Development of a Modified Gentamicin Protection Assay to Investigate Interactions between *Campylobacter jejuni* and *Acanthamoeba castellanii*

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Dedications

Dedicated to my wife Monique and my son Jaxon whose unconditional love and support has driven me to work that much harder in everything I do.
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# Table of Contents

List of Tables ................................................................................................................... vii  
List of Figures .................................................................................................................. viii  
Abstract ............................................................................................................................. ix  

Chapter 1: Introduction .................................................................................................1  
  1. *Campylobacter jejuni* ..............................................................................................1  
     1.1 Characteristics of *Campylobacter jejuni* ..........................................................1  
     1.2 Campylobacteriosis ............................................................................................2  
     1.3 *Campylobacter jejuni* Pathogenicity .................................................................3  
        1.3.1 Infection in Humans ....................................................................................3  
        1.3.2 Flagella .......................................................................................................4  
        1.3.3 Adherence and Invasion .............................................................................5  
        1.3.4 Intracellular Trafficking and Survival .......................................................7  
     1.4 Persistence of *Campylobacter jejuni* ..................................................................8  
  2. Poultry Processing: Potential Sources of *Campylobacter jejuni* Contamination ....8  
  3. Water and *Campylobacter jejuni* ...........................................................................9  
  4. Chemical Treatments in Poultry Processing Facilities .............................................9  
     4.1 Sodium Hypochlorite (Chlorine) ......................................................................10  
     4.2 Trisodium Phosphate (TSP) .............................................................................11  
     4.3 Cetylpyridinium Chloride (CPC) .....................................................................12  
  5. Current Problems with *Campylobacter jejuni* in Poultry ......................................12  
  6. Amoebae ..................................................................................................................12  
     6.1 *Acanthamoeba* spp ..........................................................................................14  
     6.2 *Acanthamoeba* spp. and Endosymbiotic Bacteria ............................................16  
     6.3 *Acanthamoeba* spp. and Pathogens ................................................................18  
        6.3.1 *Legionella pneumophila* ..........................................................................22  
        6.3.2 *Vibrio* spp ..............................................................................................23  
        6.3.3 *Mycobacterium* spp ...............................................................................24
6.3.4 Order Chlamydiales .................................................................25
6.3.5 Francisella tularensis. ...............................................................26
6.3.6 Salmonella spp. .......................................................................26
6.3.7 Listeria monocytogenes. ............................................................27
6.3.8 Escherichia coli .........................................................................28
6.3.9 Shigella spp. .............................................................................29
6.3.10 Acanthamoeba spp. and Other Pathogenic Bacteria. ..........29

6.4 Acanthamoeba spp. and Campylobacter jejuni. .......................30

7. The Gentamicin Protection Assay (GPA) ......................................33
8. Conclusion ...........................................................................................35

Chapter 2: Development of a Modified Gentamicin Protection Assay to Investigate the Interactions between Campylobacter jejuni and Acanthamoeba castellanii ......37

Abstract.................................................................37
Introduction........................................................................38
Methods..............................................................................41
Results.....................................................................................46
Discussion..............................................................................56
Conclusions..............................................................................60

Chapter 3: Interaction of Campylobacter jejuni Strains with Acanthamoeba castellanii ..............................................................62

Abstract.................................................................63
Introduction........................................................................42
Methods..............................................................................65
Results.....................................................................................69
Discussion..............................................................................70
Conclusions..............................................................................75

Chapter 4: Assessment of the Susceptibility of Acanthamoeba castellanii and Campylobacter jejuni to Antimicrobial Agents used in Poultry Processing ..........76

Abstract.................................................................76
Introduction........................................................................77
Methods..............................................................................79
Results..................................................................................................................................................82
Discussion..............................................................................................................................................87
Conclusions............................................................................................................................................90

Chapter 5: Overall Discussion and Conclusion..................................................................................92
List of References..................................................................................................................................106
Appendix A: Raw Data.........................................................................................................................120
Appendix B: Curriculum Vitae..............................................................................................................193
Vita.........................................................................................................................................................197
List of Tables

**Table 1.1**: Compilation of Protozoan species the bacteria that they harbor or protect .....19

**Table 2.1**: Results of modified gentamicin protection assay using standardized *Acanthamoeba castellanii* cultures ........................................................................................................53

**Table 2.2**: Examination of the impact of *Acanthamoeba castellanii* growth phase and nutrient status on internalization of *Campylobacter jejuni* ........................................55

**Table 3.1**: *Campylobacter jejuni* strains used in Chapter 3 ..............................................68

**Table 3.2**: Rate of internalization for *Campylobacter jejuni* strains .................................70

**Table 4.1**: Reduction of *Campylobacter jejuni* by chlorine, TSP, and CPC .....................87
List of Figures

**Figure 1.1**: Hypothetical model for *Campylobacter jejuni*-induced signaling events ........6

**Figure 1.2**: Image of *Acanthamoeba castellanii* trophozoite and cyst.......................14

**Figure 1.3**: Internalization of *Campylobacter jejuni* by *Acanthamoeba castellanii* ........32

**Figure 2.1**: Standard Curve of *Campylobacter jejuni* NCTC 11168 ................................49

**Figure 2.2**: *Campylobacter jejuni* NCTC 11168 Sensitivity to Gentamicin....................49

**Figure 2.3**: *Acanthamoeba castellanii* ATCC 30010 Sensitivity to Gentamicin..............50

**Figure 2.4**: *Campylobacter jejuni* NCTC 11168 Sensitivity to Triton X-100 ..............51

**Figure 2.4**: *Acanthamoeba castellanii* ATCC 30010 Sensitivity to Triton X-100 ..........52

**Figure 4.1**: Recovery of *Acanthamoeba castellanii* following exposure to sodium hypochlorite (chlorine)........................................................................................................84

**Figure 4.2**: Recovery of *Acanthamoeba castellanii* following exposure to cetylpyridinium chloride (CPC)........................................................................................................85

**Figure 4.3**: Recovery of *Acanthamoeba castellanii* following exposure to trisodium phosphate (TSP)........................................................................................................86
Abstract
Development of a Modified Gentamicin Protection Assay to Investigate Interactions between *Campylobacter jejuni* and *Acanthamoeba castellanii*
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*Campylobacter jejuni* is one of the leading causes of bacterial foodborne illness in the U.S. and the leading bacterial cause of diarrheal illness worldwide. *C. jejuni* is persistent on poultry products despite its sensitivity to many environmental factors. Protozoa have been studied for their involvement in the internalization and subsequent protection of bacterial pathogens including *C. jejuni*. In the work presented here a modified gentamicin protection assay (mGPA) was developed to further investigate the internalization, and/or protection of *C. jejuni* using the amoebal species *Acanthamoeba castellanii*. This mGPA utilizes tubes instead of the commonly used cell culture plates. Using tubes allows for the inclusion of a *C. jejuni* only control to quantify *C. jejuni* survival through the assay independent of the presence of amoebae. Additionally by not working with an amoebal monolayer, this assay is able to provide a more consistent amoeba inoculum and more stable washing step. Previous studies employing a traditional GPA determined internalization by the recovery of *C. jejuni* at the conclusion of the assay. This mGPA determines internalization by the recovery of higher levels of *C. jejuni* in the presence of amoebae compared to *C. jejuni* controls in the absence of amoebae. Using this mGPA, it was determined that *C. jejuni* NCTC 11168 does survive the assay independent of the presence of amoebae. Taking into account this *C. jejuni* survival, it was determined that internalization of *C. jejuni* by amoebae occurs but not
consistently. Further investigation provided evidence that internalization may be strain specific and that possible virulence factors may be involved in the occurrence and extent of internalization. Finally the sensitivity of *A. castellanii* to antimicrobial compounds commonly used in poultry processing was investigated. Results indicated that *A. castellanii* is susceptible to these antimicrobials at concentrations acceptable for use in poultry processing; however their resistance is higher than *C. jejuni*. This resistance increases with the addition and increase of organic load, supporting the potential for amoebae to provide a protection to *C. jejuni*. Overall *A. castellanii* has shown the ability to provide a protective effect to *C. jejuni* and may aid in environmental survival.
Chapter 1: Introduction

1. *Campylobacter jejuni*

1.1 Characteristics of *Campylobacter jejuni*

*Campylobacter jejuni* belongs to the family *Campylobacteraeae*. Included in this family is the genus *Campylobacter* consisting of 18 species and subspecies and the genus *Arcobacter* containing four species [1]. *Campylobacter jejuni* subspecies *jejuni* (hereby referred to as *C. jejuni*) and to a lesser extent, *Campylobacter coli* are the most common *Campylobacter* species associated with human illness [1, 2]. *C. jejuni* is a gram-negative, curved or S-shaped spiral rod bacterium 0.2μm to 0.9μm wide and 0.5μm to 5μm long [1, 2]. Strains of *C. jejuni* are commonly thermophilic organisms and are motile by single, polar, unsheathed flagella at both ends. *C. jejuni* is an obligate microaerophilic (microaerobic) organism, requiring 5% oxygen and 10% carbon dioxide [1]. These organisms are very susceptible to a variety of environmental conditions that make it difficult to survive for extended periods of time outside of the host. *C. jejuni* does not grow at temperatures below 30°C, and is sensitive to desiccation, high oxygen conditions, and low pH [1, 3].

1.2 Campylobacteriosis

*Campylobacter jejuni* is one of the leading causes of foodborne illness and the leading cause of bacterial diarrheal illness worldwide [4]. The illness caused by *C. jejuni*, Campylobacteriosis, can range from asymptomatic to severe. Symptoms can include: fever, abdominal cramping, muscle pain and diarrhea (sometimes bloody). The
incubation period ranges from 1-7 (mean 3) days with symptoms, if present, lasting several days to a week. Symptoms are usually self-limiting, however relapses occur in 5-10% of patients [1-3, 5, 6]. Severe cases can be treated with antibiotics including erythromycin and ciprofloxacin [1-3]. Possible sequelae or secondary disorders that can follow a C. jejuni infection include: hemolytic uremic syndrome, bacteremia, bursitis, urinary tract infection, meningitis, acute cholecystitis, endocarditis, peritonitis, erythema nodosum, pancreatitis, abortion, neonatal sepsis, and reactive arthritis [1-3]. C. jejuni has also been reported to cause Guillain Barré (GBS). GBS is an autoimmune disorder of the peripheral nervous system. The lipooligosaccharide (LOS) of C. jejuni has various LOS structures resembling human neuronal gangliosides and this similarity is thought to lead to the development of this autoimmune disorder [6, 7]. C. jejuni was the cause of 30% of GBS cases; the chance of developing GBS after being infected with C. jejuni is 1 in every 1058 cases [7].

In 2011 Campylobacter spp. were estimated to affect 845,024 individuals in the U.S. leading to 8,463 hospitalizations and 76 deaths [8]. The Centers for Disease Control (CDC) Foodborne Disease Active Surveillance Network estimates the incidence of Campylobacter spp. infection in 2010 to be 13.52 infections per 100,000 population; the 2nd leading cause of bacterial foodborne illness behind Salmonella spp. [9]. More than 90% of cases are sporadic, with the consumption (and cross-contamination) of undercooked poultry identified as a risk factor [10-12]. Fewer than 500 organisms are needed to infect a person, meaning that even one drop of juice from a contaminated piece of chicken is enough to infect an individual [1-3, 13, 14]. Additionally, strains of antimicrobial resistant C. jejuni have been found in raw retail meat. These strains have
been found to be resistant to the common treatments for campylobacteriosis such as a fluoroquinolone like ciprofloxacin. These resistant *C. jejuni* strains generally result in a more prolonged, severe illness [14].

### 1.3 *Campylobacter jejuni* Pathogenicity

#### 1.3.1 Infection in humans

The mechanism(s) involved in the virulence and pathogenicity of *Campylobacter jejuni* are not fully understood and there are many conflicting studies in the literature [6, 15-17]. The main reason for the lack of knowledge of *C. jejuni* pathogenicity is that lack of typical pathogen adhesions, toxins, or secretions systems in the genome of *C. jejuni* [16]. It has been proposed that the reason for these conflicting reports may be due to differences in unique virulence factors of infecting strains, host innate immune response, experimental design/assays used, experimental conditions, and/or host cell behavior [15-17]. Infection begins with the ingestion of contaminated food or water containing *C. jejuni* [6]. The pathogen travels through the stomach, where the acidic environment may kill most of the *C. jejuni*, the remaining *C. jejuni* then enters the intestines [5, 6]. Infection begins in the small intestines and later affects the colon and rectum [5]. Once in the intestines *C. jejuni* must penetrate the mucosal layer of the intestinal epithelial cells to be able to interact with the cells [6]. Two mechanisms have been proposed to describe how *C. jejuni* causes disease. The first is that *C. jejuni* adhere to intestinal cells or mucus, reproduce and produce toxin(s), altering fluid absorption capacity of intestines, resulting in diarrhea [5]. The cytolethal distending toxin (CDT) is believe to enter the cell and lead to apoptosis by causing double–stranded DNA breaks and cell-cycle arrest.
The second proposed mechanism is that *C. jejuni* invade and reproduce within intestinal cells and cause an inflammatory response [5]. *C. jejuni* invade intestinal epithelial cells by inducing phagocytosis in the typically non-phagocytic cell [6]. Once internalized, *C. jejuni* contained in vacuoles move to the perinuclear region of the cell [6]. This scenario is further supported by a study reporting that *C. jejuni* does not cause apoptosis but instead causes oncosis (necrosis) independently of the production of CDT [19]. Various *C. jejuni* factors are involved in epithelial cell invasion process including flagellum/motility/chemotaxis, outer membrane proteins, O- and N-linked glycosylation, capsular synthesis, and Lipooligosaccharide (LOS) [6, 16] (Figure 1.1).

### 1.3.2 Flagella

In *Campylobacter jejuni* the flagella play a key role in causing disease. Each flagellum, consisting of a basal body, hook, and filament is employed as an export apparatus [15, 20]. The flagellum injects virulence proteins into eukaryotic cells similarly to a type III secretion system [15-17, 20]. Proteins secreted by *C. jejuni* upon co-cultivation with epithelial cells are termed *Campylobacter* invasion antigens (Cia) [15]. Proteins secreted include CiaB, CiaC, FspA, FlaA, and FlaC [15, 16].

In addition to the use of flagella as export apparatus motility and a complex chemotaxis system also play a key role in *Campylobacter jejuni* infection [16]. To establish infection *C. jejuni* must bypass physical and immunological barriers of the gastrointestinal tract [21]. The mucus layer in the intestines protects underlying epithelial cells by trapping and clearing pathogens by gut movement and mucus turnover. In addition, antimicrobial peptides in mucus and natural gut flora are directly antagonistic to
pathogens. The corkscrew-like shape and short O-side chains of their lipooligosaccharide allow *C. jejuni* to avoid binding of glycoproteins in the mucus. *C. jejuni* are believed to have adapted to the mucus environment and evolved mechanisms to breech the mucus barrier and attach to the surface of the underlying epithelial cells [21]. *C. jejuni* has shown an increased chemotaxis behavior toward mucus as well as up-regulation of virulence factors in the presence of mucus [21]. In addition, highly viscous solutions (similar to mucus) have been reported to increase *C. jejuni* motility [21].

### 1.3.3 Adherence and Invasion

Adherence and invasion may play key roles in disease development of *Campylobacter jejuni* and is mediated by several outer membrane proteins (OMPs) [22]. Fibronectin binding proteins (CadF and FlpA) have been proposed to work together to bind to epithelial cells [16] Jlpa, a lipoprotein aids also aids in binding and elicits a pro-inflammatory response [16, 21, 22]. Other proteins of note are CapA an autotransporter, the major outer membrane porin PorA, and Peb proteins [16, 22].
Peb proteins are surface molecules proposed to be involved in adherence and invasion but it has been difficult to identify their specific mechanisms of action [16]. Peb1 has a dual role as it is also found in the periplasm where it binds aspartate and glutamic acid [16]. Peb4 (cj0596), a periplasmic chaperone may also play a key role in
adhesion by exporting proteins to the outer membrane [16]. It is an abundant periplasmic protein indicated in OMP assembly inhibiting protein folding and aggregation by sequestration of unfolded protein [22]. The mutant \textit{C. jejuni} Δcj0596 expressed the alteration of several virulence related phenotypes [24, 25]. Mutants showed altered levels of eight outer membrane proteins, five proteins increasing in abundance including two flagella proteins and three decreasing in abundance. The \textit{C. jejuni} Δ0596 mutant expressed increased motility and invasion of epithelial cells [25]. Surface related characteristics were also changed in the \textit{C. jejuni} Δcj0596 mutant allowing for faster auto-agglutination and decreased biofilm formation [25].

Both the capsule and Lipooligosaccharide (LOS) of \textit{C. jejuni} are important factors involved in \textit{C. jejuni} virulence. In addition to playing a role in epithelial cell adherence [16] they both also confer resistance to serum and cationic antimicrobials, allowing \textit{C. jejuni} to by-pass the host’s innate immune response [26].

\section*{1.3.4 Intracellular Trafficking and Survival}

\textit{Campylobacter jejuni} enters intestinal epithelial cells and is localized in a \textit{C. jejuni}-containing vacuole (CCV). This vacuole is a specific compartment in the cytoplasm that is distinct from the lysosome. The CCV deviates from the canonical endocytic pathway avoiding the lysosome. CiaI has been proposed as one of the \textit{C. jejuni} factors involved in intracellular trafficking [16].
1.4 Persistence of *Campylobacter jejuni*

Rabbits, rodents, wild birds, insects, sheep, horses, cattle, pigs, poultry, and domestic pets are all reservoirs of *Campylobacter jejuni*. *C. jejuni* is commonly found to colonize the mucosal layer of the intestines of many of these reservoirs, especially chicken [27]. *C. jejuni* even appears to favor avian intestines as a commensal environment [28]. *C. jejuni* can also be found on vegetables, seafood, and water. In non-chlorinated water *C. jejuni* can be found dormant in a viable but non-culturable state [1, 3]. *C. jejuni* is primarily a poultry contaminate in the U.S. and has a prevalence of over 80% in chickens with 60% of retail raw chicken contaminated at 1-3 log<sub>10</sub> CFU/carcass [13, 29, 30]. The intestinal tract of chicken may harbor 5-9 log<sub>10</sub> CFU/g of *C. jejuni* [31].

2. Poultry Processing: Potential Sources of *Campylobacter jejuni* Contamination

Human traffic, animals (wild and domestic), and insects can be sources of *Campylobacter jejuni* contamination throughout poultry processing [32]. Poultry processing is a complex production process with the most common commercial poultry produced being broiler or fryer, which are chickens 6-8 weeks of age [32]. Chickens are raised in large groups (20,000 chickens) in enclosed broiler houses and the large number of animals in a relatively small space can lead to the spread of *C. jejuni* throughout flocks due to the coprophagic nature of chicken [32]. Untreated drinking water has also been shown as a source of contamination [12, 28]. Feed withdrawal prior to transport as well as contaminated transport containers have also been implicated in the transmission of *C. jejuni* to chicken prior to slaughter [32, 33]. Processing of commercial poultry involves a production line made up of stations for slaughter, scalding, feather removal, multiple
stations for evisceration, and chilling of the carcass prior to packaging and distribution [32]. Each station has the potential for becoming contaminated and potentially causing cross-contamination of \textit{C. jejuni} to subsequent carcasses [32].

3. Water and \textit{Campylobacter jejuni}

Water has been found to be a significant source of \textit{Campylobacter jejuni} contamination and is also used in large volumes throughout poultry processing. It is thought that chicks can be infected by untreated or contaminated drinking water [12, 28]. \textit{C. jejuni} has been recognized to survive in water in suspension or through attachment in biofilms to submerged surfaces [12, 28]. These aquatic biofilms may harbor pathogens through a variety of mechanisms including uptake by protozoa. While inside the protozoa, the bacteria may be protected, to an extent, from disinfection [12, 28]. Snelling et al., 2005 found \textit{C. jejuni} inside of a variety of protozoa in a broiler house. Their study showed that \textit{in vitro} \textit{C. jejuni} had increased resistance to disinfection techniques. This would suggest that the interactions of \textit{C. jejuni} and protozoa present new challenges in sanitation, and increase the potential of contamination on carcasses [12]. There are commercially available water treatment products that can reduce biofilms and foodborne pathogens [32, 33] however it is unknown if these treatments are commonly poultry processing and to what extent they can reduce the protozoal population.

4. Chemical Treatments in Poultry Processing Facilities

One approach to reduce pathogens from the surface of poultry is using “generally regarded as safe” (GRAS) chemicals. These chemicals are used throughout poultry
processing to not only remove debris from the carcass but also to reduce pathogens on the surface. Some of the most common GRAS compounds used in poultry processing are sodium hypochlorite (chlorine), trisodium phosphate (TSP), and cetylpyridinium chloride (CPC).

