaded at 3.7% (Fig. 3). The environmentally collected isolates, *L. gormanii* and *Legionella* strain D4482, displayed reduced invasion ability into *A. castellanii* as compared to *L. pneumophila* as well, invading the host at less than 5% (Fig. 3). No difference in invasion ability was observed between the clinically collected isolates to the environmentally collected isolates ($p>0.05$).

Planktonic replication assessment

Like invasion ability, the capability to replicate inside a host is also vital for proliferation of *Legionella.* The replication ability of each of the five *Legionella* species was measured in the two *Acanthamoebae* species (Fig. 4). Replication of the bacteria inside a host allows for amplification of the bacteria creating a potential exposure risk for human infection if this occurs in man-made water systems. Replication abilities of *L. pneumophila* were equivalent in both amoebae hosts ($p>0.05$). *Legionella* strain D4482 showed higher fold replication within *A. castellanii* than within *A. polyphaga,* with over a 500 fold replication in *A. castellanii* as compared to less than a 1 fold replication in *A. polyphaga.* This was the only instance in which there was a difference between the host species for any non-*pneumophila* species of *Legionella. Legionella* strain D4482 also demonstrated a higher fold replication in *A. castellanii* than *L. pneumophila,* even after a significantly lower invasion rate.

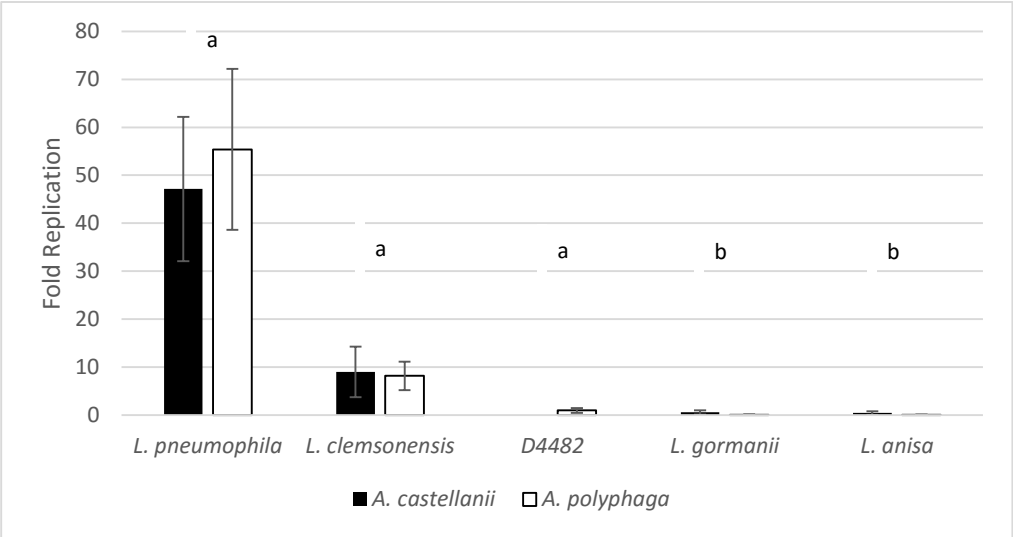

Figure 4. Replication ability of *Legionella* species in both *A. castellanii* (A) and *A. polyphaga* (B). There were no significant changes in replication of *Legionella* species between hosts. *L. pneumophila* had significantly higher fold replication than both *L. gormanii* and *L. anisa* in both *Acanthamoebae* hosts ($p<0.05$). n=3

Clinical and environmental isolates were again compared, this time for replication. Within *A. castellanii, L. pneumophila* showed significantly higher replication ability than the clinical *L. anisa* isolate (Fig. 4)*. L. clemsonensis* had a 9 fold increase over 48 hours in *A. castellanii*, and an 8.2 fold replication in *A. polyphaga*, showing no significant change between the two hosts, and also showing reduced replication compared to *L. pneumophila* in both species (*A. castellanii:* 47.1, *A. polyphaga:* 55.4*)* ($p>0.05$). *L. pneumophila* also showed higher replication in *A. polyphaga* as compared to these two clinical isolates, again being significantly more than *L. anisa* ($p<0.05$). Both *L. gormanii* and *L. anisa* demonstrated extremely limited replication in both *Acanthamoebae* species with less than 0.5 fold increase over 48 hours. This was significantly less than *L. pneumophila* ($p<0.05$).

Amoebae-Biofilm interaction

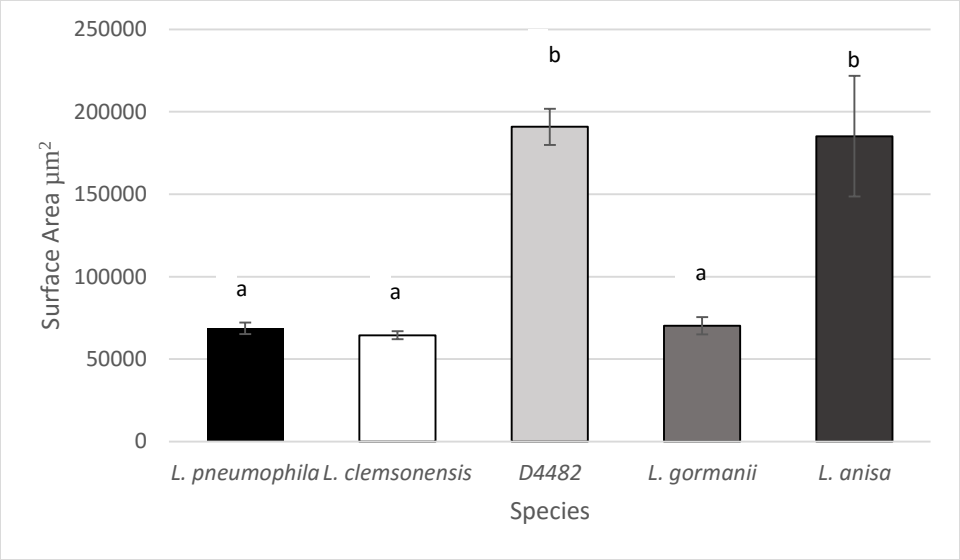

Figure 5. Biofilm surface area of all five *Legionella* species. *L. pneumophila, L. clemsonensis,* and *L. gormanii* were similar in surface area, and were all significantly less than *Legionella* strain D4482 and *L. anisa* ($p<0.05$).

