

**The Roles of Calprotectin and Calgranulin C in
Campylobacter jejuni Infection**

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

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December 2017

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ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Jeremiah Johnson, for providing me with the resources I needed to make this thesis possible.

Thank you to my committee members, Dr. Sarah Lebeis and Dr. Todd Reynolds, for your valuable insight.

I would also like to thank my post-doc, Dr. Tylor Johnson, for helping to make lab such an enjoyable experience. Without your support and encouragement, this would not have been possible.

Thank you to Brittni Kelley for not letting me give up, no matter how overwhelming graduate school seemed. You are a wonderful friend and coworker and you helped make this possible.

Thank you to Nicole Yackley for helping emotionally support me through graduate school. You are an amazing friend and an excellent procrastinator.

I would like to thank my family, particularly my mother, Victoria, my father, Gregory, my aunt, Renee, and my two very energetic dogs, JJ and Archer. Your patience and enthusiasm has helped me accomplish so much. Thank you all for always being there for me.

Lastly, I would like to give a special thanks to the Upward Bound program in Loretto, Pennsylvania. I learned a lot through this program and it has helped me to achieve so much. I especially want to thank Anne, Kristin, and Leo who were excellent mentors. I would not be where I am today without the three of you.

ABSTRACT

Campylobacter jejuni is a leading cause of bacterial-derived gastroenteritis worldwide. In the developed world, campylobacteriosis is most commonly acquired following the consumption of cross contaminated foods or undercooked poultry meat, since the bacterium asymptotically colonizes chickens. In the developing world, infection most commonly occurs through drinking contaminated water. Following ingestion, *Campylobacter* adheres to the intestinal epithelium and mucus layer, causing toxin-mediated inflammation and inhibition of fluid reabsorption. Currently, the bacterial mechanisms behind colonization and disease are relatively unknown. Thus, it is important to identify factors that influence the development of these sequelae during and after initial *C. jejuni* infection.

Ferrets (*Mustela putorius furo*) have been previously used as an animal model of human campylobacteriosis, but intensive investigation into this model has yet to be performed. Thus, in this study, we non-invasively examined for disease in ferrets and determined whether there were any effects on development following infection with *C. jejuni*. Ferrets were effectively colonized by *C. jejuni* with peak fecal loads observed at day 3 post-infection, and with full resolution by day 12 post-infection. Infected male ferrets had reduced weight when compared to uninfected males early in infection, but this was resolved by the conclusion of the experiment. All infected ferrets exhibited reduced activity and minor changes in fecal consistency. Cytokine levels in serum increased in

response to infection, with significance observed for IL-10 and TNF α (tumor necrosis factor alpha). Occult blood was observed in both uninfected and infected cohorts. Additionally, in response to infection, the neutrophil protein calgranulin C (S100A12) was found to be increased in the feces of both ferrets and humans infected with *C. jejuni*, while calprotectin (another neutrophil protein) was not. The addition of either purified S100A12 or of calprotectin to *in vitro* cultures of *C. jejuni* was found to inhibit growth in a zinc-dependent manner. These results suggest that upon infection with *C. jejuni*, neutrophils that are trafficked to the intestine release S100A12 and possibly calprotectin as a mechanism for inhibiting *C. jejuni* growth in the intestine.

TABLE OF CONTENTS

Chapter One Introduction and General Information.....	1
<i>Campylobacter</i>	1
<i>C. jejuni</i>	3
<i>C. coli</i>	4
Other Strains	5
<i>Campylobacter</i> Mechanism of Infection	6
Antibiotic Resistance in <i>Campylobacter</i>	8
Mechanisms of Antimicrobial Resistance	11
Additional Complications of Antibiotic Resistance	12
Chapter Two Literature Review	14
S100 Proteins	14
Calprotectin	18
Calgranulin C	20
The Role of Zinc in <i>Campylobacter</i>	22
Ferrets as a Model for Gastrointestinal Illness	23
Chapter Three Materials and Methods	24
Animals	24
Bacterial Cultures and Inoculation	24
Sample Collection and <i>Campylobacter</i> Enumeration	25
Serum Assay	26
Fecal Assays	27
<i>C. jejuni</i> Growth in the Presence of S100 Proteins in vitro	28
Restoration of <i>C. jejuni</i> Growth Following Treatment with S100 Proteins.....	29
RNA seq Analysis of S100A12-Treated <i>C. jejuni</i>	29
Chapter Four Results and Discussion	32
Sample Collection and <i>Campylobacter</i> Enumeration	32
Serum Assay	35
Fecal Assays	38
<i>C. jejuni</i> Growth in the Presence of S100 Proteins in vitro	44
Restoration of <i>C. jejuni</i> Growth Following Treatment with S100 Proteins.....	46
RNA seq Analysis of S100A12-Treated <i>C. jejuni</i>	48
Chapter Five Conclusions and Recommendations.....	51
References	55
Vita	83