### 4.1 Sodium Hypochlorite (Chlorine)

Chlorine is the most common chemical treatment in poultry processing [32]. Traditionally chlorine, in the form of sodium hypochlorite or calcium hypochlorite, has been added to water used for washing carcasses because of its availability, efficiency and low cost [13, 34, 35]. Chlorine is limited by initial bacterial concentration, water level, temperature, pH, trace minerals, and organic load. Chlorine is only effective as a disinfectant when it is in its free form. When a carcass is rinsed with water with chlorinated water, the free/reactive chlorine binds to organic matter such as blood, tissue fragments, feces, etc. Due to the heavy organic load, much, if not all, of the reactive chlorine is used up and no longer an effective disinfectant [10, 13, 35]. To overcome this obstacle more chlorine would need to be added to the water but USDA regulations only allow poultry processing plants to use a concentration of 50ppm which is far exceeded by the organic load present [10, 35, 36]. Higher concentrations of chlorine have been reported to be more effective in reducing the level of pathogens from the surface of poultry carcasses. High concentrations of chlorine can have an adverse effect on creating a wholesome produce, resulting in discoloration, and change in odor and taste. Additionally, chlorine at high concentration not only corrodes metal but also poses a health concern for workers [32]. Blaser et al., 1986 showed that current methods of
treating water using chlorine are adequate for the elimination of *Campylobacter jejuni* [37].

### 4.2 Trisodium Phosphate (TSP)

Trisodium Phosphate (TSP) is a GRAS food additive that can affect the skin of the carcass allowing for bacteria to be washed from the surface more effectively with no alteration of any sensory aspects [32]. The mode of action of TSP includes exposure of microorganisms to high pH, effecting cell membrane components, sequestration of metal ions, and acting as a surfactant, enhancing detachment of bacteria from food surfaces by removing a thin layer of lipid from the chicken skin surface [38, 39]. TSP is approved for use by the USDA at levels of 8-12% and has been reported to reduce *Campylobacter jejuni* [38-40]. It has been reported that 5% TSP resulted in a 2.1 log$_{10}$ CFU reduction while 10% TSP resulted in a 2.2 log$_{10}$ CFU reduction [39]. Tripotassium phosphate (TPP) a similar compound to TSP has also been shown to significantly reduce the number of C. *jejuni* on the surface of chicken especially when mixed with lauric or myristic acid [34]. The concern with these compounds is that with such a high pH there is a likelihood of altered food properties, wear of industrial equipment, and environmental damage [38, 39]. To replace the need for high concentrations of TSP, Carniero et al., 1998 reported that the use of lower concentrations of TSP followed by treatment with lysozyme or nicin finding was effective in reducing pathogens [38].
4.3 Cetylpyridinium chloride (CPC)

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound. It kills bacteria by cetylpyridinium ions interacting with acid groups of the bacteria and inhibiting cell metabolism. It has been demonstrated to kill 0.9-1.7 log$_{10}$ CFU of *Salmonella* without affecting the quality of the food [41]. Another study reported CPC to reduce *Salmonella* 1.5 – 1.9 log$_{10}$ CFU at concentrations of 0.1 to 0.5% CPC respectively [39].

5. Current Problem with *Campylobacter jejuni* in Poultry

As mentioned *Campylobacter jejuni* is a major foodborne pathogen and primarily found on raw poultry. *C. jejuni* is sensitive to numerous environmental factors including high oxygen, low temperature, and low pH. All of these conditions can be found during poultry processing yet *C. jejuni* is able to survive. In addition, many interventions are in place during poultry processing to reduce *C. jejuni* from the surface of raw poultry but fail at completely eliminating this pathogen from the final product. It may be that other factors are involved that have been overlooked by current research. One factor in particular is the possible involvement of amoebae in the survival of *C. jejuni* under unfavorable environmental conditions as well as throughout poultry processing.

6. Amoebae

Amoebae are a group of organisms belonging to the kingdom Protista. Protists are a diverse group of eukaryotic organisms. They often referred to as Protozoa which is an unofficial group of heterotrophic species of Protists. All protozoa that are motile by
means of a finger-like projection called a pseudopod are referred to as amoebae. They consume bacteria, yeast, algae and other Protists by means of the receptor-dependent process phagocytosis and engulf large amounts of solutes and food particles by non-specific pinocytosis [42-44]. They may eat several types of prey, but tend to exhibit selectivity toward certain types of food [45]. Amoebae undergo asexual reproduction by binary fission. The life cycle of many amoebae consists of a vegetative trophozoite stage where they actively feed on bacteria and a dormant; cellulose based double-walled cyst stage when conditions are unfavorable. Conditions such as extremes in temperature, pH, salinity, and lack of food may stimulate encystation (formation of cyst). During the process of encystation excess food, water, and particulate matter are expelled and the trophozoite condenses into a precyst. The precyst then matures into a resistant double-walled cyst with the outer wall protecting the cyst from the hostile environment. Cellular levels of RNA, proteins, triglycerides, and glycogen decline significantly reducing cell volume and dry weight. While in the cyst stage the amoebae are protected from desiccation, starvation, disinfectants and antimicrobials, anoxia, extremes in temperature and pH, UV radiation, and lack of nutrients. Cysts can become airborne allowing the spread of amoebae throughout the environment. Cysts possess ostioles which are pores that monitor environmental changes. When conditions are once again favorable the trophozoite will reemerge from the cyst structure leaving behind the outer shell and continuing their life cycle (Figure 1.2) [42-44, 46, 47].
6.1 *Acanthamoeba* spp.

The genus *Acanthamoeba* is one of the most common genera of amoebae [42, 44]. Protists from this group are important predators that can control and alter the microbial communities that they reside in [48, 49]. *Acanthamoeba* species are characterized by acanthopodia which are spine-like structures on the surface of the trophozoite [42]. The purpose of these structures is not fully understood but they could aid in adhesion to surfaces, movement, or capturing prey. These organisms are ubiquitous in the environment and can be found in a wide range of temperatures, salinity, and pH conditions. *Acanthamoeba* spp. has been found in a wide variety of environmental habitats including fresh water, brackish water, sea water, beach sand, sewage, air, dust, compost, vegetables, and a range of soil types. In homes, *Acanthamoeba* spp. have been isolated from flowerpot soil, aquariums, humidifiers, water taps, contact lens cases and solutions, swimming pools, bottled water, and sink
drains. In hospitals they have been recovered from showerheads, ventilators, hydrotherapy baths, surgical equipment, dialysis units, eye wash stations, cooling towers and heating/ventilation/air systems [42, 44, 48]. *Acanthamoeba* spp. have been isolated from human cavities, pharyngeal swabs, lung tissue, skin lesions, corneal biopsies, and cerebrospinal fluid [42-44]. These organisms are human pathogens that can cause an ocular infection, *Acanthamoeba* keratitis, commonly associated with contact lens solution. They also can cause granulomatous amoeba encephalitis (GAE) in immunocompromised individuals as well as animals [43, 44].

*Acanthamoeba* can be cultured in xenic (with bacteria) or axenic (without bacteria) cultures. In xenic cultures they can be easily grown on non-nutrient agar or media containing low concentrations of nutrients i.e. PYG (peptone, yeast extract, and glucose) with the addition of live or killed bacteria. Common bacteria choices are *Klebsiella pneumonia*, *Enterobacter* spp., and *E. coli*. The typically avoid bacterial species with capsules because the presence of mucoid capsules around bacteria impedes phagocytosis. The concentration of glucose in particular is important in growing amoebae because in the presence of bacteria, too much glucose will lead to overgrowth of the bacteria, impeding amoeba growth [43]. Conversely, the lack of preferred prey or low prey densities can limit the ability of the amoebae to thrive [45]. To grow amoebae in axenic culture, media with higher concentrations of nutrients supplemented with antibiotics (penicillin, gentamicin, and streptomycin), to kill bacteria in culture is used. Although there are a number of media types used for the growth of *Acanthamoeba* axenically, they primarily are different variations of PYG [45].
6.2 *Acanthamoeba* spp. and endosymbiotic bacteria

Many species of protozoa including many amoebal species have been found to harbor a wide variety of endosymbiotic bacteria including human pathogens [43, 44, 50-52]. These bacteria are phagocytized but not digested [43, 44, 50-52]. The pressures of predation, harsh environments, and nutrient availability have shaped complex defense strategies allowing some bacteria to survive and sometimes replicate inside of amoebae [43, 44, 50-52]. When bacteria are inside of amoebae, they are protected from desiccation, antimicrobials, atmospheric conditions, and chemicals and are possibly provided with nutrients [43, 50, 51]. Some bacteria have also been shown to reside inside of amoebal cysts which further protect them from environmental conditions, enabling spread to new habitats via the airborne cyst [44, 51, 53-55]. It is believed that amoebae act as reservoirs, allowing some bacterial pathogens to persist under unfavorable conditions and facilitating the dispersal of these bacteria in the environment thereby potentially increasing disease transmission [50]. It is not known if amoebae benefit from this association but they may acquire nutrients [45]. It has also been reported that certain endosymbiotic bacteria may affect motility of the amoebae [56]. This endosymbiosis may result in one of three outcomes: 1) bacteria that multiply intracellularly and eventually lyse the amoeba, (i.e. *Legionella* spp. [57, 58], *Salmonella* spp. [59, 60], *Campylobacter jejuni* [23]); 2) bacteria that multiply intracellularly but do not cause cell lysis, (*Vibrio cholerae* [53], *Shigella* spp. [61]); and 3) bacteria that survive intracellularly without replication (*Mycobacterium* spp. [62]) [46, 55]. Symbiosis with pathogens often results in damage or death to the amoebae but many non-pathogens may cause little or no harm to the amoebae [45]. In addition, while inside of amoebae,
intracellular bacteria, particularly *Legionella* spp. and *Chlamydia* spp., have been reported to exchange genes [63]. Amoebae have also been reported to support the growth of certain pathogens by secretion of extracellular factors. *Vibrio parahaemolyticus* has been reported to avoid phagocytosis by *Acanthamoeba castellanii* but was able to survive in culture longer in the presence of the amoeba [64]. Similar results were reported with *Listeria monocytogenes* where *L. monocytogenes* was able to utilize materials released from amoebae to survive extracellularly [65]. *C. jejuni* was reported to survive outside of *A. castellanii* due to the amoebae reducing harmful dissolved oxygen levels in the media [66].

Amoebae are “primordial phagocytes” with similar features as mammalian phagocytes such as macrophages. The development of these strategies or mechanisms that allow for intracellular survival within protozoa may have primed these amoebae associated bacterial pathogens for invasion into macrophage, facilitating human illness [50]. This has led to the description of amoebae as pathogen “training grounds” because the pathways of destruction the bacteria need to avoid/survive internalization by amoebae are similar to what is needed to avoid/survive the human immune response [45]. Cirillo et al., 1999 demonstrated that *Legionella pneumophila* grown in *Acanthamoeba castellanii* reported increased replication in monocytes and was more virulent [57]. Similarly Feng et al., 2009 reported an up-regulation of *Salmonella* virulence genes while inside of *Acanthamoeba rhysodes* [59].

A common feature of many non-pathogenic endosymbionts found inside of amoebae is their close phylogenetic relationship to other obligate intracellular pathogens
Phylum-level diversity of these endosymbionts suggests that this exploitation of amoebae has evolved independently with the type of interaction varying between lineages [46, 48, 51]. Five evolutionary lineages have been identified as endosymbionts of amoeba. These lineages include the Alphaproteobacteria, Betaproteobacteria, and Gamaproteobacteria classes (phylum Proteobacteria) along with the phyla Bacteroidetes and Chlamydiae [46, 48].

6.3 Acanthamoeba spp. and Pathogens

Initial studies investigated the role of protist species in the environmental survival and pathogenicity of the pathogen Legionella pneumophila and other Legionella spp. This Protist: L. pneumophila interaction has led to extensive investigation into what other species of bacteria can survive inside of protozoa (Table 1.1). Each pathogen interacts with amoebae differently so it is important to compile and understand what is currently known of the interactions of all of the different pathogens with amoebae to provide better insight into what may be happening during the interaction of Acanthamoeba castellanii and Campylobacter jejuni.
<table>
<thead>
<tr>
<th>Host</th>
<th>Bacteria</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba astronyxis</td>
<td>Burkholderia pseudomallei</td>
<td>[52]</td>
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<tr>
<td>Acanthamoeba culbertoni</td>
<td>Legionella feeleii, Legionella pneumophila</td>
<td>[52]</td>
</tr>
<tr>
<td>Acanthamoeba griffin</td>
<td>Legionella pneumophila</td>
<td>[52]</td>
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<td>Acanthamoeba lenticulata</td>
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<td>[52]</td>
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<td>Acanthamoeba palestinensis</td>
<td>Legionella pneumophila</td>
<td>[52]</td>
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<tr>
<td>Acanthamoeba rhysodes</td>
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<td>[59, 60, 79]</td>
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<td>Acanthamoeba royreba</td>
<td>Legionella pneumophila</td>
<td>[52]</td>
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Table 1.1 (continued)

| Balamuthia mandrillaris | Legionella pneumophila | [52] |
Table 1.1 (continued)

<table>
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<tr>
<th>Organism</th>
<th>Pathogens</th>
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<tr>
<td>Dictyostelium discoideum</td>
<td><em>Legionella pneumophila, Legionella</em> – like amoebal pathogens, <em>Mycobacterium avium, Psuedomonas aeruginosa, Salmonella, Vibrio cholerae</em></td>
<td>[52, 103-109]</td>
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<td>Echinamoeba exundans</td>
<td><em>Legionella pneumophila</em></td>
<td>[52]</td>
</tr>
<tr>
<td>Entamoeba dispar</td>
<td><em>Escherichia coli, Shigella</em> spp.</td>
<td>[110]</td>
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<tr>
<td>Hartmannella cantabrigiensis</td>
<td><em>Legionella pneumophila</em></td>
<td>[52]</td>
</tr>
<tr>
<td>Hartmannella vermiformis</td>
<td><em>Campylobacter lari, Campylobacter coli, Campylobacter hyointestinalis, Campylobacter jejuni, Escherichia coli, Legionella-like ameobal pathogens, Neochlamydia hartmannellae, Sarcobium lyticum</em></td>
<td>[49, 51, 52, 54, 79, 101]</td>
</tr>
<tr>
<td>Naegleria australiensis</td>
<td><em>Legionella pneumophila</em></td>
<td>[52]</td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td><em>Bacillus licheniformis, Legionella pneumophila</em></td>
<td>[52]</td>
</tr>
<tr>
<td>Naegleria gruberi</td>
<td><em>Legionella pneumophila, Legionella spp., Vibrio cholera</em></td>
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</tr>
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<td>Naegleria Iovaniensis</td>
<td><em>Legionella pneumophila</em></td>
<td>[52]</td>
</tr>
<tr>
<td>Naegleria jadini</td>
<td><em>Legionella pneumophila</em></td>
<td>[52]</td>
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<td>Platyamoeba placida</td>
<td><em>Legionella pneumophila</em></td>
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<td>Saccamoeba spp.</td>
<td><em>Ehrlichia-like, Legionella pneumophila</em></td>
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Table 1.1 (continued)

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<th>Legionella pneumophila</th>
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<tr>
<td>Vexillifera spp.</td>
<td>Legionella pneumophila</td>
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<td>[52]</td>
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<tr>
<td>Willaertia spp.</td>
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<td>[52]</td>
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</table>

### 6.3.1 Legionella pneumophila

The first protozoa/pathogen interactions to be studied at length was the interaction of different species of protozoa with *Legionella pneumophila* [58]. *Legionella pneumophila* is the causative agent of a severe pneumonia known as Legionnaire’s disease as well as a milder infection referred to as Pontiac fever [54, 116]. This organism can be found in freshwater habitats where it can infect a wide range of protozoan species [116, 117]. While in protozoa, *L. pneumophila* can be aerosolized and inhaled by humans i.e. via air conditioning units [54, 116]. *L. pneumophila* has developed strategies to evade the transport to the lysosome after phagocytosis and establish a unique endoplasmic reticulum-derived organelle [106, 116]. Once inside of the protozoan *L. pneumophila* replicates and when nutrients begin to become limited, they induce necrosis in the amoeba, lysing the host cell to allow for invasion into new eukaryotic hosts [88, 106, 116]. This process is also seen when *L. pneumophila* infect human alveolar macrophages and it is believed that similar survival strategies are being employed [54, 116, 117]. Virulent strains have been reported to be more successful in surviving and multiplying inside of the host [54]. *L. pneumophila* grown in *Acanthamoeba castellanii*
showed an enhanced monocyte entry, an increased intracellular replication in monocytes, and increased virulence in mice compared to \textit{L. pneumophila} cultured in growth media \cite{57}. A second study comparing \textit{A. castellanii}, cultured \textit{L. pneumophila}, and agar cultured \textit{L. pneumophila} demonstrated a 100-fold increase in epithelial cell invasion and a 10-fold increase in macrophage entry of bacteria cultured in \textit{A. castellanii} \cite{72}. Growth inside of aquatic protozoa seems to have generated a pool of virulent traits during evolution, which has allowed for this infection into human cells \cite{117}. The genome sequence of \textit{Legionella pneumophila} encodes a high number of eukaryotic like proteins which may be used to interfere with different stages of the intracellular cycle in their host by mimicking functions of eukaryotic proteins \cite{117}.

Protozoan hosts for \textit{Legionella pneumophila} include 15 species of amoeba such as \textit{Acanthamoeba spp.}, \textit{Hartmannella spp.}, \textit{Vahlkamphia spp.}, \textit{Echinamoeba spp.}, and \textit{Dictyostelium discoideum} as well as some ciliate Protists \cite{54, 88, 103}. It has been shown that \textit{Acanthamoeba castellanii} is the most efficient in supporting growth of \textit{L. pneumophila} \cite{54}, and \textit{Acanthamoeba spp.} have been used in co-culture to isolate \textit{Legionella} spp. from environmental and clinical samples originally negative for \textit{Legionella} spp. by culturability tests \cite{118, 119}. \textit{Acanthamoeba polyphaga} has also been reported to protect internalized \textit{Legionella pneumophila} from exposure to sodium hypochlorite \cite{120} and other biocides \cite{55}.

\textbf{6.3.2 Vibrio spp.}

\textit{Vibrio cholerae} is a waterborne pathogen common in underdeveloped countries. \textit{V. cholerae} cultures grown in the presence of \textit{Acanthamoeba castellanii} showed an
increase in population over 2 weeks, while cultures without amoeba were non-detectable after 4 days [53]. Microscopic evaluation revealed *A. castellanii* trophozoites had *V. cholerae* cells localized in vacuoles and multiplication was observed in the cytoplasm. *V. cholera* cells were also discovered in amoebal cysts both inside the cyst as well as in the space between the double-walls of the cyst [53]. It has been reported that *V. cholerae* uses a type six secretion system to be able to infect amoebae [109].

*Vibrio mimicus* in co-culture with *Acanthamoeba castellanii* had a reported increase in survival compared to *V. mimicus* cultured without *V. mimicus* [78]. *V. mimicus* was found localized in the cytoplasm of trophozoites for 14 days [78]. *V. mimicus* was also reported to survive in *A. castellanii* cysts where they were protected from high levels of gentamicin [78].

In contrast, *Vibrio parahaemolyticus*, a foodborne pathogen found in seafood, is predated on by *Acanthamoeba castellanii* [64]. Interestingly, although predated upon, *V. parahaemolyticus* survives extracellularly in the presence of *A. castellanii* by means of secreted factors by the amoebae. These factors allow *V. parahaemolyticus* to exist in a non-proliferative state under non-favorable conditions while in the presence of amoeba [64].

6.3.3. *Mycobacterium* spp.

Multiple species of the genus *Mycobacterium* have been investigated to determine their interaction with amoeba species. *Mycobacterium tuberculosis* and *Mycobacterium marinum* have been reported to survive phagocytosis by the amoeba *Dictyostelium* spp.
25. Instead of lysing the amoebal host like many other pathogens these organisms replicate and utilize the host’s processes to eject themselves from inside of host the cell, allowing them to spread to another host cell [107]. Another study investigating a bovine pathogen, Mycobacterium bovis showed that the organism was able to survive in Acanthamoeba castellanii cysts [83]. It was reported that a virulent strain of M. bovis was able to survive in amoebae more effectively when compared to the attenuated vaccine strain [83]. Mycobacterium avium has also been reported to infect multiple strains of Acanthamoeba spp., protecting them from Monochloramine [100]. M. avium subspecies paratuberculosis has been reported to be internalized and replicate into both A. castellanii and Acanthamoeba polyphaga where they were protected from chlorine exposure [77]. M. ulcerans was reported to survive inside A. polyphaga for at least two weeks [89].

Adekambi et al., 2006 investigated the ability of twenty six environmental Mycobacteria spp. to survive internally inside of A. polyphaga and reported that all species survived in the amoeba trophozoites over 5 days and in amoebal cysts for over 15 days [87]. Phylogenic analysis comparing Mycobacterium spp. and other amoebae-resistant bacteria has revealed eight Mycobacteria open-reading frames that were probably acquired by horizontal gene transfer while inside of amoebae [121].