Typically, amoebae – *Legionella* interactions have been studied by adding the bacteria to amoebae monolayers. However, in the environment, the interaction likely occurs with the bacteria on the surface as a biofilm and the amoebae contacting the bacteria from above. We established an amoebae grazing assay to determine if this difference affected *Legionella*-amoebae interactions. To test the preference of a grazing host for specific *Legionella,* we grew biofilms of the *Legionella* and calculated the surface area removed by each *Acanthamoebae* species. In our standard biofilm growth assay, *L. pneumophila* biofilms covered similar amounts of surface area as both *L. clemsonensis* and *L. gormanii,* while covering a significantly smaller surface area than

both *Legionella* strain D4482 and *L. anisa* biofilms ($p<0.05$) (Fig. 5). When amoebae were added to the biofilms, *A. castellanii* removed equal amounts of surface area from all *Legionella* biofilms ($p>0.05$) (Fig. 6). *A. castellanii* grazed on average 30% of the biofilms in 48hrs, with *L. anisa* being grazed least by *A. castellanii*, having only 12.8% of the surface area grazed.

A. polyphaga grazed *Legionella* biofilms equally with an average of approximately 35% of each biofilm being grazed in 48 hours (Fig. 6). In comparing biofilm grazing ability of the two amoebae species, both *Acanthamoeba* species showed similar grazing activity on *L. pneumophila, L. gormanii* and *Legionella* strain D4482 biofilms (~30% for all species), but *A. castellanii* consumed less than *A. polyphaga* of both *L. anisa* (*A. polyphaga:* 47.1%, *A. castellanii:* 12.8%) and *L. clemsonensis* biofilms (*A. polyphaga:* 35.8%, *A. castellanii:* 21.8%) ($p<0.05$).

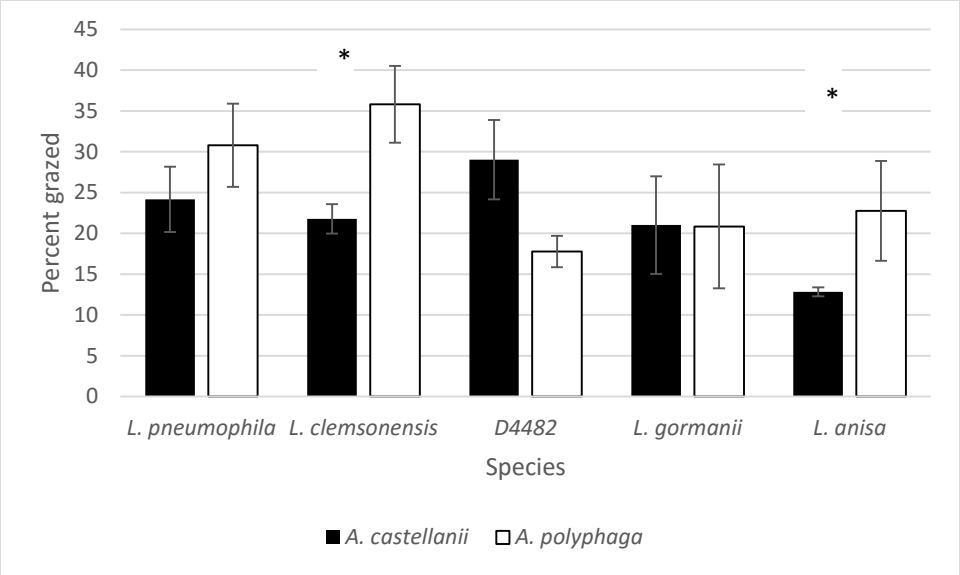

Figure 6. The percentage of biofilms of all *Legionella* species grazed by *A. castellanii.* Similar grazing occurred between both hosts except in the case of *L. clemsonensis* and *L. anisa,* both of which were grazed significantly less by *A. castellanii* ($p<0.05$).

Amoebae viability

If *Legionella* are effectively replicating within an amoeba host, lysis of the amoeba will eventually occur, therefore amoeba loss can be correlated to the ability of the *Legionella* successfully using an amoebae species as a host for amplification. Of the initial $1x10^6$ *A. castellanii* added to the biofilms, less than 25% survived grazing, regardless of *Legionella* species (Fig. 7). Grazing on *L. anisa* resulted in slightly higher, but not significant, amoebae survival compared to other *Legionella* species ($p>0.05$).

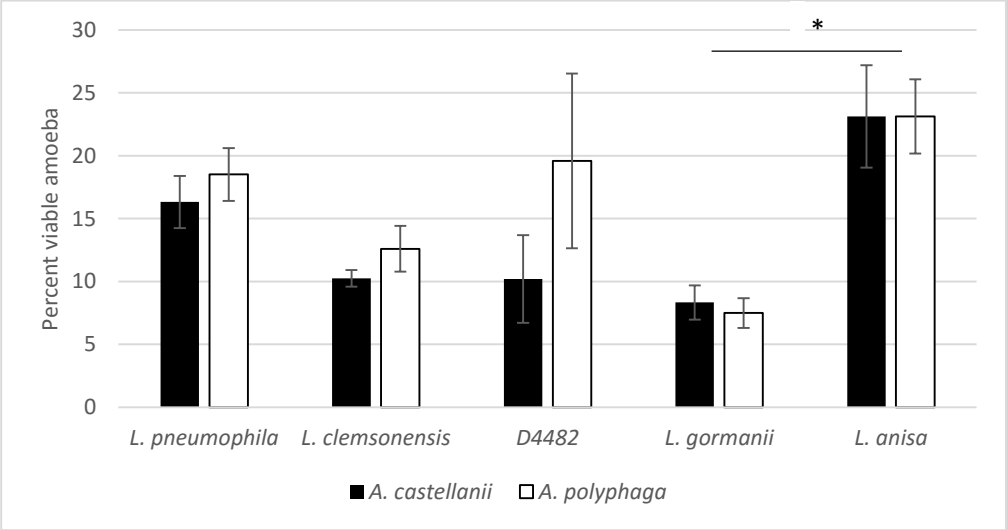

Figure 7. After grazing on *Legionella* biofilms, the percentage of *A. castellanii* remaining was consistent between all five species of *Legionella. L. anisa* displays a slightly increased level of amoebae survival post grazing.

Similar to *A. castellanii,* there were no differences in *A. polyphaga* survival between all *Legionella* biofilms being assessed (Fig. 7). Again, the highest rate of *A. polyphaga* survival occurred on *L. anisa* biofilms with 23.125% survival. Comparing the two amoebae species shows that both are equally susceptible to replication and lysis by *Legionella* species when the *Legionella* are acquired from the biofilm.

Amoebae reinfection assessment

Bacterial passage through a host can lead to an increase in virulence and invasion potential (46, 47). Once we had a baseline for the initial interaction of planktonic culture and amoebae, we then investigated if a reinfection into the same host would increase the *Legionella'*s ability to invade the host or replicate once inside of

it. When reinfection was assessed, *L. pneumophila* invaded at similar rates in both the initial infection and the re-infection. However, *L. clemsonensis* showed increased invasion abilities in *A. castellanii* after passage through the amoebae host ($p<0.05$) (Fig. 8). *L. pneumophila* and *L. clemsonensis* both showed a reduction in replication ability after a passage through *A. castellanii* (Fig. 9).

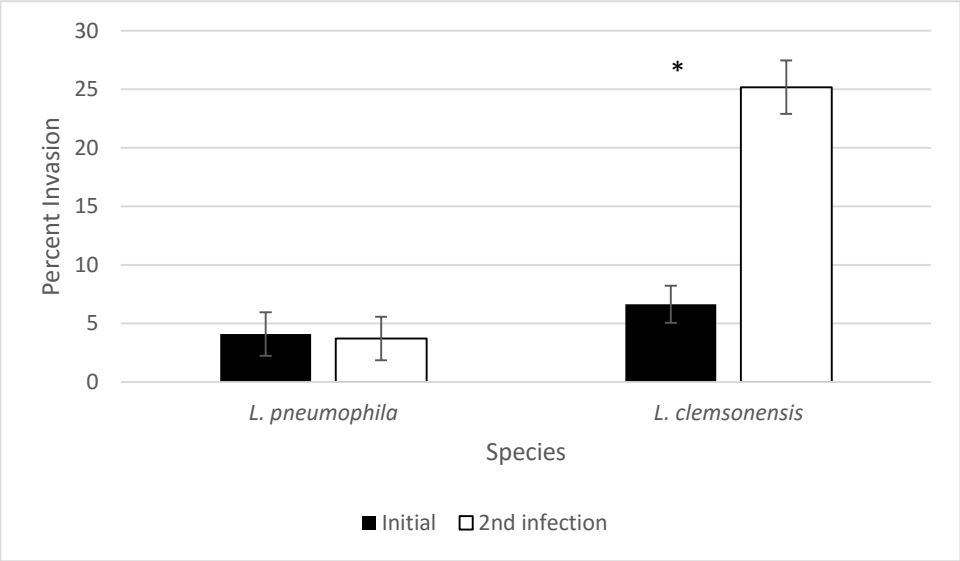

Figure 8. Reinfection of *A. castellanii* with amoebae grown *L. pneumophila* and *L. clemsonensis.* *A. castellanii* grown *L. pneumophila* invaded at similar amounts the second time. *L. clemsonensis* invaded at higher levels upon reinfection ($p<0.05$).

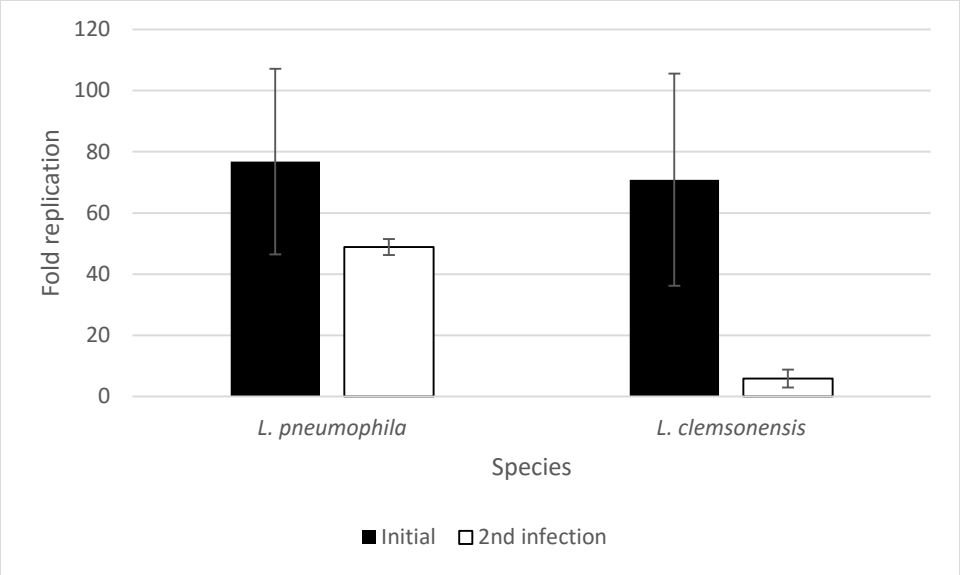

Figure 9. Replication after initial invasion and a second invasion of *L. pneumophila* compared to *L. clemsonensis* in *A. castellanii. L. pneumophila* replicated at lower amounts when passed into the same species of amoebae a second time. *L. clemsonensis* also showed reduced replication when passed through *A. castellanii.*

Amoebae grown biofilm assessment

Environmental biofilm formation will likely come from bacteria that have first been acquired from a biofilm, replicated within a host, and then been released in a new environment. To investigate if there are differences between culture inoculated biofilms and biofilms originating from bacteria after passage through amoebae, we tested the ability of *Legionella* to form biofilms after being released from *Acanthamoebae* species*.* We also tested how much of an amoebae grown biofilm the *Acanthamoebae* would graze. Biofilms of *L. pneumophila* and *L. anisa* were grazed by *A. castellanii* and the bacteria from those amoebae were collected. These *Legionella* were then used to inoculate new biofilms. All of these second generation biofilms covered similar surface areas as the initial biofilms established from culture grown *Legionella*. When amoebae

grazed these second generation biofilms however, grazing was reduced. *L. pneumophila* and *L. anisa* biofilms both demonstrated that after passage through a host, biofilms established from these bacteria saw reduced grazing by amoebae (Fig. 10).