6.3.4 Order Chlamydiales

Many organisms in the order Chlamydiales have been shown to survive inside of amoebae [122, 123]. Simkania negevensis has been reported to survive and replicate within Acanthamoeba polyphaga for more than 15 days [123]. It was found to survive
both inside of the cyst as well as between the double walls of the amoebal cyst [123]. Similarly *Parachlamydia acanthamoeba* has been reported to utilize *A. polyphaga* as part of their life cycle [122]. *Protochlamydia* has also been reported to increase the motility of *A. castellanii* while inside of the amoebae [56].

### 6.3.5 *Francisella tularensis*

*Francisella tularensis*, the causative agent of tularemia (Rabbit Fever) has been reported to not only survive inside of *Acanthamoeba castellanii*, but to also cause rapid encystation of the amoebae [102]. This encystation is essential for *F. tularensis* to survive adverse conditions, and they have been shown to survive in these cysts for up to 3 weeks [102].

### 6.3.6 *Salmonella* spp.

Evidence has shown that the relationship between *Salmonella* spp. and survival inside of amoebae as well as other protists is related to strain, serotype, and virulence of the *Salmonella* strain [59, 60, 113, 114, 124]. Invasive strains were recovered in higher concentrations from amoebae than non-invasive strains. There has also been a reported preferential uptake of certain *Salmonella* serotypes by *Acanthamoeba* spp. [60, 124]. It has been reported that feed preferences of different *Salmonella* O serovars is reflected by the environment in which the protist was isolated more than the protist’s taxonomic relationship [124]. Once inside of *Acanthamoeba rhysodes*, *Salmonella* spp. begin to replicate at 6 hours post infection, virulence genes are up-regulated and after 24 hours the amoebae begin to die by a *Salmonella* mediated apoptosis like cell death [59].
*Acanthamoeba polyphaga*, *Salmonella* was reported to have a lag phase upon entry into amoebae followed by intracellular replication [92]. *Salmonella* has also been reported to survive inside of the ciliate *Tetrahymena* spp. where they are taken from an undigested food vacuole to a vesicle that is released. This vesicle has been shown to protect *Salmonella* from chlorine [59]. While inside of *Tetrahymena* spp., *Salmonella* up-regulates genes involved in anaerobic metabolism, acid resistance, and many genes utilized for human epithelial and macrophage invasion [114]. *Salmonella enterica* Typhi has an increase in survival when co-cultured with *A. castellanii* but showed no cytotoxic effect on the amoebae [73].

### 6.3.7 *Listeria monocytogenes*

The interaction between *Listeria monocytogenes* and *Acanthamoeba* spp. has been reported with conflicting results. One study reported that *Acanthamoeba castellanii* does not actively kill *L. monocytogenes* and populations can be maintained for at least 96 hours [69]. A second study reported intracellular survival for the duration of the study (72 hours) [92]. There was no difference in survival when comparing serotypes or other *Listeria* spp. [69]. In addition, mutants lacking the *hly* gene, a virulence gene showed decreased survival [69]. A second study reported that *Listeria monocytogenes* could not survive or replicate in *Acanthamoeba polyphaga*, *A. castellanii*, or *Acanthamoeba lenticulatta*, and once phagocytized, only survived a few hours before being digested in the lysosome [65]. When comparing virulent strains containing the *hly* gene with avirulent strains lacking *hly*, there was no difference in survival. Interestingly, although unable to survive internalization, *L. monocytogenes* was able to grow for extended
periods of time in the presence of *Acanthamoeba* spp. compared to *L. monocytogenes* cultured in the absence of amoebae. It was reported that *L. monocytogenes* survives by utilizing materials released by the amoebae [65, 92]. Another study reported that *L. monocytogenes* did not survive intracellularly and, in addition, reported the observation of a unique “backpacking” strategy employed by *Acanthamoeba* spp. [74]. Observations of this study reported aggregations of bacteria on the surface of the amoebae where they were immobilized and held together by filaments thought to be created by the amoebae [74]. The amoebae were seen phagocytizing the “backpack” and repeating the process. It was indicated that the formation of this “backpack” is likely not *Listeria* spp. specific and is independent of motility, pathogenicity, or virulence of the bacterial strain [74].

### 6.3.8 Escherichia coli

Although *Escherichia coli* is a common food source for amoeba [43, 101] virulent strains have been shown to survive in *Acanthamoeba polyphaga* [94] and *Acanthamoeba castellanii* [70]. In a study involving *Acanthamoeba polyphaga*, *E. coli* O157:H7 survived over 35 days at 25°C in co-culture with amoebae compared to 10 days in the absence of amoebae [94]. Viable *E. coli* were discovered first inside vacuoles and then being expelled into the environment [94]. *E. coli* cells were also seen inside of amoebal cysts [94]. It was reported that at both 4°C and 25°C some *E. coli* was digested but not at a rate to eliminate all bacteria from the inside of *A. polyphaga* [94]. Further investigation with enterohemorrhagic *E. coli* (EHEC) reported that more viable cells were recovered after three weeks when co-incubated with *A. castellanii* and less cells shifted to a viable but non-culturable state [125]. In addition it was reported that shiga toxins (stx) are not
involved in this relationship [125]. Although shiga toxins are not involved, another
virulence factor, the phosphate regulon (pho), which allows for adaptation to low
concentrations of phosphate, is required for internalization into *A. castellanii* [125].

Another study investigated the relationship of *A. castellanii* and *E. coli* K1
(capsule serogroup 1), an invasive strain, and K12 strains, a non-invasive strain [70]. It
was reported that *E. coli* K1 showed a higher association with *A. castellanii* and increased
invasion of/uptake by *A. castellanii* [70]. *E. coli* K1 remained inside of the amoebae and
multiplied, while *E. coli* K12 was quickly digested by the amoebae [70].

### 6.3.9 *Shigella* spp.

*Shigella dysenteriae* and *Shigella sonnei* have been shown to reside in both
*Acanthamoeba castellanii* trophozoites and cysts for up to 3 weeks [61]. Co-cultures of
*S. dysenteriae* and *S. sonnei* with *A. castellanii* showed enhanced growth when compared
with cultures in the absence of amoebae, 100-fold and 10-fold after 3 days, respectively
[61]. Internalized *Shigella* spp. grew at the same rate at temperatures of 20°C and 30°C
and did not adversely affect the amoebae [61]. A different study reported inhibition of *A.
castellanii* at 30°C [126]. At 37°C however, virulence genes on the invasion plasmid of
*Shigella* spp. are expressed and the amoebae are killed [61, 126].

### 6.3.10 *Acanthamoeba* spp. and Other Pathogenic Bacteria

*Enterobacter faecalis* [92] and *Staphylococcus aureus* [91, 92, 99] have been
reported to persist and grow intracellularly in *Acanthamoeba polyphaga*. Both
methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) strains of *S. aureus* were
able to bind and invade *Acanthamoeba castellanii* similar to their interactions with macrophage-like cells, surviving encystation [99]. *Aeromonas hydrophila* and *Yersinia enterocolitica* were reported to show internalization into *A. polyphaga*, followed by an initial replication and probably a subsequent digestion represented by reduced recovery or absence of bacterial recovery after 48-72h [92]. *A. castellanii* has also been reported to serve as an amplifier for *Bacillus anthracis* (Anthrax) spores [98]. Anthrax spores are able to germinate within amoebal phagosomes even when environmental conditions are unfavorable. The vegetative cells then multiply within the amoebae, eventually causing their death [98]. The vegetative cells, now outside of the protective environment, re-sporulate [98].

### 6.4 *Acanthamoeba* spp. and *Campylobacter jejuni*

Previous studies have already investigated the interaction of *Campylobacter jejuni* and *Acanthamoeba* spp. and have provided evidence to support further investigation of the relationship between these two organisms. *C. jejuni* has been reported to survive and replicate inside of *Acanthamoeba polyphaga* ([Figure 1.3]) [23, 86], *Acanthamoeba castellanii* [12, 67, 75], and *Acanthamoeba rhyhodes* [79]. It has been reported that not only can *C. jejuni* survive phagocytosis by *Acanthamoeba polyphaga* but also that it survives for a longer duration in the presence of amoebae [23]. Similar results were reported with *A. castellanii* [12]. Additionally *A. polyphaga* was able to resuscitate cultures that were previously negative by culturability tests and have been shown to enrich a culture starting with only 10 cells of *C. jejuni* [23, 86]. While in *A. polyphaga* the relatively fragile *C. jejuni*, being microaerophilic and extremely sensitive to
atmospheric levels of oxygen, are able to be cultured and enriched at low temperatures under normal atmospheric conditions [23, 86]. Microscopic studies performed while *C. jejuni* was co-cultured with *A. polyphaga* revealed that initially *C. jejuni* aggregate at specific sites near the cell wall of the amoeba where they seemed attached to the cell wall. One hour after inoculation *C. jejuni* could be seen moving within amoebic vacuoles. At 37°C under microaerophilic conditions, *C. jejuni* begins to divide rapidly resulting in the lysis of the amoeba (**Figure 1.3**) [23, 75]. A different study reported that co-culturing of *A. castellanii* and *C. jejuni* did not result in an increase in either organism but did show a delayed decline in *C. jejuni* populations as well as an increased long-term survival [75]. It has also been reported that low invasive strains (based on epithelial cells) of *C. jejuni* had higher survival rates compared to more invasive *C. jejuni* strains [75].

*C. jejuni* has shown a greater resistance to chlorine [67] and Virudine, an iodine-based disinfectant, while inside amoebae [12]. While in *A. polyphaga*, *C. jejuni* was able to survive at a pH as low as 2 for 5 hours [93]. Interestingly at a pH of 4-5 *C. jejuni* motility increased as well as adhesion and internalization into amoebae [93]. Snelling et al., 2008 have also reported that *C. jejuni* internalized in *A. castellanii* could still colonize broiler aged chickens [68].
Figure 1.3: Internalization of *Campylobacter jejuni* by *Acanthamoeba polyphaga*.

Early in the infection model, *C. jejuni* cells aggregated at certain positions on *A. polyphaga* cell walls (A and B), and after some time, live bacterial cells were observed in amoebic vacuoles (C and D). Subculturing of culture-negative samples together with fresh amoebae at 37°C resulted in lysis of the amoebae, after which live *C. jejuni* cells could be retrieved (E). Obtained from [23].

A more recent study by Bui et al., 2012, employing slightly different methods reported conflicting results compared to the previously mentioned studies. In this study it
was reported that *C. jejuni* does not survive internalization into amoebae but survives extracellularly [66]. Due to the microaerophilic nature of *C. jejuni*, oxygen at high levels is lethal, however when co-incubated with *A. castellanii* it was reported that the amoebae reduced the dissolved oxygen levels in the media allowing for *C. jejuni* to survive longer under non-microaerophilic conditions [66].

7. The Gentamicin Protection Assay (GPA)

The gentamicin protection assay (GPA) is a useful tool to investigate internalization of bacteria into eukaryotic cells [127]. Briefly, the gentamicin protection assay begins with a co-incubation step to allow for the bacteria to enter the eukaryotic host. The samples are then washed to remove most of the extracellular bacteria leaving only bacteria that were either internalized by the eukaryotic host or attached to the eukaryotic cell. Gentamicin is then added to the samples to kill any remaining extracellular bacteria, including those that are attached. Eukaryotic cells are more resistant to gentamicin than bacterial cells and are able to protect any internalized bacteria. The samples are then washed again to remove any residual gentamicin and eukaryotic cells are lysed to release intracellular bacteria. This research focuses on *C. jejuni* and *A. castellanii* therefore experimental variables were compared among other studies investigating the interaction of bacterial pathogens with *Acanthamoeba* spp.

The co-incubation step in the GPA can vary by what the samples is being co-incubated in. Previous studies have employed 6-well [60, 66, 69], 12-well [23, 79, 86, 93], or 24-well [64, 70, 71, 76] tissue culture plates or 75cm² tissue culture flasks [53, 61, 73] for co-incubation of *Acanthamoeba* spp. with bacteria. For those used in gentamicin
protection assays, amoebae needed to be seeded days prior to the experiment to obtain a confluent monolayer and amoebae counts can estimated by sacrificing wells prior to the experiment or in some cases not counted until later in the experiment [70, 71, 76]. Other studies with known amoebae concentrations being added had to be incubated 2-24h to allow attachment to the bottom [60, 66].

Multiplicity of infection or MOI (ratio of bacterial cells: amoebal cells) is also variable among studies. The density of bacteria in co-culture with A. castellanii has shown to have an influence on the growth and survival of A. castellanii with higher levels of bacteria being more inhibitory [128]. It is not known whether this effect can be seen at shorter time points as employed in some studies since this study tested the effect at 48h. Common multiplicities of infection used included 1:1 [12, 53], 10:1 [64, 73, 79], 100:1 [61, 66, 75, 91], and 1,000:1 [61, 91] C. jejuni: Acanthamoeba spp.

The duration of co-incubation as well as the co-incubation temperature also varies among studies. For those performing gentamicin protection assays the co-incubation times included 30m [73], 1h [60], 2h [60], and 3h [66]. Co-incubation time may be an important factor due to the amount of time it takes for bacteria to enter amoebae as well as the amount of time it takes amoebae to digest the bacteria that can be digested. If co-incubation times are too short it may lead to the belief that a bacteria is not internalize when in fact more time was required for sufficient internalization. In addition, if amoebae are lysed without sufficient time for lysosomal breakdown of bacteria that can be utilized as a food source it may give the impression that the bacteria survived internalization. Temperature is an interesting variable because lower temperatures are
more representative of most environmental interactions of these organisms; however other studies used 37°C which is an optimal temperature for most human pathogens. It may be that higher temperatures such as 37°C may activate factors in certain bacteria causing a different result. With *C. jejuni* co-incubation at 25°C showed survival but no replication [12, 23] but at 37°C replication and eventual lysis of *A. polyphaga* can be shown [86]. Additionally at 37°C many of these bacterial pathogens may be more metabolically active, depleting nutrients faster which may alter the interaction with amoebae.

8. Conclusion

*Campylobacter jejuni* is a major foodborne pathogen. Despite many efforts to eliminate *C. jejuni* from poultry it remains a persistent cause of foodborne illness. It may be that unidentified factors, such as of free-living amoebae, could be aiding in the persistence of *C. jejuni*. Amoebae such as *Acanthamoeba castellanii* are eukaryotic cells that tend to be much more resilient to environmental factors and many chemical disinfectants than bacterial cells. There has been extensive research on the role of protozoa in the life cycle and pathogenicity of *Legionella pneumophila* and subsequent investigations of protozoa with other bacterial pathogens. This extensive work, combined with studies already supporting a role of amoebae in *C. jejuni* protection suggests a need for further investigation into this interaction.

Current research investigating the interactions of bacterial pathogens and protozoa employs a traditional gentamicin protection assay (GPA) that utilizes cell culture plates and a protozoa monolayer. Briefly, this assay involves the co-incubation of bacteria with
the protozoa to allow internalization. Following co-incubation gentamicin is applied to eliminate all bacterial cells outside of the protozoan host. The antibiotic is then washed away and the protozoa are lysed to release internalized bacteria. The sample is then plated to determine either the presence or the amount (CFU) of internalized bacteria.

Although this traditional GPA is a useful tool to investigate the internalization of bacteria by amoebae it has some limitations. The traditional GPA uses a monolayer of amoebae in tissue culture plates. When using a monolayer there is no way to control for the amount of amoebae used in the assay. Typically wells are sacrificed prior to experiments to obtain an estimation of the amount of amoebae in the wells and bacterial inoculum is adjusted based on this estimation. The monolayer can also be easily washed away, requiring wash steps to be performed by gently pipetting liquid, taking care not to disturb the monolayer. Most importantly, by using cell culture plates there is not effective way to employ a bacteria only control to determine the survival of the bacteria in the absence of amoebae. Bacteria are easily washed out of cell culture plates without a monolayer to attach to. Without bacteria only controls investigators rely on preliminary experiments to determine the sensitivity of the bacteria to gentamicin and assume that bacteria survival (if any) is negligible. In the absence of bacteria only controls, internalization is determined by the presence of bacteria at the conclusion of the assay.

The primary goal of this study was to modify the traditional GPA to address these limitations and then employ this assay to investigate in the interaction of *C. jejuni* and *Acanthamoeba castellanii*. This modified gentamicin protection assay may provide additional insight into this interaction that was not available using the traditional assay.
Chapter 2: Development of a Modified Gentamicin Protection Assay to Investigate the Interaction between *Campylobacter jejuni* and *Acanthamoeba castellanii*

Prepared for Submission to Journal of Microbiology Methods

Abstract

*Campylobacter jejuni* is one of the leading causes of foodborne illness in the United States and is persistent on poultry products despite its challenges to grow under atmospheric conditions and current strategies to control it during poultry processing. A number of bacterial pathogens including *C. jejuni* have been shown to survive predation by free-living amoebae such as *Acanthamoeba castellanii*, where the bacteria are phagocytized by the protozoa but survive internally. It has been shown that *C. jejuni* internalized by *Acanthamoeba* spp. are protected from harmful environmental factors and are able to survive for longer periods of time during unfavorable conditions. In this study a modified gentamicin protection assay (mGPA) was developed to address limitations in the traditional (GPA). The mGPA utilizes tubes compared to cell culture plates, allowing for the use of *C. jejuni* only controls to determine the survival of *C. jejuni* throughout the assay in the absence of *A. castellanii*. Previous studies employing a traditional GPA determined internalization by the recovery of *C. jejuni* at the conclusion of the assay. This mGPA determines internalization by the recovery of higher levels of *C. jejuni* in the presence of *A. castellanii* compared to *C. jejuni* controls in the absence of *A. castellanii*. Using the mGPA it was determined that *C. jejuni* survives the assay independently of *A.
castellani and quantification of this survival indicated variation in the internalization of 
*C. jejuni* by *A. castellanii* The age of the amoebae culture and nutrient depletion were 
examined to determine if these variables had an effect on internalization of *C. jejuni* by 
*A. castellanii*. This mGPA assay was able to demonstrate that internalization of *C. jejuni*
into *A. castellanii* is not a consistent event. While early growth phase cultures as well as 
nutrient deprived cultures of *A. castellanii* appeared to internalize *C. jejuni* more 
consistently, internalization varied. Older maintained monolayer amoebae cultures 
consistently failed to internalize *Campylobacter jejuni*. This modified gentamicin 
protection assay is able to quantify the level of *Campylobacter jejuni* that survives the 
assay allowing for lack of internalization to be determined, a control step which is 
lacking from current assays. This mGPA has proven to be a useful tool in further 
providing insight into the interaction of bacterial pathogens with free-living amoebae.

**Introduction**

*Campylobacter jejuni* is one of the leading causes of foodborne illness in the U. S. 
and is a common poultry contaminate [3, 9]. Despite efforts to control and eradicate *C. jejuni* during processing, it is still persistent on poultry. *C. jejuni* is relatively fragile in 
both the environment and laboratory settings due to its sensitivity to pH, desiccation, and 
its requirement for a microaerophilic environment (85% N₂, 10% CO₂, 5% O₂) [3, 23] yet 
*C. jejuni* is able to survive poultry processing [3]. The factors involved in this 
persistence remain unclear leading investigation into different areas of research.

*Acanthamoeba castellanii* is a species of free-living protozoa that is ubiquitous in 
the environment. This amoeba has been isolated from many sources including fresh,
stagnant, and chlorinated water [42], as well as poultry houses [12, 129] and meat cutting plants [130]. *Acanthamoeba* spp. feed on bacteria by phagocytosis and numerous studies have reported intracellular survival of bacterial pathogens after ingestion. Some bacterial pathogens of note include *Legionella pneumophila* [116], *E. coli* [67, 70, 71, 76], *Salmonella* spp. [60, 67, 73], *Shigella* spp. [61, 67], *Vibrio cholerae* [53], *Staphylococcus aureus* [91], *Enterobacter faecalis* [92] and *Campylobacter jejuni* [12, 23, 67, 75, 79, 86, 93].

Previous studies investigating the interaction of *Acanthamoeba* spp. and *C. jejuni* have reported delayed decline of *C. jejuni* cultures and a longer survival period at both sub-optimal temperatures as well as under aerobic conditions [23, 75, 79]. Co-incubation with *Acanthamoeba polyphaga* has also been reported to resuscitate *C. jejuni* cultures which previously resulted in negative culturability tests [23, 86], and this co-incubation has been reported as an effective *C. jejuni* aerobic enrichment method when compared to conventional enrichment procedures [86]. In addition to survival from unfavorable environmental conditions, the co-incubation of *C. jejuni* with *Acanthamoeba* spp. has been reported to provide *C. jejuni* with increased acid tolerance [93] as well as protection from chlorine [67] and other chemical disinfectants [12].