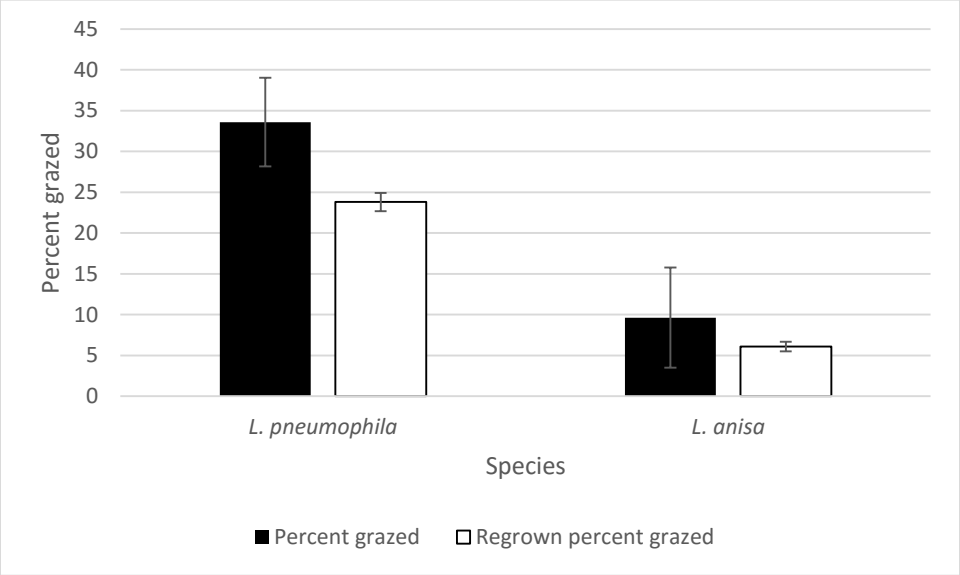

Figure 10. Percentage of *Legionella* biofilms grazed after initial biofilm formation and biofilm formation after one passage through *A. castellanii. L. pneumophila* showed reduced biofilm grazing by *A. castellanii* after one passage as did *L. anisa.* ($p>0.05$)

DISCUSSION

Host interactions are vital for the survival and proliferation of many species of bacteria. *Legionella* in particular requires a host cell for replication. While *Legionella* exists as part of a biofilm for periods of time, the use of a host cell allows for replication and propagation of the species (64). *Legionella* can replicate within 20 species of amoebae, two ciliates, and one slime mold (65). But not all species of *Legionella* have equally favorable interactions with these hosts. In certain cases, the host will ingest and destroy the *Legionella* as opposed to the more outcome of bacterial amplification.

Legionella amplification in a host allows for the continued persistence the bacteria. If entry into a host is detrimental to the *Legionella,* that particular species would not survive in an environment with that host (24)

Construction of man-made water systems has provided new environments in which *Legionella,* along with many other bacteria, thrive. Addition of many new buildings requires the need for more man-made water systems to keep the buildings cool and running. These new systems are then an environment for bacterial growth. As the weather temperature increases as well, the water systems are increasingly used to cool the buildings, leading to more aerosolization of the water inside them (66,67). Certain water systems will eventually aerosolize the water inside them, which can contain *Legionella* and other organisms. If *Legionella* are in this water that is aerosolized, they can then be inhaled by humans, leading to LD. Because of this chain of events and the new environments that we have constructed, there is much higher risk for outbreaks of LD. Along with the new environments, better healthcare and hygiene practices have increased lifespan not only for healthy individuals but for the immune compromised as well. The percentage of elderly within the US population has increased by 15.1% since 2000 alone. The number of people living with immune compromising illnesses such as HIV/AIDS, organ transplants and cancer has increased to 10 million people (69). Both of these groups serve as susceptible hosts for *Legionella*. Together, the increased environmental presence of the bacteria and the increased numbers of

possible human hosts, suggest a future with an overall increase in LD exposure, risks, and case numbers.

LD exposure from water systems is in part dependent on the amplification of *Legionella* numbers within these systems. This amplification is dependent on the host-pathogen interactions that take place. The number of protozoan hosts in water systems positively correlates with the amount of bacterial biofilm (70). Therefore, a number of protozoan hosts for *Legionella* are likely to be found within the same environments. While we know that protozoan hosts and *Legionella* are commonly found within the same system, there is a gap in the current *Legionella* knowledge base regarding what actual species level host-pathogen interactions are occurring.

***L. pneumophila – Acanthamoebae* interactions**

The interactions of *L. pneumophila* and *Acanthamoebae* are well characterized, but limited knowledge exists regarding the interactions of non-*pneumophila Legionella* species and protozoa. The two most commonly used *Acanthamoeba* species in *Legionella* research – *A. polyphaga* and *A. castellanii* both support *L. pneumophila* replication (18, 71). *L. pneumophila* increases eight fold over 72 hours in *A. polyphaga,* and has been shown to increase 4 fold over 72 hours in *A. castellanii* (72, 15). We used this knowledge as a baseline to compare the four experimental species of *Legionella.* The *Acanthamoebae* host interactions of non-*pneumophila* species are not well characterized, so whether or not they replicate inside *Acanthamoeba* is not known.

Characterization of host-pathogen interactions could allow a predictive risk assessment for man-made water systems to be implemented. *L. pneumophila* invades *Acanthamoebae* hosts at rates from 40-80% of the initial inoculum (71, 73)**.** We found lowered invasion rates of *L. pneumophila* into *Acanthamoebae* species*.* Approximately 13.5% of the initial bacteria invaded *A. castellanii* as opposed to only 5% in *A. polyphaga*. We saw similar replication ability of *L. pneumophila* into these two hosts. Reported replication within *A. castellanii* typically occurs at a log 3-4 fold increase over 48 hours (63, 73). In our comparison we saw a 47 fold increase in *A. castellanii* and a 55 fold increase in *A. polyphaga.* As we had lower invasion numbers than published literature, it follows that our replication numbers would also be lower.

Although amoebae may encounter and consume planktonic *Legionella*, the more likely scenario is acquisition from biofilm grazing. The biofilm forming ability of *Legionella* plays a role in the virulence of the species, as well as in its ability to persist is the environment (46, 74). Due to the need for a protocol that allowed the interaction of biofilms and amoebae hosts, our lab designed a protocol allowing this to happen (58). By growing mature biofilms and then adding on the amoebae, we simulate an environment in which a mature biofilm containing *Legionella* are suddenly grazed by a host. Our results show that *L. pneumophila* forms biofilms with a surface area of approximately 95000 μm^2. As *L. pneumophila* is commonly found in man-made water systems and is used in a majority of *Legionella* studies, its capacity to form biofilms can be used as a baseline for comparison of non-*pneumophila* species.

Our lab has developed a protocol to assess biofilm grazing by amoebae hosts. Using this assay to assess grazing ability, we have found that on average 30% of the *L. pneumophila* biofilm was consumed by both species of *Acanthamoebae.* This percentage was then used as a comparison for non-*pneumophila* species. *Legionella g*razing by hosts has not been well characterized, but is an immensely important part of the *Legionella* life cycle. This interaction between the biofilm and grazing protozoa and how this interaction affects survival, replication and persistence is essential for understanding *Legionella* ecology. Natural biofilms would have *Legionella* on the surface of the biofilm (22), so from a mixed culture biofilm, a high proportion of the colonized *Legionella* could be taken into the amoebae host, and depending on the host, amplify more into the environment. Specific amoebae species solely graze the surface of biofilms, as is the case with *A. polyphaga* (75). Hosts such as this will more closely interact than one that would graze the whole biofilm.

Legionella species are characterized by their biphasic lifestyle, alternating between an infectious stage and an intracellular replicative stage. The trademark of transmissive phase *Legionella* is the presence of a flagella (76). As part of a biofilm, *Legionella* are unflagellated, so would therefore be considered avirulent. However we found that *Legionella* grown as part of a biofilm are virulent, and can invade and kill amoebae at high numbers. When we planktonically infected amoebae with the five *Legionella* species, we saw invasion and replication of the bacteria, as expected. To test if *Legionella* were virulent during their non-infective, replicative stage, we grow biofilms

and allowed for amoebae grazing. We found that all *Legionella* species were infective when grazed from a biofilm. At least 66.5% of the amoebae allowed to graze the *Legionella* biofilms died. Amoebae viability after grazing on biofilms indicates the infectivity of the *Legionella* biofilm. This is in comparison to amoebae grazing on *E. coli* biofilms. *A. castellanii* grazing on *E. coli* showed some host death, but in much more limited numbers than when grazing on biofilms of *Legionella* species (77). *L. pneumophila* killed between 75-85% of the amoebae that were allowed to graze. While some normal amoebae death will occur, this high percentage indicates that *L. pneumophila* is in fact replicating within both *A. castellanii* and *A. polyphaga*, and lysing the host cell.

Non-*pneumophila* and *Acanthamoebae* interactions

We hypothesized that *L. pneumophila* and non-*pneumophila Legionella* species would exhibit differences in infection ability for different amoebae hosts. In infections using in vitro grown cultures of *Legionella*, non-*pneumophila* species showed less than 8% of the invasion ability of *L. pneumophila* in *A. castellanii.* In *A. polyphaga* also, *L. pneumophila* invaded at higher percentages than the non- *pneumophila* species, though the invasion rates of the non-*pneumophila* species were closer to that of *L. pneumophila* within this host. *L. pneumophila* was able to invade at 6.9% as compared to the next highest *Legionella* strain D4482, which invaded at 4.9%. Planktonic invasion of a host is less likely to happen in the environment, as *Legionella* will more often be acquired from

a biofilm, but planktonic invasion experiments give insight into the *Legionella* ability to invade and replicate within host cell. These assays showed whether or not the pairing of a specific *Legionella* – amoebae interactions are significantly different based of species.

L. clemsonensis replicated, although to a lesser degree than *L. pneumophila,* in both *Acanthamoebae* species. As *L. clemsonensis* was a clinically collected isolate, it clearly has the ability to cause disease. This makes it unsurprising that it would replicate within *Acanthamoebae,* and would also therefore likely replicate within macrophages.

Studies have shown that *L. gormanii* does not invade or survive well inside of *Acanthamoebae* (78). In our experiments as well, *L. gormanii* demonstrated an inability to replicate within both *Acanthamoebae* species in our study. The lack of replication here could explain the low number of *L. gormanii* LD cases. Although invasion and uptake of *L. anisa* was observed, no replication in either species of *Acanthamoebae* was detected. Amoebae phagocytize and degrade bacteria for food. The less than one fold replication of *L. anisa* and *L. gormanii* suggests that degradation could be occurring, suggesting that *Acanthamoebae* is not the typical host for *L. anisa* or *L. gormanii* in the environment. This lack of replication may indicate one reason for the prevalence of *L. pneumophila* over other species within systems where *Acanthamoebae* predominate**.** The temperature of incubation could also affect the replication abilities; some temperatures allow for faster replication of *Legionella* within the host (19). Our

experiments were done at 35°C but a lower temperature could have been more favorable for the interaction of *L. anisa* or *L. gormanii* and *Acanthamoebae* (19).

Legionella strain D4482 actually exhibited more replication in *A. castellanii* compared to *L. pneumophila,* but showed extremely limited replication in *A. polyphaga.* In *A. castellanii, Legionella* strain D4482 had over a 500 fold replication, whereas in *A. polyphaga* had less than 1 fold replication, similar to *L. gormanii* and *L. anisa,* implying that *Legionella* strain D4482 was being destroyed inside *A. polyphaga.* As the D4482 strain mip sequence suggests that this is a novel strain (<93% mip sequence match to any known *Legionella* species), assessing the ability of this strain to replicate in macrophages would determine if there is a chance that this novel strain could be causing cases of LD. These results also display the vastly different response of *Legionella* in different hosts. *Legionella* strain D4482 demonstrates that even within the same host genus (*Acanthamoebae)*, *Legionella* amplification can vary and alter the analysis of the danger of each particular strain.