The gentamicin protection assay (GPA) is a traditional method commonly used to determine the internalization of *C. jejuni* into human intestinal epithelial cells [127] and has also been used to determine internalization of other bacteria into *Acanthamoeba* spp. [60, 70]. Briefly, the goal of the GPA is to eliminate extracellular bacteria with gentamicin leaving only internalized bacteria (protected inside of the eukaryotic cell).
Previous studies have employed a variety of tissue culture plates or 75cm² tissue [23, 53, 60, 64, 66, 69-71, 73, 76, 79, 86, 93] for co-incubation of Acanthamoeba spp. with bacteria. For those used in gentamicin protection assays, amoebae needed to be seeded days prior to the experiment to obtain a confluent monolayer, and the amount of amoebae in each well is estimated by sacrificing wells prior to the experiment or, in some studies, not determined until later in the experiment [70, 71, 76]. Other studies which attempted to more accurately account for amoeba concentration added known amounts of amoebae to cell culture plates, however these plates needed to be incubated 2-24h to allow attachment to the bottom of the plate [60, 66]. Following co-incubation the wells are washed by removing the liquid by pipetting and replacing it with a buffer prior to the addition of media containing gentamicin. The wells are then washed again and the amoebae are lysed to release intracellular bacteria. Samples are then plated to determine either the presence or amount (CFU) of bacteria internalized.

Using tissue culture plates and flasks may be problematic for three reasons. In utilizing a confluent monolayer, exact number of amoebae may vary among samples and/or trials. Second, an amoebal monolayer may not be completely stable leading to partial loss of sample during the wash steps. Finally, with the cell culture plate/flask method there is no way to employ an effective control to determine the fate of C. jejuni in the absence of amoebae because bacteria are easily washed away from wells without an amoebal monolayer to attach to.

The goal of the present study was to modify the currently used GPA to allow for more accurate A. castellanii inoculum in the co-culture and a more suitable C. jejuni only
control, while decreasing the amount of unwanted loss due to washing steps. This mGPA was then used to investigate the interaction of \textit{C. jejuni} and \textit{A. castellanii} under different \textit{A. castellanii} culture conditions.

**Methods**

\textbf{Strains and Growth Conditions.} \textit{Campylobacter jejuni} NCTC 11168 was obtained from the American Type Culture Collection (ATCC Manassas, VA) and stored at -80°C in Mueller Hinton Broth (MHB) with 10% glycerol. All \textit{C. jejuni} cultures were incubated at 37°C under microaerophilic conditions (85% N\textsubscript{2}, 10% CO\textsubscript{2}, and 5% O\textsubscript{2}) in a Binder (Bohemia, NY) CB150 incubator. Frozen working stocks were streaked for isolation onto Mueller Hinton Agar with 5µg/mL trimethoprim (MHA) and incubated 48h followed by two 24h passages with sterile swabs onto fresh MHA plates. Prior to experiments MHA plates containing \textit{C. jejuni} were swabbed and resuspended in MHB and CFU/mL estimated using a standard curve based on absorbance at 600nm (Figure 2.1). \textit{C. jejuni} inoculum was adjusted to 1 x 10\textsuperscript{9} CFU/mL by centrifugation at 3220 x g for 20m at 4°C and resuspended in Proteose Peptone-Yeast Extract-Glucose medium (PYG). \textit{Acanthamoeba castellanii} ATCC 30010 was obtained from ATCC (Manassas, VA). \textit{A. castellanii} cultures were maintained at room temperature as a monolayer in 75cm\textsuperscript{2} tissue culture flasks containing 15mL PYG.

**Determination of \textit{Campylobacter jejuni} Sensitivity to Gentamicin.** In 1.5mL centrifuge tubes 1mL of adjusted \textit{C. jejuni} inoculum (1 x 10\textsuperscript{9} CFU/mL) were centrifuged at 10,000 x g for 2 min and resuspended in 1mL PYG medium containing 200µL/mL gentamicin and incubated at room temperature 2hrs. Samples were washed three times in
1mL Page’s Amoebal Saline (PAS) by centrifugation at 10,000 x g for 2 min and resuspended in MHB. Samples were serial diluted in MHB, plated onto MHA containing 5µg/mL trimethoprim, and incubated 48h at 37°C under microaerophilic conditions. Samples were performed in triplicate with control samples being resuspended in PYG without gentamicin.

**Determination of Acanthamoeba castellanii Sensitivity to Gentamicin.**

*Acanthamoeba castellanii* cultures were harvested by removing the liquid from the 75cm² flasks and replacing it with 3ml PAS. Flasks were then incubated in a freezer (-20°C) for 10m to detach amoebae [86]. Amoebae were further detached by repeated pipetting of the liquid over the monolayer. The liquid from multiple flasks was pooled and washed three times with PAS by centrifugation at 600 x g 5m at 4°C and resuspended in PAS. *A. castellanii* trophozoites (vegetative amoebal cells) were counted using a hemocytometer with trypan blue dye (10X) added to allow for exclusion of any non-viable amoebae [65]. Amoebae inoculum was adjusted to a concentration of 1 x 10⁶ amoeba/mL in PAS. In 1.5mL centrifuge tubes 1mL of adjusted *A. castellanii* inoculum (1 x 10⁶ CFU/mL) were centrifuged at 600 x g for 5 min and resuspended in 1mL PYG medium containing 200µL/mL gentamicin and incubated at room temperature 2hrs. Samples were washed three times in 1mL Page’s Amoebal Saline (PAS) by centrifugation at 600 x g for 5 min and resuspended in 900µL PAS, mixed with 100µL trypan blue, and counted to determine the average total viable amoebae trophozoites recovered.

**Determination of Campylobacter jejuni Sensitivity to Triton X-100.** In 1.5mL centrifuge tubes 1mL of adjusted *C. jejuni* inoculum (1 x 10⁹ CFU/mL) were centrifuged
at 10,000 x g for 2 min and resuspended in 1mL PYG medium containing 0.3% Triton X-100 and incubated at 15min at room temperature in a shaking incubator (200rpm).

Samples were serial diluted in MHB, plated onto MHA containing 5µg/mL trimethoprim, and incubated 48h at 37°C under microaerophilic conditions. Samples were performed in triplicate with control samples being resuspended in PYG without Triton X-100.

**Determination of *Acanthamoeba castellanii* Sensitivity to Triton X-100.**

*Acanthamoeba castellanii* cultures were harvested by removing the liquid from the 75cm² flasks and replacing it with 3ml PAS. Flasks were then incubated in a freezer (-20°C) for 10m to detach amoebae [86]. Amoebae were further detached by repeated pipetting of the liquid over the monolayer. The liquid from multiple flasks was pooled and washed three times with PAS by centrifugation at 600 x g 5m at 4°C and resuspended in PAS. *A. castellanii* trophozoites (vegetative amoebal cells) were counted using a hemocytometer with trypan blue dye (10X) added to allow for exclusion of any non-viable amoebae [65]. Amoebae inoculum was adjusted to a concentration of 1 x 10⁶ amoeba/mL in PAS. In 1.5mL centrifuge tubes 1mL of adjusted *A. castellanii* inoculum (1 x 10⁶ CFU/mL) were centrifuged at 600 x g for 5 min and resuspended in 1mL PYG medium containing 0.3% Triton X-100 and incubated at 15min at room temperature in a shaking incubator (200rpm). Samples were centrifuged at 600 x g for 5 min and resuspended in 900µL PAS, mixed with 100µL trypan blue, and counted to determine the average total viable amoebae trophozoites recovered.
Modified Gentamicin Protection Assay. Acanthamoeba castellanii cultures were harvested by removing the liquid from the 75cm² flasks and replacing it with 3ml PAS. Flasks were then incubated in a freezer (-20°C) for 10m to detach amoebae [86]. Amoebae were further detached by repeated pipetting of the liquid over the monolayer. The liquid from multiple flasks was pooled and washed three times with PAS by centrifugation at 600 x g 5m at 4°C and resuspended in PAS. A. castellanii trophozoites (vegetative amoebal cells) were counted using a hemocytometer with trypan blue dye (10X) added to allow for exclusion of any non-viable amoebae [65]. The amoeba inoculum was adjusted to a concentration of 1 x 10⁷ amoebae/mL in PAS.

In a 1.5mL centrifuge tube, 1mL of adjusted C. jejuni inoculum (1 x 10⁹ CFU/mL) was mixed with 100µL adjusted A. castellanii culture (1 x 10⁷ amoebae/mL) giving a Multiplicity of Infection (MOI) of 1000:1 C. jejuni: A. castellanii. Control samples contained 1mL of C. jejuni inoculum and were mixed with 100µL PAS in place of A. castellanii inoculum. Samples were incubated for 2h at room temperature to allow C. jejuni to enter amoebae [65].

Following co-incubation samples were washed three times in PAS by centrifugation at 600 x g 5m at room temperature to remove extracellular C. jejuni. They were then resuspended in 1mL PYG containing 200µg/mL gentamicin [64]. Samples were incubated for 2h at room temperature to remove any remaining extracellular and attached C. jejuni [64]. The gentamicin incubation was followed by three washes in PAS to remove residual gentamicin. Samples were then resuspended in 1mL PYG containing 0.3% Triton X-100 to lyse amoebae, releasing intracellular C. jejuni [64, 65].
Samples were incubated on their side for 15m at room temperature in a shaking incubator (200rpm). Prior to the addition of Triton X-100 half of the samples containing both C. jejuni and A. castellanii were resuspended in 900µL PAS, mixed with 100µL trypan blue, and counted to determine the average total viable amoebae trophozoites recovered throughout the assay. Following incubation with Triton-X 100 control samples (C. jejuni without amoebae) and remaining samples containing both C. jejuni and A. castellanii were serially diluted in MHB, plated onto MHA containing 5µg/mL trimethoprim, and incubated 48h at 37°C under microaerophilic conditions to estimate C. jejuni internalization.

**Determining Rate of Internalization.** The rate of internalization (ratio of C. jejuni internalized: A. castellanii recovered) was calculated by subtracting the average CFU of C. jejuni recovered from control samples (CFU of C. jejuni that survived the assay without A. castellanii) from the average CFU of C. jejuni recovered from samples co-incubated with A. castellanii. The CFU of internalized C. jejuni was then divided by the average A. castellanii recovered. When C. jejuni recovered from control samples (C. jejuni without amoebae) were approximately the same or higher than C. jejuni recovered from samples co-incubated with A. castellanii, the CFU of C. jejuni internalized resulted in a low negative value. These low or negative values were considered samples where the amoebae conveyed no protective effect for C. jejuni.

**Evaluation of A. castellanii Culture Age and Nutrient Deprivation on Rate of Internalization of C. jejuni.** Three days prior to experiments, 1mL of culture was removed from A. castellanii stock flasks and used to inoculate new 75cm² flasks
containing 15mL PYG. Twenty four hours prior to experiments a portion of the flasks had the liquid removed and replaced with 15mL PAS. Flasks containing PYG for 3 days were termed “early growth phase” cultures of *A. castellanii*, those resuspended in Page’s amoebal saline were termed “nutrient deprived” cultures of *A. castellanii*. Older maintained monolayer stock flasks were considered “monolayer” cultures of *A. castellanii*. The mGPA was then employed as previously described to determine the rate of internalization of *C. jejuni* for each culture condition of *A. castellanii* (early growth phase, monolayer, and nutrient deprived).

**Statistical Analysis.** T-tests were performed to determine significance between control (*C. jejuni* only) and *C. jejuni* co-incubated with *A. castellanii* samples. A p-value $<0.05$ was determined statistically significant.

**Results**

A standard curve was created for *Campylobacter jejuni* NCTC 11168 to adjust *C. jejuni* cultures prior to experiments (Figure 2.1). Preliminary studies to determine the sensitivity of *C. jejuni* to gentamicin have shown that *C. jejuni* was reduced $1.5 \times 10^8$ total CFU after exposure to gentamicin. This was a significant (p $\leq 0.05$) reduction in *C. jejuni* when compared to controls not exposed to gentamicin (Figure 2.2). Preliminary studies to determine the sensitivity of *Acanthamoeba castellanii* showed no significant difference (p $\geq 0.05$) in recovery of *A. castellanii* between control samples and samples exposed to gentamicin (Figure 2.3).
Preliminary studies to determine the sensitivity of *Campylobacter jejuni* to triton X-100 showed no significant difference (p $\geq 0.05$) in recovery of *C. jejuni* between control samples and samples exposed to Triton X-100 (**Figure 2.4**). Preliminary studies to determine the sensitivity of *Acanthamoeba castellanii* to triton X-100 have shown that *A. castellanii* was reduced $7.56 \times 10^5$ total amoebae after exposure to triton X-100. No *A. castellanii* (detection limit 1250 total amoebae) after exposure to triton X-100. This was a significant (p $\leq 0.05$) reduction in *A. castellanii* when compared to controls not exposed to triton X-100 (**Figure 2.5**).

Initial trials did not account for *A. castellanii* culture status. Amoebae flasks were harvested at various ages to obtain a sufficient number of amoebal cells needed to perform the assay. Results varied among trials with 8 trials showing a protective effect (rate of internalization $>0.1$ *C. jejuni: A. castellanii*), suggesting amoebal internalization of *C. jejuni* and 9 trials possibly showing a slight protective effect (rate of internalization $<0.1$ but $>0.01$ *C. jejuni: A. castellanii*). Six trials showed no protective effect (Rate of internalization $<0.01$ *C. jejuni: A. castellanii*) (**Table 2.1**).

These results led to the investigation of factors which could be involved in this variation as *C. jejuni* culture preparation was standard for each trial but *A. castellanii* cultures were not. To test for an effect of culture condition, *A. castellanii* flasks were harvested at various ages to obtain the concentration of amoebae needed for each experiment. Experiments consisted of *C. jejuni* co-incubated with *Acanthamoeba castellanii* “monolayer”, *A. castellanii* “early growth phase”, and *A. castellanii* “nutrient deprived”. Five trials were performed with all trials showing no internalization or
protective effect for \textit{C. jejuni} co-incubated with “monolayer” amoebae cultures, represented by a higher recovery of \textit{C. jejuni} from control samples without amoebae then when co-incubated with “monolayer” amoebae cultures. One trial (trial 1) showed a larger CFU \textit{C. jejuni} internalized for nutrient deprived \textit{A. castellanii} cultures, two trials (trials 2 and 4) showed a larger CFU \textit{C. jejuni} internalized for early growth phase \textit{A. castellanii} cultures, and two trials (trial 3 and 5) showed a lack of internalization for all \textit{A. castellanii} culture conditions (Table 2).

The average percent of \textit{A. castellanii} inoculum recovered from each trial based on an estimated \textit{A. castellanii} starting concentration of $1 \times 10^6$ was 78.1\% (+/- 17.1). Rate of internalization was calculated: \((\text{CFU \textit{C. jejuni} recovered from co-incubation with \textit{A. castellanii} - CFU \textit{C. jejuni} recovered from no \textit{A. castellanii} controls})/\textit{A. castellanii} recovered\). Two trials showed internalization of \textit{C. jejuni} by the nutrient deprived amoebae (trials 1 and 2) with rates of internalization of 4.89 and 0.19 (\textit{C. jejuni}: \textit{A. castellanii}) respectively. Three trials showed internalization of \textit{C. jejuni} by early growth phase amoebae (trials 1, 2, and 4) with rates of internalization of 0.55, 2.1, and 1.5 (\textit{C. jejuni}: \textit{A. castellanii}) respectively. When the rate of internalization resulted in a value <0.1 it was considered a lack of internalization or amoebae protection of \textit{C. jejuni} and was reported as “(-)” for rate of internalization (Table 2.2).
Figure 2.1: Standard Curve of *Campylobacter jejuni* NCTC 11168.

\[
y = 8.6081E+08x - 2.4505E+07 \\
R^2 = 9.9877E-01
\]

Figure 2.2: *Campylobacter jejuni* NCTC 11168 Sensitivity to Gentamicin.
The Sensitivity of *C. jejuni* NCTC 11168 to gentamicin was assessed to determine if gentamicin exposure (200µg/mL for 2hr) was effective in significantly reducing *C. jejuni*. Results indicate that this gentamicin treatment significantly (p < 0.5) reduced *C. jejuni* compared to controls that were not exposed to gentamicin. Gentamicin reduced *C. jejuni* $1.5 \times 10^8$ total CFU, however *C. jejuni* was still present after exposure to gentamicin.

Figure 2.3: *Acanthamoeba castellanii* ATCC 30010 Sensitivity to Gentamicin.

The Sensitivity of *A. castellanii* ATCC 30010 to gentamicin was assessed to determine if gentamicin exposure (200µg/mL for 2hr) would have any adverse effects on *A. castellanii*. Results indicate that there was no significant difference (p > 0.05) in *A. castellanii* recovery between control samples or samples exposed to gentamicin.
Figure 2.4: *Campylobacter jejuni* NCTC 11168 Sensitivity to Triton X-100.

The Sensitivity of *C. jejuni* NCTC 11168 to triton X-100 was assessed to determine if triton X-100 exposure (0.3% for 15min) would have any adverse effects on *C. jejuni*. Results indicate that there was no significant difference (p > 0.05) in *C. jejuni* recovery between control samples or samples exposed to triton X-100.
The Sensitivity of *A. castellanii* ATCC 30010 to triton X-100 was assessed to determine if triton X-100 exposure (0.3% for 2hr) was effective in significantly reducing *A. castellanii*. Results indicate that this triton X-100 treatment significantly (p < 0.5) reduced *A. castellanii* compared to controls that were not exposed to gentamicin. *A. castellanii* was reduced 7.56 x 10^5 total amoebae and no *A. castellanii* was recovered from samples exposed to triton X-100 (detection limit 1250 amoebae).

**Figure 2.5: Acanthamoeba castellanii* ATCC 30010 Sensitivity to Triton X-100.**
Table 2.1: Results of modified gentamicin protection assay using non-standardized *Acanthamoeba castellanii* cultures.

The recovery of *C. jejuni* NCTC 11168 (CFU) from an initial inoculum of ~ 1 x 10^9 (CFU) for controls samples (C. jejuni only) and C. jejuni co-incubated with A. castellanii. The rate of internalization (ROI) (C. jejuni internalized: A. castellanii recovered) was calculated for each trial. Each trial consisted of three samples of C. jejuni NCTC 11168 and A. castellanii and three control samples (C. jejuni only). Based on the rate of internalization the trials were grouped by “Internalization” (ROI > 0.1), “Possible Internalization” (ROI > 0.01), and “Lack of Internalization” (ROI <0.01). Standard error of the mean is denoted by “( )”.

<table>
<thead>
<tr>
<th>Internalization (protective effect)</th>
<th>C. jejuni control (no A. castellanii)</th>
<th>C. jejuni with A. castellanii</th>
<th>Rate of internalization</th>
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<td>3.4 x 10^3 (1.9 x 10^3)</td>
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<td>2.2 x 10^5 (9.2 x 10^4)</td>
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<tr>
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<td>1.2 x 10^5 (4.2 x 10^3)</td>
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<tr>
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<td>0.17^b</td>
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<td>1.9 x 10^4 (1.9 x 10^3)</td>
<td>1.3 x 10^4 (2.3 x 10^3)</td>
<td>0.11^b</td>
<td></td>
</tr>
<tr>
<td>Possible Internalization (slight protective effect)</td>
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<td>5.8 x 10^4 (1.7 x 10^3)</td>
<td>0.07</td>
</tr>
<tr>
<td>2.6 x 10^2 (3.29 x 10^3)</td>
<td>4.6 x 10^4 (5.5 x 10^3)</td>
<td>0.07^b</td>
<td></td>
</tr>
<tr>
<td>2.9 x 10^4 (4.9 x 10^3)</td>
<td>6.6 x 10^4 (1.7 x 10^3)</td>
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<td></td>
</tr>
<tr>
<td>7.7 x 10^2 (2.6 x 10^3)</td>
<td>5.5 x 10^4 (3.2 x 10^3)</td>
<td>0.05^b</td>
<td></td>
</tr>
<tr>
<td>4.7 x 10^1 (5.1 x 10^0)</td>
<td>3.4 x 10^4 (4.2 x 10^3)</td>
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</tr>
<tr>
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<td>1.1 x 10^4 (5.0 x 10^3)</td>
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<td>7.4 x 10^2 (3.1 x 10^2)</td>
<td>0.014^b</td>
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Table 2.1 (continued)

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<tr>
<th>Lack of Internalization (No Protective effect)</th>
<th>C. jejuni control (no A. castellanii)</th>
<th>C. jejuni with A. castellanii</th>
<th>Rate of internalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 x 10^4 (8.5 x 10^2)</td>
<td>1.6 x 10^4 (1.7 x 10^2)</td>
<td>(-)^a</td>
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</tr>
<tr>
<td>2.1 x 10^2 (7.6 x 10^1)</td>
<td>5.7 x 10^2 (1.2 x 10^2)</td>
<td>(-)^a</td>
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<tr>
<td>1.7 x 10^4 (3.58 x 10^3)</td>
<td>1.7 x 10^4 (2.51 x 10^3)</td>
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<tr>
<td>8.8 x 10^2 (4.62 x 10^2)</td>
<td>2.5 x 10^2 (1.34 x 10^3)</td>
<td>(-)^a</td>
<td></td>
</tr>
<tr>
<td>3.0 x 10^5 (1.1 x 10^3)</td>
<td>2.3 x 10^5 (4.8 x 10^3)</td>
<td>(-)^a</td>
<td></td>
</tr>
<tr>
<td>6.2 x 10^5 (1.5 x 10^3)</td>
<td>3.8 x 10^5 (9.4 x 10^4)</td>
<td>(-)^a</td>
<td></td>
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</tbody>
</table>

^a Rate of internalization <0.01

^b Statistically significant (p < 0.05) compared to control
Table 2.2: Examination of the impact of *Acanthamoeba castellanii* growth phase and nutrient status on internalization of *Campylobacter jejuni*.