For all of the experiments, *L. pneumophila* had lowered infection and replication abilities compared to currently published work (18). Less work has been published regarding the other *Legionella* species and their host interactions. Previously, *L. gormanii* has been shown to increase in number when in co-culture with *A. castellanii,* but our data differs (63). There have been no publications on the two novel *Legionella* species. *L. clemsonensis* seems to follow similar patterns to the other *Legionella* species,

but *Legionella* strain D4482 differs from what *L. pneumophila* is normally believed to do in regards to host interactions, specifically in the vast difference in replication between hosts. Most *Legionella*/host interactions will result in amplification of bacteria, but usually between 3-4 log increase (63, 18) *Legionella* strain D4482 showed much higher replication in *A. castellanii* and much lower replication rates in *A. polyphaga.*

Biofilm formation

L. pneumophila, L. clemsonensis, and *L. gormanii* all formed biofilms resulting in similar surface area coverage after five days. Both *Legionella* strain D4482 and *L. anisa* formed significantly larger biofilms in the same time period. This data suggests that these *Legionella* species could more efficiently form biofilms directly on a solid surface, versus *L. pneumophila* that colonizes existing biofilm more efficiently. If these species with larger biofilms are better capable of persistence, they have a higher likelihood of amplifying in a water system and infecting humans, and as biofilms of *Legionella* have been implicated in most outbreaks of LD, they could cause more disease (48). Environmental conditions and the microbes present in water systems will affect the biofilm colonization and establishment of *Legionella* species, but in pure culture, all five *Legionella* species formed stable biofilms. Once in a multi-species culture environment, the biofilm formation may differ, also altering likelihood of host interactions.

No *Legionella* biofilm lost more than half of its biofilm through *Acanthamoebae* grazing. The largest amount of biofilm grazed was *L. clemsonensis* when it was grazed by

A. polyphaga, with 42.3% of its surface area removed by the amoebae in 48 hours*. L. anisa* only lost 12.8% of its surface area when grazed by *A. castellanii.* This limited grazing by the host would imply that something about *L. anisa* inhibits grazing by *A. polyphaga*. The bacteria morphology of *L. anisa* could make it more difficult for the amoebae to graze. Various species of *Legionella* have been shown to have different morphologies, including needle-like microcolonies, wool-like microcolonies and serpentine chains. While some of these morphologies were seen in replicating *Legionella,* the wool-like morphology made replication within a host more difficult (78). *L. anisa* could have a similar morphology that is making its uptake and replication more difficult. Alternatively, the ligands on the surface of *L. anisa* could be changing the effectiveness of the amoebae uptake mechanism. Certain protozoa have been shown to contain a Gal/GalNAc lectin that is involved in the uptake of *Legionella.* In the presence of anti-lectin antibodies or concentrations over 100 mM of Gal or Gal/NAc, the host was not able to adhere to the bacteria (79). Uptake of *Legionella* into monocytes is mediated through various complement receptors, and while is it unclear exactly what causes *Legionella* uptake into protozoa, it is believed to be receptor mediated endocytosis (80). Unlike in macrophages, uptake into protozoa is not microfilament mediated (81).

When the *Legionella* biofilms were grazed by *A. castellanii, L. clemsonensis* was the most grazed, losing approximately 36% of the biofilm surface area. Combined with the information from the planktonic assay showing the reduced rate of *L. clemsonensis* replication within amoebae as compared to *L. pneumophila*, the increased grazing rate

also supports the use of *L. clemsonensis* as a preferred food source for *Acanthamoebae.* This increased loss of surface area would support the reasoning behind *L. pneumophila* being the most common *Legionella* species collected from water systems as opposed to the non-*pneumophila* species. If the non-*pneumophila* species are grazed more than *L. pneumophila*, and furthermore do not replicate within hosts, there would be reduced numbers of that species within a man-made water system. This could also be due to a cytotoxic effect that *Legionella* have on both protozoa and macrophages (82). This cytotoxicity is due to various *icm* genes found in *L. pneumophila.* These genes may also be turned on in *L. clemsonensis,* leading the lack of replication, and also the loss of amoebae after grazing.

Amoebae viability after grazing on all *Legionella* biofilms was reduced. Based on current knowledge of *Legionella*, this should not happen. *Legionella* grown as part of a biofilm do not have flagella, and should be then considered avirulent (83). This implies that they should not have the ability to infect and lyse hosts, and yet that is not the case. Fewer *Acanthamoebae* survived after grazing on *L. clemsonensis* compared to *L. pneumophila*. *L. clemsonensis* did not replicate within *Acanthamoebae* as well, but were able to reduce amoebae survival. This suggests that *L. clemsonensis* could be lysing the host cell even without replicating. *L. gormanii* exhibited similar amoebae killing ability. The novel *Legionella* strain D4482 did not lyse as many *A. polyphaga* as the other *Legionella* species. This is consistent with the replication results.

Increased virulence in *Legionella* passed through host

Previous studies have shown that bacterial passage through a host can increase the virulence of that bacteria into the same host (46, 47). Our study shows after passage through *A. castellanii*, *L. pneumophila* displayed a similar invasion ability with 3.7% of amoebae grown *Legionella* invading the amoebae in 2 hours compared to 4.1% of broth grown *Legionella.* The clinical isolate *L. clemsonensis* significantly increased invasion ability after one passage through the host ($p<0.05$). Both *L. pneumophila* and *L. clemsonensis* replication ability was reduced after passage through *A. castellanii.* This could in part be due to the initial invasion rates. A higher number of bacteria/amoebae restricts host nutrients and organelles available for use in each replication vacuole.

The natural state of the bacteria when they interact with amoebae hosts is likely to be as part of a biofilm (1). While passage through a host could increase infectivity of *Legionella*, passage through a host once could also make a *Legionella* species more likely to be grazed by either the same or a different host. *Francisella tularensis* not only evades immune responses after passage through a macrophage, but less of the bacteria are ingested upon a second interaction. This is believed to be due to changes to the bacteria during growth in the host (47). *Legionella* could experience similar changes that cause the host to recognize it less. Our experiments show that *A. castellanii* grazed higher proportions of both *L. clemsonensis* and *L. gormanii* after the bacteria used to establish the biofilms were harvested from amoebae. The other three *Legionella* species

were grazed less after the amoebae grown *Legionella* were used for biofilm initiation. Protozoan hosts do show preference to specific organisms when grazing (84). Various factors including bacterial size, bacterial toxin production, and Gram identity affect the predation of protozoa on organisms (85, 86, 87).