Comparison of *C. jejuni* recovered (CFU) and rate of internalization of internalization (*C. jejuni* internalized: *A. castellanii* recovered) when co-cultured with different *A. castellanii* inoculums. These inoculums included older established *A. castellanii* monolayers, fresh early growth phase *A. castellanii* cultures, and fresh early growth phase *A. castellanii* cultures that had nutrients deprived 24h prior to experiment by replacing the growth medium with PAS. Standard error of the mean is denoted by "( )".

<table>
<thead>
<tr>
<th>C. jejuni control (no <em>A. castellanii</em>)</th>
<th>Monolayer</th>
<th>Early Growth Phase</th>
<th>Nutrient Deprived</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni Recovered (CFU)</td>
<td>C. jejuni Recovered (CFU)</td>
<td>ROI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C. jejuni Recovered (CFU)</td>
</tr>
<tr>
<td><strong>Trial 1</strong></td>
<td>3.7 x 10&lt;sup&gt;6&lt;/sup&gt; (4.5 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>2.94 x 10&lt;sup&gt;5&lt;/sup&gt; (2.2 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>(-)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td>1.5 x 10&lt;sup&gt;6&lt;/sup&gt; (2.6 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>8.8 x 10&lt;sup&gt;5&lt;/sup&gt; (8.01 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>(-)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Trial 3</strong></td>
<td>4.1 x 10&lt;sup&gt;5&lt;/sup&gt; (4.0 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>2.8 x 10&lt;sup&gt;4&lt;/sup&gt; (8.01 x 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>(-)&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Trial 4</strong></td>
<td>1.1 x 10&lt;sup&gt;6&lt;/sup&gt; (1.7 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>9.9 x 10&lt;sup&gt;5&lt;/sup&gt; (3.8 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>(-)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Trial 5</strong></td>
<td>7.5 x 10&lt;sup&gt;5&lt;/sup&gt; (1.3 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>1.8 x 10&lt;sup&gt;5&lt;/sup&gt; (1.3 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>(-)&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rate of internalization

<sup>b</sup> Rate of internalization <0.01

<sup>c</sup> Statistically significant (p < 0.05) compared to control
Discussion

In this study the commonly used gentamicin protection assay was adapted to address limitations with currently used methods. By using 1.5 mL centrifuge tubes, both amoebae and bacteria can be added at known and consistent levels reducing variability. Additionally, washes were performed by low speed centrifugation instead of removing and re-applying liquid to a monolayer. By eliminating the use of a monolayer the risk of losing samples (in particular amoebae) is reduced. Most importantly, *C. jejuni* only controls can be utilized to determine the level of *C. jejuni* that remain throughout the entire assay. Preliminary investigation of the sensitivity of *C. jejuni* (Figure 2.2) has shown that although gentamicin significantly reduces *C. jejuni*, it fails to completely eliminate all of the bacteria. It is assumed in most assays that the gentamicin will remove most of the bacteria and that the amount of bacteria remaining is negligible. If internalization occurs at a high level this is a true statement. However, as demonstrated in this study, if internalization does not occur or occurs at a low rate, failure to take into account a *C. jejuni* control may result in the appearance that *C. jejuni* is internalized when it may be a result of *C. jejuni* survival independent of the presence of amoebae.

Initial experiments with *C. jejuni* showed variation in both the rate and occurrence of internalization (Table 2.1). These results led to further investigation of *A. castellanii* culture age and nutrient status to determine if these factors influenced this variation. Results indicate that age of the amoebae may have an influence on internalization of *C. jejuni* with older maintained monolayers consistently showing no internalization. However, although both early growth phase and nutrient deprived *A. castellanii* cultures
showed internalization in some trials they showed a lack of internalization in other trials (Table 2.2). These results differ from other studies where the internalization of C. jejuni by amoebae has been assumed to be a consistent event [12, 23, 67, 75, 79, 86, 93]. It is interesting to note that in two trials (trial 3 and 5) using older maintained monloyers of A. castellanii the C. jejuni recovery was significantly higher in C. jejuni only control samples compared to samples co-incubated with A. castellanii. This finding suggests the possibility that C. jejuni was internalized but was actually digested by A. castellanii. This was also seen in two trials (Trial 3 and 4) using nutrient deprived A. castellanii. Conversely, in Trial 1 nutrient deprived A. castellanii had a statistically higher recovery of C. jejuni when co-incubated with A. castellanii suggesting intracellular survival.

Although early growth phase A. castellanii showed a positive rate of internalization for three trials there were no trials showing a significant different in C. jejuni recovery compared to control samples.

Conflicting results of the ability of a bacterial pathogen to be internalized by Acanthamoeba spp. have been reported in previous studies for Campylobacter jejuni [12, 23, 66, 75, 79, 86, 93] and other bacterial pathogens including Enterobacter faecalis [91, 92], Salmonella enterica [60, 91], and Listeria monocytogenes [65, 69, 91, 92]. This variation in results may be due to differences seen in experimental design or approach as well as differences in bacterial strains and Acanthamoeba species used.

Campylobacter jejuni, a microaerophilic organism has been reported to survive under aerobic conditions when co-incubated with Acanthamoeba polyphaga and this co-incubation has been reported to be an effective C. jejuni enrichment procedure under
aerobic conditions [23, 86]. In contrast Bui et al., 2012, using *A. castellanii* have reported that *C. jejuni* did not survive internalization by *A. castellanii* but did survived for extended periods of time extracellularly [66]. It has been proposed that the reason for this extracellular survival is the depletion of harmful dissolved oxygen in the media by the amoebae that allows *C. jejuni* to survive under aerobic conditions[66]. Extracellular survival of bacterial pathogens has also been reported for *Listeria monocytogenes* [65, 91] and *Vibrio parahaemolyticus* [64] where both pathogens were reported to survive on materials released by the amoebae.

There could be many reasons why the study of the interaction of bacterial pathogens with amoebae has shown variable results. The use of different strains species of both bacteria and amoebae may be chief among them. It has also been shown in that different species of the same genus i.e. *Vibrio cholerae* [53]and *Vibrio parahaemolyticus* [64] as well as different strains of the same species i.e. *E. coli* yield different results in internalization [71]. It is has also been shown that similar species in the genus *Acanthamoeba* have different feeding preference [131] and in the case of conflicting reports for *Salmonella enterica* and *Campylobacter jejuni* different *Acanthamoeba* spp. were used. Previous studies showing internalization of *C. jejuni* used different *C. jejuni* strains [12, 23, 75, 79, 86, 93], and either different *Acanthamoeba* spp. [23, 79, 86, 93]or a different strain of *A. castellanii* [12, 75]. Interestingly the strain of *C. jejuni* (NCTC 11168) used in this study was likewise used by Bui et al., 2012 where it was reported that *C. jejuni* did not survive internalization by *A. castellanii* although a different strain of *A. castellanii* was used.
Another possibility in the variation of results could be a result in experimental approach and design. As previously mentioned there is a large amount of variation in the initial set up of co-incubation in both the vessel used as well as the preparation of amoebae prior. Multiplicity of infection or MOI (ratio of bacterial cells: amoebal cells) may also play a key role. The density of bacteria in co-culture with \textit{A. castellanii} has shown to have an influence on the growth and survival of \textit{A. castellanii} with higher levels of bacteria being more inhibitory [128]. It is not known whether this effect can be seen at shorter time points as employed in some studies since this study tested the effect at 48h. Common multiplicities of infection used included 1:1 [12, 53], 10:1 [64, 73, 79], 100:1 [61, 66, 75, 91], and 1,000:1 [61, 91] \textit{C. jejuni: A. castellanii}. In this study a MOI of 1:1000 to allow for sufficient recovery by culture plating.

The duration of co-incubation as well as the co-incubation temperature also varies among studies. For those performing gentamicin protection assays the co-incubation times included 30m [73], 1hr [60], 2hr [60], and 3hr [66]. Co-incubation time may be an important factor due to the amount of time it takes for bacteria to enter amoebae as well as the amount of time it takes amoebae to digest the bacteria that can be digested. If co-incubation times are too short it may lead to the belief that a bacteria is not internalize when in fact more time was required for sufficient internalization. In addition, if amoebae are lysed without sufficient time for lysosomal breakdown of bacteria that can be utilized as a food source it may give the impression that the bacteria survived internalization. Temperature is an interesting variable because lower temperatures are more representative of most environmental interactions of these organisms; however, other studies used 37°C which is an optimal temperature for most human pathogens. It
may be that higher temperatures such as 37°C may activate factors in certain bacteria causing a different result. With *C. jejuni* co-incubation at 25°C showed survival but no replication [12, 23] but at 37°C replication and eventual lysis of *A. polyphaga* can be shown [86]. Additionally at 37°C many of these bacterial pathogens may be more metabolically active, depleting nutrients faster which may alter the interaction with amoebae [75]. In comparing this study with Bui et al., 2012 the time of the experiment from co-culture to sampling was much shorter. Bui et al., 2012 reported that at similar conditions to this study (25°C aerobic conditions) after at 5h post gentamicin *C. jejuni* could still be seen in *A. castellanii* however 24h post gentamicin treatment no *C. jejuni* could be seen microscopically or recovered using a gentamicin protection assay [66].

The experimental approach differed between studies that were looking for amounts of bacteria internalized, which, like this study, typically used shorter co-incubation times and employed a gentamicin protection assay to determine internalization. Other studies investigated the long-term survival of the bacteria when co-incubated with amoebae and were investigating the presence/absence of bacteria instead of quantifying the amount of bacteria internalized [23, 75, 86, 93]. Long term survival of bacteria in co-incubation may lead the investigator to assume that intracellular survival is taking place when in fact it may be due to other factors such as what has been reported with *L. monocytogenes* [65, 91], *Vibrio parahaemolyticus* [64], and *C. jejuni* [66].

Conclusions

The assay developed in this study has been successful in showing internalization and survival of *Campylobacter jejuni* by *Acanthamoeba castellanii* giving further insight
into the interaction of these organisms. By using this assay with a C. jejuni only control it has been demonstrated that C. jejuni survives the assay independently of the presence of A. castellanii. The ability to quantify the level of C. jejuni survival without A. castellanii allowed for the determination that internalization or some protection of C. jejuni by A. castellanii does take place but not consistently and at varied levels. It is interesting to note that although results varied by trial, individual samples within each trial were consistent for either internalization (or protection of C. jejuni by A. castellanii) or lack of internalization (or protection of C. jejuni by A. castellanii). In four trials older A. castellanii showed no significant difference between C. jejuni only control samples and samples co-incubated with amoebae which may be due to the fact that A. castellanii was likely in a stationary phase of growth. Other factors for both A. castellanii and C. jejuni may be involved in these interactions that are not fully understood. More importantly the development of this mGPA provides another a useful tool to further investigate the interaction of bacterial pathogens with protozoa species.
Chapter 3: Interaction of *Campylobacter jejuni* strains with *Acanthamoeba castellanii*

Abstract

*Campylobacter jejuni* is one of the leading causes of foodborne illness in the U.S. and has been reported to be internalized by free living amoebae and survive intracellularly. Little is known about this interaction and the mechanism(s) by which *C. jejuni* is able to invae and/or survive inside amoebae is unknown. Virulence studies of *C. jejuni* in humans have investigated internalization of the bacteria into monolayers of human epithelial cells. Many studies investigate mutant strains to aid in the discovery of factors involved in *C. jejuni* virulence. In this study, three wild-type *C. jejuni* strains were investigated to determine if there is variation of internalization by *A. castellanii*. Three mutant *C. jejuni* strains with altered virulence (with epithelial cells) were also investigated to determine if there was any relationship between the virulence of a strain in epithelial cells and internalization by amoebae. Previous work (Chapter 2) developed a modified gentamicin protection assay to investigate the relationship between *A. castellanii* and *C. jejuni* NCTC 11168. *C. jejuni* NCTC 11168 appeared to be internalized in 74% of experiments but in 26% of experiments the strains did not appear to be internalized at all. In this study we employed the same assay with multiple strains of wild-type and mutant *C. jejuni* strains to determine if other strains showed more consistent internalization or lack of internalization. Wild-type strains *C. jejuni* 81168, *C. jejuni* 81-176, and *C. jejuni* ATCC 43502 as well as the mutant strain *C. jejuni* NCTC 11168 ΔflgK showed little or no internalization by amoebae. Mutant strains *C. jejuni* 81-176 Δcj0596 and 81-176 ΔcsrA both showed higher internalization by *Acanthamoeba*
The results of this study provide further insight into the interaction between *C. jejuni* and amoebae by demonstrating differences among wild-type strains as well as specific genes that may play a role in internalization. Future studies are needed to determine what differences between wild-type strains account for this variation in internalization. Additionally, investigating more mutants with different virulence genes altered may also aid in further understanding of the relationship between *C. jejuni* and *A. castellanii*.

**Introduction**

There have been numerous studies reporting an association between *C. jejuni* and free living amoebae in the genus *Acanthamoeba* [12, 23, 66, 67, 75, 79, 86, 93]. Many studies report an internalization of *C. jejuni* into *Acanthamoeba* spp. and survival intracellularly [12, 23, 67, 75, 79, 86, 93]. *Legionella pneumophila* is another pathogen reported to survive internalization by amoebae [54, 106, 117]. *L. pneumophila* is believed to use similar strategies and mechanisms to survive and replicate in both amoebae and human macrophages [54, 106, 117]. It has been reported that there is a direct relationship between *L. pneumophila* virulence in human cells and successful internalization by amoebae [54]. The mechanism(s) for which *C. jejuni* enter and/or survive into amoebae are not known. Amoebae are phagocytic cells and a part of *C. jejuni* invasion into human intestinal epithelial cells involves inducing phagocytosis in these cells [6]. It may be that similar strategies or mechanisms are employed by *C. jejuni* to enter and/or survive in amoebae.
Campylobacter jejuni is one of the leading causes of foodborne illness in the U. S. [9]. C. jejuni virulence in humans and mechanisms used to invade human intestinal epithelial cells are still not fully understood [6, 15-17]. It has been reported that strains of C. jejuni vary in invasiveness with cultured epithelial cells [132, 133] leading to the suggestion that different C. jejuni strains employ different strategies or mechanisms to enter epithelial cells [132]. The flagellum of C. jejuni is an important virulence factor required for motility, chemotaxis, attachment, and to inject factors into the host similar to a type III secretion system, used for internalization into epithelial cells [6, 134]. The C. jejuni NCTC 11168 Δflgk mutant has truncated flagella causing this strain to have diminished motility and has been reported to have a reduction in adherence and internalization compared to the wild-type strain [134]. Additionally mutant strains C. jejuni 81-176 ΔcsrA and Δcj0596 were investigated [24, 25, 135]. In C. jejuni, csrA is a global posttranscriptional regulator and is suspected to play a vital role in the regulation of stress responses and virulence determinates [135]. The C. jejuni 81-176 ΔcsrA mutant strain has been reported to be more susceptible to oxidative stress, decreased motility and adherence to intestinal epithelial cells, but increased intestinal epithelial cell invasion of adhered bacterial cells. Cj0596 is a periplasmic chaperone that plays a role in outer membrane protein (OMP) folding [24, 25]. The C. jejuni 81-176 ΔCj0596 mutant was reported to have changes in cell surface related characteristics and showed increased motility and invasion of epithelial cells.

The purpose of this study was to investigate the relationship between C. jejuni and the amoeba Acanthamoeba castellanii by using a modified gentamicin protection assay (mGPA) and multiple strains of C. jejuni. The research described here examined whether
wild-type and mutant strains of \textit{C. jejuni} which demonstrate different rates of epithelial cell invasion, also demonstrate different rates of internalization by \textit{Acanthamoeba castellanii}. In this study the common laboratory strains \textit{C. jejuni} 81116 [136, 137], \textit{C. jejuni} 81-176 (a hyper-invasive strain) [132], and clinical isolate \textit{C. jejuni} ATCC 43502 were investigated.

**Methods**

**Strains and Growth Conditions.** \textit{Campylobacter jejuni} strains used in this study and where they were obtained are summarized in Table 3.1. All \textit{C. jejuni} strains were stored at -80°C in Mueller Hinton Broth with 10% glycerol. All \textit{C. jejuni} cultures were incubated at 37°C under microaerophilic conditions (85% N$_2$, 10% CO$_2$, and 5% O$_2$) in a Binder (Bohemia, NY) CB150 incubator. Frozen working stocks were streaked for isolation onto Mueller Hinton Agar with 5μg/mL trimethoprim (MHA) and incubated 48h followed by two 24h passages with sterile swabs onto fresh MHA plates. Prior to experiments MHA plates containing \textit{C. jejuni} were swabbed and resuspended in Mueller Hinton Broth (MHB) and CFU/mL was estimated using a standard curve based on absorbance at 600nm (Figure 2.1). \textit{C. jejuni} inoculum was adjusted to 1 x 10$^9$ CFU/mL by centrifugation at 3,220 x g for 20m at 4°C and resuspended in Proteose Peptone-Yeast Extract-Glucose medium (PYG). \textit{Acanthamoeba castellanii} ATCC 30010 was obtained from the American Type Culture Collection (ATCC Manassas, VA). \textit{A. castellanii} cultures stocks were maintained at room temperature in a monolayer in 75cm$^2$ tissue culture flasks containing 15mL PYG medium. Three days prior to the experiment1mL of
culture was removed from the *A. castellanii* stock flasks and used to inoculate new 75cm² flasks containing 15mL PYG medium.

**Modified Gentamicin Protection Assay.** *Acanthamoeba castellanii* cultures were harvested by removing the liquid from the 75cm² flasks and replacing it with 3ml Page’s Amoebal Saline (PAS). The flasks were then incubated in a freezer (-20°C) for 10m to detach amoebae [86]. The amoebae were further detached by repeated pipetting of the liquid over the monolayer. The liquid from multiple flasks was pooled and washed three times with PAS by centrifugation at 600 x g 5m at 4°C and resuspended in PAS. *A. castellanii* trophozoites (vegetative amoebal cells) were counted using a hemocytometer with trypan blue dye (10X) [64, 65] added to allow for exclusion of any non-viable amoebae. Amoebae inoculum was adjusted to a concentration of 1 x 10⁷ amoebae/mL in PAS.

In a 1.5mL centrifuge tube 1mL of adjusted *C. jejuni* inoculum (1 x 10⁹ CFU/mL) was mixed with 100µL adjusted *A. castellanii* culture (1 x 10⁷ amoebae/mL) giving a Multiplicity of Infection (MOI) of 1000:1 *C. jejuni: A. castellanii*. Control samples contained 1mL of *C. jejuni* inoculum and were mixed with 100µL PAS in place of *A. castellanii* inoculum. Samples were incubated for 2h at room temperature to allow *C. jejuni* to enter amoebae [65].

Following co-incubation of *C. jejuni* and *A. castellanii*, samples were washed three times in PAS by centrifugation at 600 x g 5m at room temperature to remove extracellular *C. jejuni* and samples were resuspended in 1mL PYG containing 200µg/mL gentamicin, and samples were incubated an additional 2h at room temperature to remove
any remaining extracellular and/or attached *C. jejuni* [64]. The gentamicin incubation was followed by three washes in PAS to remove residual gentamicin and samples were resuspended in 1mL PYG containing 0.3% Triton X-100 to lyse *A. castellanii* releasing intracellular *C. jejuni* [65]. Samples were incubated on their side for 15m at room temperature in a shaking incubator (200rpm). Prior to the addition of Triton X-100 half of the samples containing both *C. jejuni* and *A. castellanii* were resuspended in 900µL PAS, mixed with 100µL trypan blue, and counted to determine the average total viable *A. castellanii* trophozoites recovered throughout the assay. Following incubation with Triton-X 100 control samples (*C. jejuni* without amoebae) and remaining samples containing both *C. jejuni* and *A. castellanii* were serial diluted in MHB, plated onto MHA containing 5µg/mL trimethoprim, and incubated 48h at 37°C under microaerophilic conditions to determine *C. jejuni* survival.

**Determining Rate of Internalization.** The rate of internalization (*C. jejuni* internalized: *A. castellanii* recovered) was calculated by subtracting the average CFU of *C. jejuni* recovered from control samples (amount of *C. jejuni* that survived the assay without *A. castellanii*) from the average CFU of *C. jejuni* recovered from samples co-incubated with *A. castellanii* to determine the CFU *C. jejuni* internalized. The CFU of internalized *C. jejuni* was then divided by the average *A. castellanii* recovered. When *C. jejuni* recovered from control samples (*C. jejuni* without *A. castellanii*) were approximately the same or higher than *C. jejuni* recovered from samples co-incubated with *A. castellanii* the CFU internalized resulted in a low or negative value. These low or negative values were considered samples where the amoebae conveyed no protective effect for *C. jejuni*. 
**Statistical Analysis.** T-tests were performed to determine significance between control (\(C. \textit{jejuni}\) only) and \(C. \textit{jejuni}\) co-incubated with \(A. \textit{castellanii}\) samples. A p-value \(<0.05\) was determined statistically significant.

**Table 3.1: \textit{Campylobacter jejuni} strains used in Chapter 3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type \textit{Campylobacter jejuni} strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>Commonly used, genomic sequenced strain [138]</td>
<td>American Type Culture Collection (ATCC (Manassas. VA)</td>
</tr>
<tr>
<td>81116</td>
<td>Genomic sequence strain transformable with plasmids [136, 137]</td>
<td>National Collection of Type Cultures (NCTC Salisbury, UK)</td>
</tr>
<tr>
<td>ATCC 43502</td>
<td>Clinical isolate</td>
<td>American Type Culture Collection (ATCC (Manassas. VA)</td>
</tr>
<tr>
<td>81-176</td>
<td>Contains virulence plasmid (pVir). Increased virulence strain [139]</td>
<td>Dr. Patricia Guerry U.S. Naval Medical Research Center (NMRC Silver Spring, MD)</td>
</tr>
<tr>
<td><strong>Mutant \textit{Campylobacter jejuni strains with increased epithelial cell invasion}</strong></td>
<td>Posttranscriptional Regulator causing decreased motility and adherence with increased invasion of strains adherent [135]</td>
<td>Dr. Stuart Thompson Medical College of Georgia (Augusta, Georgia)</td>
</tr>
<tr>
<td>(\Delta csrA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\Delta cj0596)</td>
<td>Periplasmic chaperone with role in OMP folding causing increased motility and invasion [24, 25]</td>
<td>Dr. Stuart Thompson Medical College of Georgia (Augusta, Georgia)</td>
</tr>
<tr>
<td><strong>Mutant \textit{Campylobacter jejuni strains with decreased epithelial cell invasion}</strong></td>
<td>Mutant strain with altered flagella causing decreased motility and invasion [134]</td>
<td>Dr. A. Potter University of Saskatchewan Saskatchewan, Canada</td>
</tr>
</tbody>
</table>
Results

Four trials (n=3) were performed for each *C. jejuni* strain. The common wild-type laboratory strains *C. jejuni* 81116 and *C. jejuni* ATCC 43502 both showed a lack of internalization or protective effect when co-incubated with *A. castellanii* as indicated by a rate of internalization (*C. jejuni* internalized: *A. castellanii* recovered) <0.01 (Table 3.2). The other commonly used wild-type laboratory strain *C. jejuni* 81-176, which has also been reported to be hyper-invasive in human epithelial cells also showed a lack of internalization or protective effect when co-incubated with *A. castellanii* (Table 3.2). The mutant strain *C. jejuni* NCTC 11168 ΔflgK, reported to have decreased virulence in human epithelial cells due to a truncated flagella and lack of motility, showed a lack of internalization by *A. castellanii* (Table 3.2). The mutant strain *C. jejuni* 81-176 ΔcsrA has been reported to express a decrease in motility and adherence in human epithelial cells but an increase in invasion of cells that are attached to epithelial cells. *C. jejuni* 81-176 ΔcsrA was internalized or protected when co-incubated with *A. castellanii* indicated by a rate of internalization of 0.88 (*C. jejuni* internalized: *A. castellanii* recovered) (Table 3.2). The mutant strain *C. jejuni* 81-176 Δcj0596 has been reported to express both increased motility and invasion of human epithelial cells and was internalized or protected when co-incubated with *A. castellanii* indicated by a rate of internalization of 0.23 (*C. jejuni* internalized: *A. castellanii* recovered) (Table 3.2).
Table 3.2: Rate of internalization for *Campylobacter jejuni* strains

The average recovery of *C. jejuni* strains (CFU) from an initial inoculum of ~ 1 x 10^9 (CFU) for controls samples (*C. jejuni* only) and *C. jejuni* co-incubated with *A. castellanii*. The rate of internalization (ROI) (*C. jejuni* internalized: *A. castellanii* recovered) was calculated from the average of four trials. Each trial consisted of three samples of *C. jejuni* NCTC 11168 and *A. castellanii* and three control samples (*C. jejuni* only). Based on the rate of internalization the trials were grouped by “Internalization” (ROI > 0.1), “Possible Internalization” (ROI > 0.01), and “Lack of Internalization” (ROI <0.01). Standard error of the mean is denoted by “( )”.

<table>
<thead>
<tr>
<th>Campylobacter jejuni Strain</th>
<th>C. jejuni control (no A. castellanii)</th>
<th>C. jejuni with A. castellanii</th>
<th>Rate of Internalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>81116</td>
<td>2.4 x 10^4 (7.6 x 10^3)</td>
<td>1.5 x 10^4 (5.1 x 10^3)</td>
<td>(-)^ab</td>
</tr>
<tr>
<td>ATCC 43502</td>
<td>2.7 x 10^4 (7.6 x 10^3)</td>
<td>9.6 x 10^3 (1.1 x 10^4)</td>
<td>(-)^ab</td>
</tr>
<tr>
<td>81-176</td>
<td>1.4 x 10^5 (6.6 x 10^5)</td>
<td>3.2 x 10^4 (9.0 x 10^3)</td>
<td>(-)^a</td>
</tr>
<tr>
<td>81-176ΔcspA</td>
<td>3.1 x 10^4 (1.4 x 10^4)</td>
<td>6.7 x 10^5 (2.1 x 10^7)</td>
<td>0.88^b</td>
</tr>
<tr>
<td>81-176 Δcj0596</td>
<td>9.9 x 10^3 (5.2 x 10^3)</td>
<td>1.5 x 10^5 (3.1 x 10^4)</td>
<td>0.23^b</td>
</tr>
<tr>
<td>NCTC 11168 ΔflgK</td>
<td>1.1 x 10^5 (3.9 x 10^5)</td>
<td>2.3 x 10^5 (5.5 x 10^5)</td>
<td>(-)^a</td>
</tr>
</tbody>
</table>

^a Rate of internalization <0.01

^b Statistically significant (p < 0.05) compared to control

Discussion

Our previous study (Chapter 2) developed a modified gentamicin protection assay to investigate the interaction of *C. jejuni* with *A. castellanii*. The study reports that internalization or a protective effect of *C. jejuni* NCTC 11168 when co-incubated with *A. castellanii*, but this result varied in occurrence (Chapter 2). This variation led to the investigation of different *C. jejuni* strains to determine if this variation is strain specific.
In this study, wild-type strains of *Campylobacter jejuni* 81116 and ATCC 43502 showed a lack of internalization or protective effect. There was a significant increase in recovery of *C. jejuni* from *C. jejuni* only control samples compared to samples where *C. jejuni* was co-incubated with *A. castellanii*. These findings may suggest that these strains may be entering the amoebae but *A. castellanii* may be digesting these strains as a food source. Further study including microscopy would be needed to provide visual evidence that *C. jejuni* is entering *A. castellanii*.

The hyper-invasive strain *C. jejuni* 81-176 showed little or no internalization by *A. castellanii*. There was no statistical difference between *C. jejuni* only control samples and samples where *C. jejuni* was co-incubated with *A. castellanii*. These findings suggest that this strain most likely was unable to enter the amoebae. In addition to being a commonly used wild-type *C. jejuni* strain 81-176 has also been reported as highly invasive and virulent strain in epithelial cells [24, 25, 132, 135, 140]. This increase in invasion is due to the presence of the plasmid *pVir* which contains genes homologous to a type IV secretion system and orthologs to genes found in a pathogenicity island in *Helicobacter pylori* [132]. It may be that these plasmid based virulence factors are solely beneficial in entrance into epithelial cells and are not required for entrance into amoebae.

Both *C. jejuni* 81-176 mutants (ΔcsrA and Δcj0596) had an increased rate of internalization or protective effect compared to other strains with results consistent among all trials. There was a significant increase in recovery of *C. jejuni* when *C. jejuni* was co-incubated with *A. castellanii* compared to *C. jejuni* only control samples. Results of increased internalization with *A. castellanii* are similar to reports of these mutants
being more invasive with epithelial cells [24, 135]. The gene *cj0596* encodes a periplasmic chaperone which affects outer membrane protein folding [24, 25]. It has been reported that the *C. jejuni Δcj0596* mutant has an altered OMP profile changing surface-related characteristics with increased motility and invasion in epithelial cells [24]. The gene *csrA* encodes a post-transcriptional regulator and the *C. jejuni 81-176 ΔcsrA* mutant was reported to have a decrease in motility and adherence to epithelial cells but increase of invasion of attached bacteria to epithelial cells [135]. It may be that *C. jejuni* does not need to adhere and that other factors that affected invasion also aided in internalization into amoeba.

*C. jejuni NCTC 11168 ΔflgK* showed no internalization or protection when co-incubated with *A. castellanii*. Similar to *C. jejuni 81-176*, there was no statistical difference between *C. jejuni* only control samples and samples where *C. jejuni* was co-incubated with *A. castellanii* suggesting that this strain also most likely was unable to enter the amoebae. This may be due to lack of motility due to the truncated flagella [134]. *C. jejuni* have been observed gathering at particular sites on the surface of *A. polyphaga* prior to phagocytosis [23] and lack of proper motility may prevent this clustering and subsequent phagocytosis. In addition the flagellum is an important virulence factor in human cells and is involved in both adherence and the secretion of virulence associated proteins into epithelial cells and may have a similar role for entrance into amoebae [6, 134].

It is interesting to note that the mutant strains used in this study that expressed an increase or decrease in epithelial cell invasiveness expressed similar results in their
ability to be internalized or protected by *A. castellanii*. In comparing results based solely from the mutant *C. jejuni* strain used in this study, it may be that motility is not a major factor. Both *C. jejuni* NCTC 11168 ΔflgK and *C. jejuni* 81-176 ΔcsrA have decreased motility however *C. jejuni* NCTC 11168 ΔflgK was not internalized or protected and *C. jejuni* 81-176 ΔcsrA had the highest rate of internalization of all of the *C. jejuni* strains. In addition, the wild-types of these mutant strains had contrasting results, with *C. jejuni* 81-176 not being internalized or protected by *A. castellanii* and *C. jejuni* NCTC 11168 being internalized in 74% of co-incubations (Chapter 2). If motility or flagella are involved in internalization or protection in *A. castellanii*, the possibility exists that this difference in internalization or protection could be due to the differences in flagella structure or motility between the two strains.

In comparing the two mutant strains that have shown internalization or protection when co-cultured with *A. castellanii*, there are a few similarities. They were both created from the wild-type strain *C. jejuni* 81-176. *C. jejuni* 81-176 has been reported to be hyper-invasive in epithelial cells due to the presence of the *pVir* plasmid. In this study the wild-type *C. jejuni* 81-176 was not internalized or protected by *A. castellanii* leading us to believe that *pVir* is most likely not involved in internalization into *A. castellanii*. Both mutant strains have been reported to have increased internalization in epithelial cells however only *C. jejuni* Δcj0596 had an increase in attachment to epithelial cells.

It is not known if *C. jejuni*, *A. castellanii*, or both are initiating this internalization. If this internalization by *A. castellanii* is being driven by *C. jejuni* it may be that particular virulence factors or other factors unrelated to epithelial cell invasion are
involved in entrance into the amoebae. It has been suggested that different strains use
different strategies or mechanisms to enter epithelial cells and this may be similar to
interactions with amoebae [132].

Alternatively if this internalization is being driven by *A. castellanii* actively
grazing on *C. jejuni* as a food source, it may be possible factors on the outside of the cell
are affecting this internalization. *Acanthamoeba* spp. have been reported to have
different feeding preferences [131]. This is supported by reports that internalization of
*Salmonella* differs by serotype [60, 124]. There are over sixty reported serotypes of *C.
jejuni* [141]. The serotypes of *C. jejuni* NCTC 11168 (O:2) [141], 81116 (O:6) [142],
and 81-176 (O:23/36) [141] have been identified in the literature however the serotype of
*C. jejuni* ATCC 43502 has not been published to our knowledge. It is interesting to note
that each strain has a different serotype and each strain showed different results regarding
their interaction with *A. castellanii*. It would be interesting to test different strains of the
same serotype to determine if there is a correlation between serotype and internalization
into *A. castellanii*. This would further support the theory that amoebae feeding
preference is the determining factor of *C. jejuni* internalization. Our results for both *C.
jejuni* 81-176 may also support this theory as both mutants exhibited changes in the
outside of the bacterial cell which may have altered the serotype and amoebae preference.
In addition *C. jejuni* NCTC 11168 Δ*flgK* most likely would have exhibited a different
serotype due to the truncated flagella. However further investigation is needed to
determine the serotype of these mutant strains.
Conclusion

Overall this study was able to reveal that although there is variation in the internalization by *A. castellanii* among the same *C. jejuni* strain, individual strains seem to have tendencies to have little or internalization or higher amounts of internalization. Similarities were observed between invasiveness with epithelial cells and internalization by *A. castellanii* for all three mutants. Future studies should investigate a more extensive library of mutants such genes known to be involved in virulence as well as genes affecting the outside surface of the cell. Virulence mutants of note would include capsule and lipooligosaccharide (LOS) mutants shown to aid in invasion/survival in epithelial cells [6, 26]. In addition different serotypes of *C. jejuni* should be tested to determine if there is a correlation between *C. jejuni* serotype and internalization into *A. castellanii*. It would also be interesting to test different amoebal strains and species to see if like human cells, invasion differs among cell lines [133]. This information is not only useful for further investigation of the interaction of *C. jejuni* and amoebae but may also be useful in studying *C. jejuni* virulence in humans by using amoebae as a model for virulence that is easier to work with than epithelial cells.
Chapter 4: Assessment of the susceptibility of *Acanthamoeba castellanii* and *Campylobacter jejuni* to Antimicrobial Agents Used in Poultry Processing

Prepared for Submission to Journal of Food Protection

Abstract

*Campylobacter jejuni* is a common poultry contaminant and one of the leading causes of foodborne illness in the U.S. Despite the environmental sensitivity of *C. jejuni* and efforts to eliminate it from poultry by various processing methods it remains persistent on the final product. Free living amoebae such as *Acanthamoeba castellanii* have been isolated from poultry houses and meat cutting plants and have been reported to harbor *C. jejuni*, potentially protecting them from adverse conditions, including chemical disinfectants. Studies have reported the sensitivity of *A. castellanii* to various chemicals but have not tested the susceptibility of *A. castellanii* to antimicrobial agents used in poultry processing. In this study the sensitivity of *A. castellanii* to sodium hypochlorite (chlorine), trisodium phosphate (TSP), and cetylpyridinium chloride (CPC) was investigated. Organic load was simulated by the addition of bovine serum albumin (BSA) at high and low levels to determine its effect on sensitivity of *A. castellanii* to each antimicrobial agent. *C. jejuni* was exposed to the highest antimicrobial concentrations to which *A. castellanii* were resistant to determine if *A. castellanii* is more resistant to these antimicrobial agents than *C. jejuni*. *A. castellanii* were resistant to chlorine in the presence of organic load but were susceptible in the absence of organic load to levels of 4.5ppm or greater. *A. castellanii* was susceptible to TSP and CPC at concentrations
much lower than commonly used in processing regardless of organic load; however organic load had an effect on decreasing biocidal activity on the antimicrobials. The results suggest that TSP and CPC are more effective than chlorine for the reduction of \textit{A. castellanii}. \textit{A. castellanii} resistance to all three antimicrobials increased with the addition and increase of organic load. \textit{C. jejuni} was typically more susceptible to each antimicrobial compared to \textit{A. castellanii}. If \textit{A. castellanii} is able to survive processing and internalizes \textit{C. jejuni} it may provide \textit{C. jejuni} with a protection from these antimicrobials aiding in \textit{C. jejuni} persistence on poultry.

\textbf{Introduction}

\textit{Campylobacter jejuni} is one of the leading causes of foodborne illness in the U.S. \cite{9}. \textit{C. jejuni} is a common poultry contaminate and can be found on a high percentage of poultry products \cite{143, 144}. \textit{Campylobacter jejuni} is relatively fragile in both the environment and laboratory settings due to its sensitivity to pH, desiccation, and its requirement for a microaerophilic environment (85\% N\textsubscript{2}, 10\% CO\textsubscript{2}, 5\% O\textsubscript{2}) \cite{3, 23}. Despite its sensitivity to the environment and many poultry processing interventions in place \cite{10, 13, 34-38, 40, 41, 145-150} \textit{C. jejuni} is still persistent on poultry. This persistence leads to questions of whether other factors may be involved in the survival of \textit{C. jejuni} during poultry processing.

Common interventions for \textit{C. jejuni} in poultry processing include antimicrobial compounds such as sodium hypochlorite (chlorine), trisodium phosphate (TSP), and cetylpyridinium chloride (CPC) \cite{13, 30, 37, 38, 41, 151-155}. Chlorine is commonly used throughout processing in water at a maximum of 50ppm \cite{35, 36}. Chlorine damages
bacteria by oxidizing cell components. The effectiveness of chlorine is extremely limited due to the presence of organic load which is found at high levels in poultry processing [13, 35, 146]. Previous studies have investigated the effectiveness of chlorine to eliminate pathogens from poultry and have reported it insufficient [10, 13, 35-37, 40, 147]. Trisodium phosphate has an alkaline pH which appears to disrupt fatty molecules in cell membranes causing leakage of intracellular fluids [38, 146]. It has been reported more effective than chlorine in reducing pathogens on chicken [13, 38, 40, 145, 150] however due to its high pH it may cause increased wear on industrial equipment as well as environmental concerns due to phosphate effluents [38, 146]. Cetylpyridinium chloride has been reported effective in reducing pathogens from poultry [145, 149]and like TSP is alkaline and reacts with the cell membrane of bacteria resulting in cell leakage [41, 146].

*Acanthamoeba castellanii* is a species of free living amoeba which is ubiquitous in the environment and has been reported to internalize *C. jejuni* [12, 23, 67, 75, 79, 86, 93]. This internalization has been reported to afford *C. jejuni* protection from atmospheric conditions and suboptimal temperature [12, 23, 67, 75, 79, 86, 93], low pH [93], and chemical disinfectants such as chlorine [12, 67] and Virudine [12]. *Acanthamoeba* spp. have been isolated from many sources including fresh, stagnant, and chlorinated water [42] and have been detected along with other protozoa in poultry houses [12, 129] and meat cutting plants [130]. Since amoebae have been reported to be isolated from poultry facilities as well as provide a protective effect to *Campylobacter jejuni* from harmful environmental and chemical factors the possibility exists that this protection is aiding in *C. jejuni* persistence in poultry. In this study the antimicrobials
commonly used in poultry processing; chlorine, TSP, and CPC commonly used in poultry processing were investigated to determine *A. castellanii* susceptibility in both the absence and presence of organic load. If *A. castellanii* is able to survive antimicrobials used in processing and internalizes *C. jejuni* it may provide a protection from these antimicrobials for the pathogen aiding in *C. jejuni* persistence on poultry. *Acanthamoeba castellanii* is a human pathogen causing eye infections with the majority studies involving sensitivity to antimicrobials investigating their sensitivity to contact lens solutions [156-158]. Further studies have examined the efficacy of disinfectants such as chlorine and the quaternary ammonia compound benzalkonium chloride against amoebae [67, 159-162]. To our knowledge this is the first study investigating the sensitivity of *A. castellanii* to antimicrobials used in poultry processing.

**Methods**

**Culture Conditions.** *Campylobacter jejuni* NCTC 11168 was obtained from the American Type Culture Collection (ATCC Manassas, VA) stored at -80°C in Mueller Hinton Broth (MHB) with 10% glycerol. All *C. jejuni* cultures were incubated at 37°C under microaerophilic conditions (85% N₂, 10% CO₂, and 5% O₂) in a Binder (Bohemia, NY) CB150 incubator. Frozen working stocks were streaked for isolation onto Mueller Hinton Agar with 5µg/mL trimethoprim (MHA) followed by two 24h passages with sterile swabs onto fresh MHA plates. Prior to experiment MHA plates containing *C. jejuni* were swabbed and resuspended in Mueller Hinton Broth (MHB) and CFU/mL was estimated using a standard curve based on absorbance at 600nm. *C. jejuni* inoculum was
adjusted to $10^9$ CFU/mL by centrifugation at 3,220 x g for 20m at 4°C and resuspended in MHB.

*Acanthamoeba castellanii* ATCC 30010 was obtained from ATCC (Manassas, VA) culture stocks were maintained at room temperature as a monolayer in 75cm$^2$ tissue culture flasks containing 15mL PYG medium (Proteose Peptone-Yeast Extract-Glucose). Fresh cultures were started by removing 1mL aliquots and placing them into new 75cm$^2$ flasks containing 15mL PYG medium. Three day old *Acanthamoeba castellanii* cultures were harvested by removing the liquid from the 75cm$^2$ flasks and replacing it with 3ml Page’s Amoebal Saline (PAS). Flasks were then incubated in a freezer (-20°C) for 10m to detach amoebae [86]. Amoebae were further detached by repeated pipetting of the liquid over the monolayer. The liquid from multiple flasks was pooled and washed three times with PAS by centrifugation at 600 x g 5m at 4°C and the pellet was resuspended in PAS. *A. castellanii* trophozoites (vegetative amoebal cells) were counted using a hemocytometer with trypan blue dye (10X) added to allow for exclusion of any non-viable amoebae [64, 65]. Amoebae inoculum was adjusted to a concentration of $1 \times 10^7$ amoeba/mL in PAS.