If after initial grazing on a *Legionella* species the amoeba hosts are being lysed, there could be some communication between the protozoa to cause the avoidance of that *Legionella* species from future biofilm grazing. Protozoan hosts often communicate via extracellular vesicles and exosomes, especially in response to environmental changes. Release of these vesicles during infection could trigger changes in uninfected protozoa and reduce grazing on certain biofilms (88). This interaction could be part of the decision of the host to choose a specific portion of biofilm to graze in the environment. In my experiments though, a second biofilm is set up in a different glass petri dish, so different amoebae are being used for the two grazing assays, but other factors could influence the lack of grazing. Various factors will affect the morphology of biofilms as they are forming. Dispersal of biofilms by nanoparticles will significantly change the makeup of the biofilm. This change was shown to affect the interaction with *A. polyphaga* (58.) Biofilm morphology will likely differ between initial biofilms and biofilms after host passage, which could lead to a change in the way hosts graze the bacteria off the biofilm surface.

Risk Assessment

Legionella on their own in a water system would pose much less of a threat for human infection than *Legionella* in the presence of protozoan hosts as they would not be able to replicate. These hosts allow for bacterial replication and release, at which point they can be aerosolized and infect humans. Since most man-made water systems will contain both *Legionella* and hosts, the need for an assessment of predictive risk is high. This assessment would utilize host-pathogen interaction background to predict which man-made systems would be at a higher risk potential based on the host species and *Legionella* that were found there. While there are current assessments for *Legionella* risk, they do not always factor in protozoan host presence, and they rely on the data about *L. pneumophila,* while in reality there will likely be many more than one *Legionella* species in a water system.

Potential risk to humans caused by interactions between *Legionella* and amoebae is not based purely on the host-pathogen interaction. Other factors such as temperature, pH, and how many bacteria and amoebae are present in the environment will also play a role, but without amplification of *Legionella* within a host, it is less likely that *Legionella* will have the chance to infect a host. Therefore, characterization of host interactions is an essential portion of *Legionella* risk assessments. Currently it is known that *L. pneumophila* replicates well in *Acanthamoebae* species and *Vermamoeba vermiformis* (90). Characterization of *L. pneumophila* in *Acanthamoebae* hosts shows

that the bacteria will amplify, and also that they will amplify significantly more than other non-*pneumophila* species such as *L. micdadei* (72). This information is limited for many of species of *Legionella,* and without this knowledge, a true risk cannot be assessed.

Factors that would affect the risk to humans of *Legionella* being in a system include the invasion and replication abilities, the amount grazed by hosts, and the change in virulence and host grazing after one passage through a host. Based on the criteria listed above, paired with both amoebae species, *L. pneumophila* could be considered high risk, as it showed high invasion and replication in both species, a large portion of its biofilm was grazed, and *L. pneumophila* also showed high ability to kill host cells. This risk, though based only on the results seen here, is not completely unsurprising. While the reported LD cases are skewed to *L. pneumophila* because of inadequate diagnostic testing, this species still causes many cases of LD each year, so should be considered a risk in any water system. *L. clemsonensis* showed some replication in the *Acanthamoebae* hosts, and some of the biofilms were grazed. The real cause for concern with *L. clemsonensis* is the increase in invasion ability after passage through *A. castellanii.* This alone would raise the risk factor for *L. clemsonensis.* Both *Legionella* strain D4482 and *L. anisa* showed either limited replication within a host or a seeming inability to invade the host. Therefore, neither would be considered much of a risk. This is again unsurprising as *L. anisa* has only been implicated in one case of LD, and *Legionella* strain D4482 is a novel species. *L. gormanii* showed extremely limited

invasion and replication potential into both hosts, as well as a reduced amount of biofilm grazing. *L. gormanii* would seem to be low risk in combination with *Acanthamoebae* species.

There will likely always be many species of *Legionella* in a given water system, along with a variety of their protozoan hosts. As previously described, water towers at the Savannah River site consistently had *Legionella* present in sample. Protozoan genera such as *Acanthamoeba* and *Vermamoeba* were detected in each of 21 water samples from a combination of environmental and man-made samples (91). This means *Legionella* will likely always be invading hosts and replicating in water systems. Understanding which interactions will be problematic can lead to a prevention of increased risk by knowing when interventions must take place. Our results show significantly higher invasion of *L. pneumophila* in *A. castellanii* compared to *A. polyphaga. A. castellanii* is the *Acanthamoebae* species most often used for *Legionella* experimentation. As *L. pneumophila* and *A. castellanii* are the most commonly used species, there is a chance that we are overestimating the impact of *L. pneumophila.* This one host-pathogen interaction, while high in *Legionella* amplification, is not the standard for *L. pneumophila* in all amoebae hosts.

Comparison to other genera

It is known that *L. pneumophila* causes a majority of cases of LD, but it is unclear what proportion of the cases of Pontiac Fever are caused by the same species. Other

non-*pneumophila* species of *Legionella* have been implicated in cases of Pontiac Fever. The large occurrence of LD caused by *L. pneumophila* begs the question, is there something about *L. pneumophila* that allows it to cause a more severe illness in humans, or is it the patient's immune system that decided the severity of the illness? Within the *Legionella* genus, there are two separate illnesses of varying severity. Due to the diversity of the species, there is a chance that only one of the species causes most of the severe cases, while the others are implicated in the lesser illness. This model of disease is not new. The genus *Salmonella* follows a similar pattern of severe versus self-limiting disease. *Salmonella enterica* subsp. *enterica* serotype Typhi (*S.* Typhi) causes Typhoid Fever. Typhoid Fever causes 21 million cases throughout the world annually, with a 12-30% mortality rate (91). *S.* Typhi is the only species within the genus known to cause such a severe illness. *S.* Typhi is a specifically human pathogen, lacking other hosts (92). The rest of the pathogenic *Salmonella* species result in gastroenteritis upon human infection. Similar to Pontiac Fever, gastroenteritis resolves on its own and therefore patients rarely go to doctors for treatment. Of all the species of *Salmonella, S.* Typhi is the only one with the capability to leave the GI tract and become systemic (93). *S.* Typhi contains a different plasmid than the rest of the genus lacks. The plasmid, pHCM2, shares common ancestry with a plasmid found in *Yersinia pestis* (94). There is a singular highly pathogenic *Salmonella* species within the genus that, due to its genes and biochemical properties, causes grave illness. There is the possibility that a similar phenomenon is happening within the *Legionella* genus. Our results showed that

between *L. pneumophila* and the non-*pneumophila* species invaded and replicated at different amounts into the *Acanthamoebae* host. This implies that there is something different about *L. pneumophila* as compared to the rest of the tested *Legionella* species. Perhaps *Legionella* and *Salmonella* follow the same pattern with one of the species in the genus causing a severe form of illness while others cause a less severe illness.