**Preparation of antimicrobial agents.** Sodium hypochlorite (chlorine) (50ppm), trisodium phosphate (TSP) (12%), and cetylpyridinium chloride (CPC) (0.8%) were prepared and diluted in sterile H$_2$O. Presence of organic load was simulated by the addition of bovine serum albumin (BSA) to a final concentration of 3g/L (high organic load) and 0.3g/L (low organic load) [162].
**Test of Acanthamoeba castellanii susceptibility to antimicrobials.** Approximately 1 x $10^6$ *Acanthamoeba castellanii* trophozoites in PAS were placed into 1.5mL centrifuge tubes and centrifuged 600 x g 5m. The pellet was resuspended in 1mL of antimicrobial and incubated at room temperature 5m. Samples were then washed 3 times in 1mL PAS by centrifugation 600 x g 5m and resuspended in 900µL PAS and 100µL trypan blue. Viable amoebae were counted using a hemocytometer. Experiments were performed in triplicate with control samples using PAS in place of antimicrobials. Three trials performed for each antimicrobial. Antimicrobial concentrations started at concentration of 50ppm chlorine, 12% TSP and 0.8% CPC and were reduced by increments of 10% by dilution in sterile water to determine the level at which *A. castellanii* exhibited resistance.

**Test of Campylobacter jejuni susceptibility to antimicrobials.** The highest concentration of each antimicrobial that *A. castellanii* were resistant without organic load, in the presence of low (0.3g/L BSA) organic load, and in the presence of high (3g/L BSA) organic load was examined to determine *C. jejuni* susceptibility. Approximately 1 x $10^9$ *C. jejuni* in MHB were placed into 1.5mL centrifuge tubes and centrifuged 10,000 x g 2m. The pellet was then resuspended in 1mL of antimicrobial at appropriate concentrations and incubated at room temperate 5m. Concentrations for chlorine included 4.5ppm for chlorine with no organic load, and 50ppm for chlorine with both low and high levels of organic load. Concentration of CPC included 0.00072% for both CPC with no organic load and low level of organic load and 0.0032% for CPC with high organic load. Concentration of TSP included 0.12% for both TSP with no organic load and low organic load and 0.24% for TSP with high organic load. Samples were washed 3 times in 1mL PAS by centrifugation 10,000 x g 2m and resuspended in 1mL MHB.
Samples were serial diluted in MHB, plated onto MHA with 5µg/mL trimethoprim and incubated 48h at 37°C under microaerophilic conditions. Each experiment was performed in triplicate with control samples using PAS in place of antimicrobial. Three trials were performed for each antimicrobial. Total CFU *C. jejuni* for each chemical treatment were averaged and compared to control samples to determine *C. jejuni* susceptibility.

**Statistical Analysis.** T-tests were performed to determine significance between control and chemical samples. A p-value ≤0.05 was determined statistically significant.

**Results**

*Acanthamoeba castellanii* susceptibility to antimicrobials. *A. castellanii* were completely eliminated by chlorine in the absence of organic load at 20ppm (detection limit 555.6 amoebae). *A. castellanii* was completely resistant (no significant difference to control samples) to chlorine without organic load at 4.5ppm chlorine. Incorporation of organic load (0.3g/L and 3.0g/L BSA) resulted in *A. castellanii* resistance to undiluted chlorine (50ppm) (*Figure 4.1*).

*A. castellanii* exposed to cetylpyridinium chloride (CPC) in the absence of organic load was completely eliminated at 0.004% CPC and the highest concentration *A. castellanii* were completely resistant to was 0.00072% CPC. For chemicals containing low levels of organic load (0.3g/L BSA) *A. castellanii* were completely eliminated at 0.008% CPC and the highest concentration *A. castellanii* were completely resistant was 0.00072% CPC. CPC containing high levels of organic load (BSA at 3g/L) amoebae
were completely eliminated at 0.48% CPC and the highest concentration *A. castellanii* were completely resistant to was 0.0032% CPC (Figure 4.2).

Trisodium phosphate in the absence of organic load completely eliminated all *A. castellanii* at a concentration of 0.48% and the highest concentration *A. castellanii* were completely resistant was 0.12% TSP. For TSP containing low levels of organic load (0.3g/L BSA) all amoebae were eliminated at 0.6% TSP and the highest concentration *A. castellanii* were completely resistant was 0.12% TSP. TSP containing high levels of organic load (3g/L BSA) completely eliminated amoebae at 0.72% TSP and the highest concentration *A. castellanii* were completely resistant was 0.24% TSP (Figure 4.3).

**Campylobacter jejuni susceptibility to antimicrobials.** Susceptibility of *C. jejuni* was examined at the highest concentration for each chemical *A. castellanii* were resistant to was investigated to determine *C. jejuni* susceptibility. Chlorine reduced *C. jejuni* by 14.3%, 11.6%, and 0% for no organic load, low organic load (0.3g/L BSA), and high organic load (3.0g/L BSA) respectively. Cetylpyridinium chloride reduced *C. jejuni* by 40.4%, 26.4%, and 0% for no organic load, low organic load (0.3g/L BSA), and high organic load (3.0g/L BSA) respectively. Trisodium phosphate reduced *C. jejuni* by 69.1%, 53.5%, and 50.5% for no organic load, low organic load (0.3g/L BSA), and high organic load (3.0g/L BSA) respectively (Table 4.1).
Figure 4.1: Recovery of *Acanthamoeba castellanii* following exposure to sodium hypochlorite (Chlorine).

Total *A. castellanii* recovered of an initial inoculum ~1 x 10^6 after exposure to chlorine in both the presence and absence of organic load (simulated by the addition of BSA). Concentrations of chlorine ranged from lowest concentration at which no *A. castellanii* was recovered after exposure to the highest chlorine concentration at which *A. castellanii* recovered after exposure was not significantly different (p>0.05) than control samples containing *A. castellanii* with PAS.
Figure 4.2: Recovery of Acanthamoeba castellanii following exposure to cetylpyridinium chloride (CPC)

Total A. castellanii recovered of an initial inoculum ~1 x 10^6 after exposure to CPC in both the presence and absence of organic load (simulated by the addition of BSA). Concentrations of CPC ranged from lowest concentration at which no A. castellanii was recovered after exposure to the highest CPC concentration at which A. castellanii recovered after exposure was not significantly different (p>0.05) than control samples containing A. castellanii with PAS.
Figure 3. Recovery of *Acanthamoeba castellanii* following exposure to trisodium phosphate (TSP)

Total *A. castellanii* recovered of an initial inoculum ~1 x 10^6 after exposure to TSP in both the presence and absence of organic load (simulated by the addition of BSA). Concentrations of TSP ranged from lowest concentration at which no *A. castellanii* was recovered after exposure to the highest TSP concentration at which *A. castellanii* recovered after exposure was not significantly different (p>0.05) than control samples containing *A. castellanii* with PAS.
Table 4.1: Reduction of *Campylobacter jejuni* by chlorine, TSP, and CPC.

Percent (%) recovery of an initial inoculum of ~1 x 10^8 *C. jejuni* NCTC 11168 after exposure to sodium hypochlorite (chlorine), cetylpyridinium chloride (CPC) and trisodium phosphate (TSP) in the presence and absence of organic load. Chemical concentrations were chosen based on the concentrations at which *A. castellanii* was resistant to each antimicrobial.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Concentration</th>
<th>Organic Load</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine</td>
<td>4.5ppm</td>
<td>N/A (0g/L)</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>50ppm</td>
<td>Low (0.3g/L)</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>50ppm</td>
<td>High (3.0g/L)</td>
<td>0</td>
</tr>
<tr>
<td>CPC</td>
<td>0.00072%</td>
<td>N/A (0g/L)</td>
<td>40.4</td>
</tr>
<tr>
<td></td>
<td>0.00072%</td>
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^a Statistically significant (p < 0.05) compared to control

Discussion

The goal of this study was to determine the likelihood that *Acanthamoeba castellanii* could be aiding in the survival of *Campylobacter jejuni* during poultry processing. *Acanthamoeba* spp. have already been reported to protect internalized *C. jejuni* from adverse environmental conditions as well as disinfectants [12, 23, 67, 75, 79, 86, 93]. To our knowledge no one has investigated this protective effect using antimicrobials used in poultry processing that are applied to the poultry itself. Due to the
variation in internalization previously reported (Chapter 2) it was decided to test *A. castellanii* sensitivity to each compound without *C. jejuni*. It has been assumed that if *A. castellanii* is unaffected by the antimicrobial then internalized *C. jejuni* should also be protected.

In the absence of organic load all three antimicrobials completely eliminated *A. castellanii* at concentrations acceptable levels for used in poultry processing (50ppm chlorine, 0.8% CPC, and 12% TSP). Although *A. castellanii* was sensitive to all three antimicrobials at acceptable levels used, poultry processing is known to contain a high level of organic load and chlorine has been reported to lose effectiveness in the presence of organic load [13, 35, 146]. Therefore the effectiveness of each antimicrobial was tested in the presence of organic load to determine if organic load would increase *A. castellanii* resistance to each antimicrobial. Organic load was simulated at low and high levels (0.3g/L and 3.0g/L BSA) similar to [162] and *A. castellanii* resistance to all three antimicrobials increased with the addition and increase of organic load. In the presence of both low (0.3g/L) and high (3g/L) organic load both CPC and TSP were effective in eliminating *A. castellanii* at acceptable levels used in poultry processing (0.8% CPC and 12% TSP) however *A. castellanii* was completely resistant to chlorine at acceptable levels used in poultry processing (50ppm). Although *A. castellanii* was still sensitive to acceptable levels of CPC and TSP organic load did decrease the effectiveness of these antimicrobials. In processing where larger amounts of organic load than this study are present this may lead to the resistance of *A. castellanii* to larger concentrations of CPC and TSP.
The sensitivity of *C. jejuni* was then tested to determine if *A. castellanii* has a greater resistance to each antimicrobial than *C. jejuni*. *C. jejuni* sensitivity was tested at the highest concentrations of each antimicrobial that *A. castellanii* was completely resistant to in both the presence and absence of organic load. All three chemicals were more effective in reducing the level of *C. jejuni* in the absence of organic load and the presence of low levels of organic load. *C. jejuni* was susceptible to chlorine in both the absence of and low levels of organic load at concentrations that *A. castellanii* was resistant. *C. jejuni* showed complete resistance to chlorine at the acceptable level used in poultry processing with high organic load but both showed resistance to chlorine in the presence of high organic load. *C. jejuni* was susceptible to CPC in both the absence of and low levels of organic load at concentrations that *A. castellanii* was resistant but both also showed resistance to CPC in the presence of high organic load. *C. jejuni* was susceptible to TSP in the presence of no organic load, low organic load, and high organic load at levels that *A. castellanii* were resistant. TSP was more effective in reducing *C. jejuni* with the highest percent reduction of *C. jejuni* in the absence of organic load as well as in the presence of low and high levels of organic load. The reduction of *C. jejuni* by most of the antimicrobials was not significantly lower compared to controls. This may be due in part by the high inoculum level used in the study.

Our results conflict slightly with Vaerewijck et al.; 2012 in that chlorine was affected by organic load [162]. However, this study varied significantly in experimental design. This study used sterile water and Vaerewijck et al.; 2012 used hard water which is more typically used in processing. Sterile water is closer to a neutral pH which may have an effect on biocidal activity [162]. This study also used higher levels of amoebae
and a different species with a contact time of five minutes where Vaerewijck et al.; 2012 investigated both five and fifteen minutes. In poultry processing TSP and CPC are commonly used as sprays or dips which are difficult to reproduce in the laboratory. A five minute contact time was selected to determine if there was an anti-amoebal effect. Another interesting difference between this study and Vaerewijck et al.; 2012 was their use of flow cytometry to count viable amoebae. One study reported a higher recover with hemocytometer counts over flow cytometry [159]and because flow cytometry stains membrane damaged cells differently it may that some of these cells are still viable and that trypan blue may not stain these “slightly” damaged cells. *A. castellanii* were more resistant to these antimicrobials when compared to *C. jejuni*.

**Conclusion**

Overall *A. castellanii* tends to be more resistant to all three antimicrobials in both the presence and absence of organic load. These results would support the possible role of *A. castellanii* in the persistence of *C. jejuni* on poultry products. If *A. castellanii* is able to survive antimicrobial treatment then *C. jejuni* internalized by *A. castellanii* will also be resistant even at levels *C. jejuni* are susceptible to extracellularly. The levels of *A. castellanii* and *C. jejuni* used in this study are likely much higher than can be found on poultry, however on poultry there is a much higher organic load and microenvironments like feather follicles which may afford more protection to both *A. castellanii* and *C. jejuni* [13, 35, 146, 163]. Results from this study support the potential for *A. castellanii* to provide *C. jejuni* a protective effect from these antimicrobials under the right circumstances.
Further studies should investigate the sensitivity of *A. castellanii* as well as other Protist \cite{67, 79} reported to harbor *C. jejuni* under more practical conditions. These conditions could include using tap water \cite{162}, chicken, and an application that is more similar to processing (i.e. sprays, dips). It would also be interesting to test the efficacy of these chemicals on *C. jejuni* that is inside of *A. castellanii* at the time of exposure.
Chapter 5: Overall Discussion and Conclusion

*Campylobacter jejuni* is one of the leading bacterial causes of foodborne illness in the U.S. and the leading bacterial cause of diarrheal illness worldwide. *C. jejuni* is a major human pathogen and despite efforts to eliminate contamination through processing methods they are still persistent on poultry products. *C. jejuni* is relatively fragile in the environment and difficult to work with in the laboratory due to their sensitivity to a number of factors including low temperature, low pH, desiccation, and high oxygen contents. It is interesting that *C. jejuni* is able to survive these challenges as well as poultry processing, resulting in a contaminated poultry product. This persistence has led many to question if there are other factors involved in the persistence of *C. jejuni* that current processing methods are not taking into account.

Previous studies in the literature have shown promise that *C. jejuni* may interact with protozoa, in particular amoebae, and that this interaction is favorable to *C. jejuni*. The ability to survive under atmospheric conditions, lower temperatures and pH, and to survive the application of chemical disinfectants such as chlorine provides *C. jejuni* with the protection needed to persist in the environment and may play a key role in *C. jejuni* persistence on poultry. However further investigation is needed to determine the extent of this interaction and its role in *C. jejuni* survival during poultry processing.

The initial goal of this study was to investigate the possible role of protozoa such as *Acanthamoeba castellanii* in the environmental survival of *Campylobacter jejuni* and its subsequent persistence on poultry products. Unfavorable environmental factors combined with processing interventions have failed to completely eliminate *C. jejuni*
from poultry products. Current interventions have not taken into account the possibility of *A. castellanii* harboring *C. jejuni* and providing protection.

Initial review of the literature for methods to investigate the interaction of *C. jejuni* and *A. castellanii* described methods that were merely detecting the presence/absence of *C. jejuni* over time under unfavorable conditions. These studies assumed that *C. jejuni* persistence was solely due to the internalization by protozoa. Microscopic evidence has also been used to prove internalization of *C. jejuni* has occurred but fail to prove survival of *C. jejuni* internally. Another methodology that has been employed in many other studies investigating internalization of bacterial pathogens by protozoa is the gentamicin protection assay. This method is commonly used in studies investigating the internalization of bacterial pathogens and epithelial cells. Gentamicin is applied to kill bacteria leaving only those bacterial cells protected inside of the eukaryotic host.

The current gentamicin protection assay being used to investigate the interaction of bacterial pathogens with protozoa is employing the same methods that are used for epithelial cells. Epithelial cells need to be grown and maintained in a confluent monolayer. The use of a maintained monolayer is problematic for three reasons. Monolayers are typically formed in cell culture plates. In using a confluent monolayer there is no way to set a consistent concentration of amoebae for the assay. Wells are typically sacrificed prior to the experiment and the concentration of amoebae for each well is estimated based on the counts from these wells. This assumes that all the wells have the same amount of eukaryotic cells which may not be the case. This cell
concentration may also vary for different experiments. Another problem with working with a monolayer is that the wash steps require repeating pipetting over the monolayer. Monolayers, in particular those formed by *Acanthamoeba* spp. are not very stable and may be easily washed away during this pipetting causing partial or full loss of sample. Most importantly bacteria only controls cannot be employed when using a cell culture plate because the bacteria are readily washed away without a monolayer to attach to. In the absence of a bacteria only control, preliminary studies need to be performed to determine the sensitivity of the bacteria to gentamicin and it is then assumed that any residual bacteria that survives the assay is negligible.

These methods failed to adapt to differences in *A. castellanii* biology compared to epithelial cells. *A. castellanii* does not need to be maintained in a monolayer and a confluent monolayer is not representative of how they would occur naturally in nature, other than possibly as members of biofilms. Therefore the main focus of this study shifted to the development of a modified gentamicin protection assay (*Chapter 2*) that takes advantages of the difference in *A. castellanii* compared to epithelial cells. The goal was an assay that has more consistent inoculum levels of *A. castellanii*, a more stable sample, and most importantly a “bacteria only” control to determine the amount of bacteria that survives the assay independent of amoebae.

In this modified gentamicin protection assay cell-culture plates were replaced with 1.5mL centrifuge tubes. These tubes allow for the inoculation of known amounts of *A. castellanii* and *C. jejuni* and this amount can be more consistent among trials. Washing is performed by low speed centrifugation which provides a more stable pellet
compared to a monolayer, decreasing the chances of a loss of sample. Control samples containing *C. jejuni* in the absence of amoebae can also be used to determine the amount of *C. jejuni* that survives the assay independent of amoeba and this value can be taken into account when determining the amount of *C. jejuni* internalized by *A. castellanii*.

Previous studies, using a traditional gentamicin protection assay, lacking these controls determined internalization by the recovery of bacteria at the conclusion of the assay. This modified gentamicin protection assay determines internalization by the recovery of higher levels of *C. jejuni* in the presence of *A. castellanii* compared to *C. jejuni* controls in the absence of *A. castellanii*. By using this *C. jejuni* only control we were able to determine that *C. jejuni* does survive the assay in the absence of *A. castellanii*. This was an important finding because the ability to quantify and account for this independent survival provided completely different results than would have been obtained using previous methods. With the modified gentamicin protection assay it was determined that *C. jejuni* NCTC 11168 is internalized by *A. castellanii* but that this internalization varies in occurrence.

This inconsistency led to the further investigation into possible variables in the modified gentamicin protection assay that were not previously accounted for. *Campylobacter* culture conditions and inoculum were determined not to be a factor. The effectiveness of the gentamicin and triton X-100 steps of the assay was verified with preliminary studies. Amoebae recovery/survival through the assay was determined not to be a variable. This led to the investigation of the *A. castellanii* culture. The *A. castellanii* culture status was investigated (Chapter 2) to determine if that may have been a cause
for the inconsistency. Results indicated that culture status of *A. castellanii* did influence internalization of *C. jejuni*. Older maintained amoebal monolayers that were harvested for inoculum failed to internalize or protect *C. jejuni*. Two trials for older maintained *A. castellanii* suggested a possible digestion of bacteria as well.

There may be many reasons for this lack on internalization or protective effect. The amoebae may primarily be in a different growth phase which could alter their need to ingest bacteria. The majority of the amoebae may be in the beginning stages of encystation due to overcrowding or lack of nutrients, preventing them from feeding or altering the ability of *C. jejuni* to enter the amoebae. It may also be that because the amoebae monolayers are maintained axenic (without bacteria) for a longer period of time they may be acclimated to obtaining nutrients from the media as opposed to bacteria.

Newer, early growth phase *A. castellanii* were also investigated and were able to internalize *C. jejuni*, but not consistently among trials. Nutrient deprived amoebae that were early growth phase *A. castellanii* that were deprived of nutrient media for 24 hours prior to the experiment showed similar results. In addition two trials of the nutrient deprived *A. castellanii* suggested possible digestion by *A. castellanii*. This suggests that this internalization of *C. jejuni* 11168 is not a consistent event. Other factors unaccounted for, may also such as fluctuations in room temperature due to the gentamicin protection assay being performed at room temperature on the bench-top. The use of sealed 1.5mL centrifuge tubes may have provided a variable by providing a different oxygen content compared to a cell culture plate that allows for the exchange of gases. It may be that the amount of oxygen in different tube altered the survival of *C. jejuni*. 
Similarly is *C. jejuni* actively enters the amoebae through the pressures of oxidative stress; the use of tubes may have decreased the need to enter the amoebae. It may also be aspects of either *C. jejuni* or *A. castellanii* biology that are not yet known to researchers.

The modified gentamicin protection assay was then used to investigate additional wild-type strains of *C. jejuni* (Chapter 3). In this chapter we determined that the internalization of *C. jejuni* by *A. castellanii* is strain specific. The wild-type strains investigated were the common laboratory strains *C. jejuni* 81116 and *C. jejuni* 81-176 (a hyper-invasive strain) as well as a clinical isolate *C. jejuni* ATCC 43502. All three wild-type *C. jejuni* strains consistently showed no internalization or protective affect by *A. castellanii*.

There was a statistically significant increase in recovery of *Campylobacter jejuni* 81116 and *C. jejuni* ATCC 43502 from *C. jejuni* only control samples compared to samples where *C. jejuni* was co-incubated with *A. castellanii*. These findings may suggest that these strains may be internalized by *A. castellanii* and digested as a food source. Further study involving microscopy to provide visual evidence would be needed to further support this finding.

*C. jejuni* 81-176 showed little or no internalization or protection by amoebae. There was no statistical difference between *C. jejuni* 81-176 *C. jejuni* only control samples and samples where *C. jejuni* was co-incubated with *A. castellanii*. These findings suggest that this strain most likely was unable to enter the amoebae. Unlike the other wild-type strains there was no negative effect when co-incubated with *A. castellanii* making it unlikely that *A. castellanii* is digesting them as a food source.
Three mutant strains with altered virulence were also investigated to determine if there is a relationship between \textit{C. jejuni} virulence in epithelial cells and internalization and survival into \textit{A. castellanii} and if so what virulence factors are involved. This has been reported for other bacterial pathogens including \textit{Legionella pneumophila} and \textit{Salmonella}. Both \textit{C. jejuni} 81-176 mutants (Δ\textit{csrA} and Δ\textit{cj0596}) had an increased rate of internalization or protective effect compared to other strains with results consistent among all trials. There was a significant increase in recovery of \textit{C. jejuni} when \textit{C. jejuni} was co-incubated with \textit{A. castellanii} compared to \textit{C. jejuni} only control samples. Results of increased internalization with \textit{A. castellanii} are similar to reports of these mutants being more invasive with epithelial cells [24, 135]. The wild-type \textit{C. jejuni} 81-176 was not internalized by \textit{A. castellanii}, therefore it is unlikely that the virulence plasmid \textit{pVir} is involved in this internalization and more likely involved with the specific mutations. The gene \textit{cj0596} encodes a periplasmic chaperone which affects outer membrane protein folding [24, 25]. It has been reported that the \textit{C. jejuni} Δ\textit{cj0596} mutant has an altered OMP profile changing surface-related characteristics with increased motility and invasion in epithelial cells [24]. The gene \textit{csrA} encodes a post-transcriptional regulator and the \textit{C. jejuni} 81-176 Δ\textit{csrA} mutant was reported to have a decrease in motility and adherence to epithelial cells but increase of invasion of attached bacteria to epithelial cells [135]. It may be that \textit{C. jejuni} does not need to adhere and that other factors that affected invasion also aided in internalization into amoeba.

\textit{C. jejuni} NCTC 11168 Δ\textit{flgK} showed no internalization or protection when co-incubated with \textit{A. castellanii}. Similar to \textit{C. jejuni} 81-176, there was no statistical difference between \textit{C. jejuni} only control samples and samples where \textit{C. jejuni} was co-
incubated with \textit{A. castellanii} suggesting that this strain also most likely was unable to enter the amoebae. The wild-type \textit{C. jejuni} NCTC 11168 (Chapter 2) was internalized by \textit{A. castellanii} but this internalization was not consistent. The lack of internalization by \textit{C. jejuni} NCTC 11168 ΔflgK may be due to lack of motility caused by truncated flagellum [134]. \textit{C. jejuni} have been observed gathering at particular sites on the surface of \textit{A. polyphaga} prior to phagocytosis [23] and lack of proper motility may prevent this clustering and subsequent phagocytosis. In addition the flagellum is an important virulence factor in human cells and is involved in both adherence and the secretion of virulence associated proteins into epithelial cells and may have a similar role for entrance into amoebae [6, 134].

It is unknown whether \textit{A. castellanii} or \textit{C. jejuni} are causing this internalization. It is possible that \textit{C. jejuni} is driving this internalization, possibly for survival under unfavorable environmental conditions. This is supported by the microscopic evidence by Axelsson-Olsson et al., 2005 where \textit{C. jejuni} cells are seen gathering at the site of phagocytosis [23]. This is further supported by another study by Axelsson-Olsson where decreases in pH increased internalization of \textit{C. jejuni} into \textit{A. castellanii} [93]. This theory is interesting because our findings in the inconsistency of internalization by \textit{C. jejuni} NCTC 11168 (Chapter 2) may have been due to the variation in environmental pressures that were not controlled for affecting \textit{C. jejuni} entrance into \textit{A. castellanii}. It may also be that this predation by amoebae and other protozoa has applied evolutionary pressure for intracellular survival, inadvertently causing virulence in other eukaryotic cells and making \textit{C. jejuni} a pathogen. If the latter is true than it would be likely that specific virulence traits would be involved in entry and survival into \textit{A. castellanii}. Our finding in
(Chapter 3) with the mutant strains that were investigated support this theory. This is difficult to assess because work with *C. jejuni* has not been as extensive as many other bacterial pathogens and many virulence factors may have not been identified. In addition it is believed that different strains of *C. jejuni* use different strategies and/or mechanisms for virulence. Findings in (Chapter 2 and 3) with different wild-type *C. jejuni* strains showed different results in internalization by *A. castellanii* supporting possible differences among strains. In addition our finding that *C. jejuni* NCTC 11168 ΔflgK was not internalized or digest may be due this mutant having a truncated flagella resulting in decreased motility and being unable to migrate and gather to a specific site on the amoebal surface.

Further study would be needed to investigate an extensive mutant library to determine specific genes involved in this internalization. Due to the possible differences among mechanisms/strategies among *C. jejuni* strains, it may be that libraries need to be created for multiple strains. The mutants could then be investigated using this modified gentamicin protection assay coupled with microscopy techniques to provide visual evidence to support the data obtained.

Alternatively this internalization may be driven by *A. castellanii* actively grazing on *C. jejuni* as a food source. *Acanthamoeba* spp. have been reported to have different feeding preferences [131]. This feeding preference is most likely due to factors on the outside of the cell. This is supported by reports that internalization of *Salmonella* differs by serotype [60, 124]. There are over sixty reported serotypes of *C. jejuni* [141]. The serotypes of *C. jejuni* NCTC 11168 (O:2) [141], 81116 (O:6) [142], and 81-176
have been found in the literature however the serotype of *C. jejuni* ATCC 43502 has not been published to our knowledge. It is interesting to note that the each strain has a different serotype and each strain showed different results regarding interaction with *A. castellanii*. It would be interesting to test different strains of the same serotype to determine if there is a correlation between serotype and internalization into *A. castellanii*. This would further support the theory that amoebae feeding preference is the determining factor of *C. jejuni* internalization. Our results for both *C. jejuni* 81-176 mutants (Chapter 3) may support this theory as both mutants exhibited changes in the outside of the bacterial cell which may have altered the serotype and amoebae preference. In addition *C. jejuni* NCTC 11168 ΔflgK most likely would have exhibited a different serotype due to the truncated flagella. However further investigation is needed to determine the serotype of these mutant strains. It may be that the gathering of *C. jejuni* cells on the outside of the amoebae reported in Axelsson-Olsson is actually caused by the amoebae. *C. jejuni* may be attracted to the site of the amoebae due to chemotaxis. Amoebae are known to phagocytize only when food is in large quantities, so this may be a strategy employed to be able to maximize food obtained by phagocytosis.

This internalization may also be caused by a combination of factors from *A. castellanii* and *C. jejuni*. It may also be caused by factors not yet known about either organism since much is still left to discover about both *A. castellanii* and *C. jejuni*. Another interesting theory is that this internalization is an artifact of experimental design. It may be that certain *C. jejuni* strains are not internalized by *A. castellanii* for reasons unknown; however the strains that are internalized may eventually be digested however this digestion is missed due to the time it is sampled. Different factors may be involved
in the time needed to phagocytize and completely digest bacteria. It may be that the variation in (Chapter 2) was an example of this variation. This theory is further supported by the differences in culture status reported in this chapter. Older *A. castellanii* cells may not be feeding at as high of a rate as younger or nutrient deprived cells and may need more time to internalize *C. jejuni*, or conversely may be feeding at a variable rate. In addition the two trials of nutrient deprived *A. castellanii* showed significantly less recovery of *C. jejuni* co-incubated with *A. castellanii* suggesting this ingestion was happening. This theory is supported by Bui et al.; 2012 where it was reported that *C. jejuni* is internalized and eventually digested over time. In response to the survival of *C. jejuni* for longer periods of time under atmospheric conditions, they believe that it is *A. castellanii* reduction of harmful dissolved oxygen that is allowing the *C. jejuni* to survive. It has also been reported with *Listeria monocytogenes* that *Acanthamoeba* spp. create “backpacks” to store bacteria until they are ready to phagocytize them. This backpacking may and the variation in when the bacteria are phagocytized may play a key role in this variation seen in rate of internalization and should be investigated further with *C. jejuni*.

**In (Chapter 4)** we revisited out original goal which was to investigate the possible role of *A. castellanii* in the persistence of *C. jejuni* on poultry. We investigated the sensitivity of *A. castellanii* to antimicrobial compounds commonly used in poultry processing. Previous studies have reported that when *C. jejuni* and other bacterial pathogens are internalized by amoebae they are provided with protection from chemical disinfectants. It appears that if the amoebae can survive exposure to the disinfectant then any bacterial inside of the amoebae are also protected. Therefore we tested the susceptibility of *A. castellanii* to sodium hypochlorite (chlorine), cetylpyridinium
chloride (CPC), and trisodium phosphate (TSP). Additionally it has been reported that the biocidal activity of many antimicrobials such as chlorine are reduced in the presence of organic load. Poultry processing is known to contain high levels of organic load. Therefore we further tested the susceptibility of *A. castellanii* to all three antimicrobials with simulated high and low levels of organic load. CPC and TSP were effective in eliminating *A. castellanii* at antimicrobial concentrations acceptable for use in poultry processing in both the absence and presence of organic load although *A. castellanii* resistance to each chemical increased as organic load increased. Chlorine was effective in eliminating *A. castellanii* at an acceptable concentration for poultry processing but *A. castellanii* was resistant as the same concentration when organic load was applied. Although *A. castellanii* was susceptible to these antimicrobials at these levels, given that increased organic load increases *A. castellanii* resistance to each antimicrobial, if levels of organic load reach a high enough concentration then *A. castellanii* may be unaffected during poultry processing.

Additionally in chapter 4 the susceptibility of *C. jejuni* to each antimicrobial was investigated at concentrations that *A. castellanii* was resistant in both the presence and absence of organic load. Results overall indicated that *C. jejuni* was more susceptible to these antimicrobials then *A. castellanii*. These results have led us to hypothesize that if *A. castellanii* is able to survive these antimicrobials during processing and if *C. jejuni* is able to be internalized by *A. castellanii*, and then the amoebae would likely protect *C. jejuni* from these antimicrobials. Further investigation using more applicable processing conditions and including exposure of *C. jejuni* internalized by *A. castellanii* are needed to
further support the theory that *A. castellanii* can protect *C. jejuni* throughout poultry processing.

Regardless of if intracellular survival is happening, this interaction may still afford protection to *C. jejuni* during processing. It may be that under the right conditions *A. castellanii* is able to phagocytize *C. jejuni*. In addition, under the right conditions *A. castellanii* may be able to survive or possibly just protect *C. jejuni* to exposure to antimicrobials. If the amoebae are killed (either by the antimicrobial or at a later processing step) or *C. jejuni* is somehow able to survive intracellularly or escape following exposure to antimicrobials then this may aid in *C. jejuni* persistence on poultry. Even if *C. jejuni* is eventually digested by *A. castellanii* normally, the application of antimicrobials may alter the ability of *A. castellanii* to effectively digest bacteria as has been reported with chlorine.

Overall this work has provided a new useful tool to investigate this interaction for future research. This modified gentamicin protection assay has addressed limitations of the traditional gentamicin protection assay, providing much different results than previously reported. *Acanthamoeba* spp. have been proven to provide protection for multiple bacterial strain however it is not clear if they provide protection to *C. jejuni*. Results from this work support that there is a chance that *A. castellanii* may be aiding in the persistence of *C. jejuni* both in the environment and on poultry. Further study is needed to better determine the likelihood and extent of this protection. The primary goal of future research should validate if *C. jejuni* is actually able to survive intracellularly inside of *A. castellanii* indefinitely or if they are eventually digested. This can be
performed by modifications to the modified gentamicin protection assay to investigate different variables including temperatures and co-incubation times. If *C. jejuni* survive intracellularly then the next goal should be to determine what organism is initiating this internalization to better focus the research approach in studying this interaction. Most importantly the modified gentamicin protection assay is a useful tool to investigate the internalization of bacteria by protozoa, taking into account differences in protozoa and epithelial cells. This modified gentamicin protection assay may be applied to investigate the interactions of numerous bacterial pathogens with multiple species of protozoa providing a major contribution to this field of study.
List of References


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### Chapter 2 Data

**Figure 2.1 Standard curve of *Campylobacter jejuni* NCTC 11168**

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Figure 2.2 *Campylobacter jejuni* sensitivity to gentamicin

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Figure 2.3 *Acanthamoeba castellanii* sensitivity to gentamicin

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**Figure 2.4 Campylobacter jejuni sensitivity to triton X-100**

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Figure 2.5 *Acanthamoeba castellanii* sensitivity to triton X-100

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Table 2.1 Results of modified gentamicin protection assay using non-standardized *Acanthamoeba castellanii* cultures

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### C. jejuni

#### Culture plating

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### C. jejuni

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**Inoculum plating**

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### C. jejuni

**Culture plating**

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| Counts                | Average        |
| Counts                | Dilution       |
| Counts                | Adj vol plated |
| Counts                | CFU/mL         |
| 32                    | 41             |
| 50                    | 41             |
| 1.0E+06               | 10             |
| 4.10E+08              |                |

<p>| Control               |                |
| Counts                | Counts         |
| Counts                | Average        |
| Counts                | Dilution       |
| Counts                | Adj vol plated |
| Counts                | CFU/mL         |
| 16                    | 21             |
| 28                    | 21.67          |
| 1.0E+01               | 10             |
| 2.17E+03              |                |
| 98                    | 25             |
| 33                    | 52             |
| 1.0E+00               | 10             |
| 5.20E+02              |                |
| 66                    | 80             |
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Inoculum plating

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### C. jejuni

#### Culture plating

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Chapter 3 Data

Table 3.1 Rate of internalization for *Campylobacter jejuni* strains

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<th>Culture plating</th>
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<th>CFU/mL</th>
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C. jejuni *flgK*

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*A. castellanii*

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**Inoculum plating**

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Chapter 4 Data

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Average: 6.62E+08
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SE: 1.36E+08
Appendix B: Curriculum Vitae

Brian P. Dirks

2931 Denise Rd.
East Norriton, PA 19403
(215)-720-5455

Drexel University
245 N 15th Street
New College Building
Philadelphia, Pa 19102
215-762-8669

EDUCATION

Drexel University, Philadelphia, PA 01/09 –
Present
Department of Biology
Ph.D. Candidate (Expected Graduation: Fall 2012)
Ph.D. Dissertation title “The Role of Acanthamoeba castellanii in Campylobacter jejuni
Environmental Survival and Pathogenicity”

Drexel University, Philadelphia, PA 01/07 – 03/10
M.S. Biology
“The effect of nonthermal dielectric barrier discharge plasma on Salmonella
enterica and Campylobacter jejuni on raw poultry”

Delaware Valley College, Doylestown, PA 08/01 – 05/05
B.S. Small Animal Science

WORK EXPERIENCE

U.S. Department of Agriculture, Wyndmoor, PA 01/10 – 09/10
Food Safety Intervention Technology Research Unit

Research Assistant
Developed a study to examine the interaction of soil fungi and the foodborne pathogens Salmonella enterica and Escherichia coli. This study investigated the role of this interaction in foodborne illness in produce.
Drexel University, Philadelphia, PA 09/05
– 12/08
Department of Biology, Dr. Jennifer J. Quinlan’s Laboratory
Laboratory Technician III
- Maintained teaching laboratories for all biology courses
- Ordered and prepared reagents and supplies for teaching laboratories
- Supervised and trained undergraduate support staff
- Re-organized and changed methods to streamline maintenance of teaching laboratories
- Designed and optimized a real-time PCR protocol for the detection of the fungi Dekkera anomala in wine and juice products. Began work on creating a genomic library for different yeast species.
- Developed a study in collaboration with Drexel’s Plasma Institute involving nonthermal dielectric barrier discharge plasma, a new technology and apply it to raw chicken to determine its effectiveness in reducing foodborne pathogens on raw chicken.

U.S. Department of Agriculture, Wyndmoor, PA 06/01
– 08/05
Microbial Food Safety Research Unit
Biological Science Aid
- Provide support for food microbiology research projects
- Molecular characterization of bacteria: Riboprinter, PFGE, PCR, Serotyping
- Recovery, isolation, and identification of bacteria

TEACHING EXPERIENCE

Drexel University, Philadelphia, PA 01/07 -
Present
Department of Biology
Adjunct Professor
- FDSC 450/550 Food Microbiology lecture: Fall/12
- FDSC 451/551 Food Microbiology laboratory: Fall/12

Teaching Assistant
- BIO 104: Biology II Genes and Heredity laboratory: Winter/09
- BIO 141: Essential Biology laboratory: Spring/09, Summer/09
- BIO 306: Biochemistry Laboratory: Fall/09
- BIO 222: Microbiology Laboratory: Fall/10, Winter/11, Summer/12
- BIO 226: Microbiology for Health Professionals Laboratory: Spring/11, Fall/11, Spring/12
- BIO 110: Biological Diversity, Ecology, and Evolution Laboratory: Winter/12

**Delaware Valley College**, Doylestown, PA
Department of Animal Biotechnology and Conservation
Teaching assistant
- SA 1105: Introduction to Small Animal Management: Fall/02 - Spring/05 (6 Semesters)

**PUBLICATIONS**


Joshua Gurtler, David Douds, **Brian Dirks**, Jennifer Quinlan, April Nicholson, John Phillips, Brendan Niemira. Survival of *Salmonella* and *E. coli* O157:H7 in Soil and Translocation into Leek (*Allium porrum*) as Influenced by Arbuscular Mycorrhizal Fungi (*G. intraradices*). Accepted to *Applied and Environmental Microbiology*.

**Brian Dirks** and Jennifer Quinlan. Development of a Modified Gentamicin Protection Assay to Investigate the Interaction between *Campylobacter jejuni* and *Acanthamoeba castellani*. In preparation for submission.

**Brian Dirks** and Jennifer Quinlan. Assessment of the susceptibility of *Acanthamoeba castellani* and *Campylobacter jejuni* to Antimicrobial Agents Used in Poultry Processing.
POSTER PRESENTATIONS


ORAL PRESENTATIONS


“Internalization Rates and Survival of Campylobacter jejuni by Acanthamoeba castellani Varies by Strains of Campylobacter” International Association for Food Protection Annual Meeting, July 2012.

PROFESSIONAL AFFILIATIONS

International Association for Food Protection 01/08 – Present
Vita

Brian Patrick Dirks was born August 31st, 1983 in Philadelphia, PA. He attended Delaware Valley College in Doylestown, PA and obtained a Bachelor’s of Science in Small Animal Science and Biotechnology in 2005. While attending college he worked at the United States Department of Agriculture’s Eastern Regional Research Center in Wyndmoor, PA. While working at the USDA Brian assisted on many intervention projects involving detection, enumeration, and reduction of *Listeria monocytogenes, Campylobacter jejuni*, *Salmonella, E. coli*, and *Bacillus anthracis* Sterne. Brian returned to the USDA for a research assistantship during his Ph.D. studies under the guidance of Dr. Joshua Gurtler, Dr. David Douds, and Dr. Brendan Niemira. This work has led to a co-authorship on a submitted manuscript for Applied and Environmental Microbiology "Survival of *Salmonella* and *E. coli* O157:H7 in Soil and Translocation into Leek (*Allium porrum*) as Influenced by Arbuscular Mycorrhizal Fungi (*G. intraradices*)" Brian completed his Master’s degree in 2010 while working as a technician for Dr. Jennifer Quinlan. While with Dr. Quinlan he has obtained two publications Stein, S.E., B.P. Dirks, and J.J. Quinlan, *Assessing and Addressing Safe Food Handling Knowledge, Attitudes, and Behaviors of College Undergraduates*. Journal of Food Science Education, 2010. 9(2): 47-52. and Brian P. Dirks et al.,. The effect of Plasma on *Campylobacter* and *Salmonella* on the surface of chicken breast and chicken skin. Journal of Food Protection, 2012. 75(1): 22-28. While completing his studies Brian has taught numerous biology laboratories and was an adjunct professor for Food Microbiology.