Bacterial risk assessment

In order to truly control case numbers and outbreaks of LD, the bacteria must be controlled within the water systems. On urgent need for water systems in the US is a standard and accountable risk assessment and management program. Risk assessment for fecal-oral pathogens such as *E. coli* and *Salmonella* within water systems follow a standardized set of guidelines. *Salmonella* is tested for using both national and international prevention methods. *Salmonella* species, like *Legionella,* are common causes of human infection, and therefore are tracked and attempts are made to remove them from the environment. Both species have risk assessment protocols in place to check for potential risk of human infection (95, 96)

Current checklists for contamination in water systems do not require the check for specific *Legionella* strains and protozoan hosts. There is no mention of the interaction of *Legionella* and amoebae, just a box to check if amoebae are visible and if there are protocols in place for regular checking for *Legionella.* This is implying that presence of *Legionella* immediately means a risk of LD (98). Descriptors for each

category of the risk assessment can be characterized as low, medium, or high risk. The current treatment plans for man-made water systems include the use of harsh chemicals including chlorine. This technique is commonly used, but there is concern about the effects on the environment and the water systems that are being decontaminated (99). When used, chlorine sits in the system for 24 hours and is then drained. If biofilms are still visible within the system after disinfection, current guidelines require the procedure be repeated. Water systems are then manually cleaned with more chlorine and flushed with water (100). When biofilms inhabit a water system, the concentration of chemical disinfectants must be higher, as biofilms are more than 100 times more difficult to remove using this method (99). UV irradiation is another decontamination method, more commonly in wastewater treatment plants. UV light kills bacteria by causing thymine dimers, therefore stopping replication of the bacteria (101). The use of metal ions have also proved effective by binding DNA (102). Physical measures as also used, such as granular filers and membrane filters. These filters clear bacteria from water systems, as well as protozoa. Sand filtration has been shown to remove between 81-100% of bacteria and 99-100% of protozoa (102). Membrane filters are pressure driven and also remove high amounts of contamination. The issue with any type of filtration is build-up of bacteria and waste that requires normal cleaning (103). Hot water flushes are also used to remove *Legionella* contamination from water systems. Temperatures of 60°C or higher should be effective to inhibit *Legionella* growth (104). Cleaning using hot water is not fully effective against

Legionella though. After a single hot water flush, *Legionella* were still persisting in water systems, using biofilms to avoid the effects of the heated water (105). In the case of *Legionella* in water systems, there are many factors to be considered, and thus most risk assessments are qualitative, and even the quantitative assessments are missing vital pieces of the puzzle (106).

Due to the 2015 LD outbreaks in New York City, new regulations have been put into place there. All man-made systems are required to be tested quarterly for the presence of *Legionella.* If *Legionella* is detected in the system, immediate cleaning is required. This requires shut down of the system, disinfection with chemicals, then a flush of the system with water before restarting the system. These requirements will become expensive quickly, and as *Legionella* is commonly found in water systems, as exhibited by collection samples at the Savannah River site, the regulations will mean constant cleaning of systems with no true effect or benefit.

Current EPA guidelines require a water system have $<10^{-4}$ *Legionella* to be considered safe (26). Presently risk assessment protocols for water systems look at temperature of the system, sources of nutrients, presence of biofilms, the design of the system (surface area available, lighting, etc.), and location in regards to humans (107). There is still currently a missing part of the assessment however, host interactions. With additional information on the microbial community, the risk prediction regarding the potential of LD cases originating from this water system could be improved. The amplification, or lack of amplification, of *Legionella* can significantly alter the risk of

disease exposure. As shown by our data and others, passage through a host can also affect the infectivity of *Legionella.* If host interactions are ignored, a large portion of what is actually happening in these aquatic systems is being missed. The interactions of the non-*pneumophila Legionella* species also need to be added to the current risk prediction assessments, and to the *Legionella* knowledge base. With the addition of these species, there can be better reporting of causative species and better predictive risk assessment to keep that reported number as low as possible.

Further Research

Our results display that within *Acanthamoebae* species, *Legionella* species will have differing infection and replication abilities. Between host species, the same *Legionella* species can also exhibit different outcomes. Further work using more host species will continue to expand the knowledge of bacterial interactions and aid in characterizing risk potential. Since *Acanthamoebae* are soil and aquatic dwelling species of amoeba, using strictly soil dwelling or aquatic dwelling species could show a difference in *Legionella* interactions. *V. vermiformis,* a water dwelling amoebae and *Dictyostelium discoideum,* a soil dwelling amoeba, are both potential hosts of *Legionella* that warrant further study. Continued investigation into the possible increase of virulence after passage through hosts could be continued using both the amoebae and macrophages, as increased virulence into macrophages would be a large concern due to its implications in human infection. The possible virulence increase could also be tested

through invasion ability into a different host than the *Legionella* species was originally passed through. An increase in invasion here could mean a higher risk in water systems overall, as well as a higher risk of human infection.

Appendix I. Media recipes

Buffered Charcoal Yeast Extract (BCYE) – 500 mL

- 5.0 g ACES
- 8.5 g agar powder
- 1.0 g charcoal
- 5.0 g yeast extract
- 0.5 g potassium alpha-ketagluterate
- 0.2 g L-cysteine
- 0.125 g iron
- 470 mL ultrapure water

Final pH of the media is 6.9. The media is autoclaved and then the cysteine and iron are sterile filtered in.

Tryptic Soy Broth (TSB) – 1 Liter

- 30.0 g TSB
- 1 L ultrapure water

The media is autoclaved for a 20 minute liquid cycle.

Peptone Yeast Glucose (PYG) – 1 Liter

- 20.0 g protease peptone
- 1.0 g yeast extract
- 1 L water
- 8 mL 0.05M $CaCl_2$
- 10 mL 0.4M $MgS0_4$ $7H_2O$
- 10 mL 0.25M Na_2HPO_4 $7H_2O$
- 10 mL 0.25 KH_2PO_4
- 1.0 g Na Citrate $2H_2O$
- 10 mL 0.005M $Fe(NH_4)_2(SO_4)_2$ $6H_2O$
- 18.0 g dextrose
- 50 mL water

Media mixed in listed order up to $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ and then autoclaved. The dextrose is dissolved in the remaining water and sterile filtered in.

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