LIST OF TABLES

Table 1. Summary of S100 proteins.....	16
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LIST OF FIGURES

Figure 1. Effect of <i>C. jejuni</i> infection on growth and development of ferrets.	33
Figure 2. <i>C. jejuni</i> loads in feces of infected ferrets.....	34
Figure 3. Cytokine levels in ferret serum.....	37
Figure 4. Occult blood found in ferret feces via Hemocult II test.....	39
Figure 5. Calprotectin concentrations in fecal samples.....	42
Figure 6. S100A12 concentrations in fecal samples.....	43
Figure 7. S100 protein inhibition of <i>C. jejuni</i> growth <i>in vitro</i>	45
Figure 8. Supplementation of media for <i>C. jejuni</i> growth restoration following S100 protein treatment.....	47
Figure 9. Differences in gene regulation of S100A12-treated <i>C. jejuni</i> and untreated <i>C. jejuni</i> , analyzed using RNA seq.....	50

CHAPTER ONE

INTRODUCTION AND GENERAL INFORMATION

Campylobacter

Campylobacter is a Gram-negative, rod-shaped member of the group Epsilonproteobacteria, and is closely related to *Helicobacter*. It is a motile organism that typically has either a single polar flagellum or bipolar flagella and moves in a corkscrew fashion (Fonseca, Fernández, & Rossi, 2016).

Campylobacter is a microaerophile that grows preferentially between 37°C and 42°C (Penner, 1988). Although this bacterium cannot grow *in vitro* at temperatures below 37°C, it is able to survive and perform basic metabolic functions at temperatures as low as 4°C (Hazeleger et al., 1998). This explains how some *Campylobacter* species can survive on stored poultry meat for extended periods of time and resume proper function once ingested. Several *Campylobacter* species reside asymptotically in the gastrointestinal tract of chickens and other domestic livestock. Chickens become colonized early in life (approximately 2-3 weeks after hatching) and are typically colonized for life (Sahin et al., 2013).

Some thermotolerant species of *Campylobacter*, such as *C. jejuni*, *C. coli*, and *C. lari*, can infect humans and cause bacterial gastroenteritis (Penner, 1988). *C. jejuni* and *C. coli* are the most common causes of *Campylobacter* infection. Campylobacteriosis typically produces symptoms such as acute mild to severe

watery or bloody diarrhea, fever, and abdominal pain. Symptoms last approximately one week and the onset of symptoms occurs 24 to 72 hours following ingestion (Man, 2011). In developing countries, infection most commonly occurs through drinking contaminated water. Children five years of age and younger are particularly susceptible to infection, which may lead to persistent colonization in the gastrointestinal tracts of these children. Although campylobacteriosis symptoms do not tend to last much longer, this persistence can cause further complications such as stunting (Amour et al., 2016). In the developed world, *Campylobacter* infection is most commonly acquired through the consumption of contaminated meat, and young adults (15-24 years old) as well as children are susceptible to infection (Friedman et al., 2000).

Campylobacter is typically self-limiting in immunocompetent patients, but can lead to more serious diseases and higher instances of death in those persistently colonized, people with compromised immune systems, young children, and the elderly (Amour et al., 2016; Coker et al., 2002). Complications such as Guillian-Barré syndrome, septicemia, reactive arthritis, and Miller Fisher syndrome can occur following campylobacteriosis (Goldstein et al., 2016).

Campylobacter has also been linked to certain irritable bowel diseases such as ulcerative colitis and Crohn's disease (Kaakoush et al., 2014). Studies have shown that up to 80% of all fresh broiler chicken meat is contaminated with *Campylobacter* (EFSA, 2014). The estimated cost of campylobacteriosis and resulting complications is approximately \$1.7 billion annually in the United States

alone (Maue et al., 2014). The high cost, serious complications, and ease of infection of *Campylobacter* makes it a priority to study.

C. jejuni

Campylobacter jejuni accounts for approximately 90% of all campylobacteriosis cases (Gillespie et al., 2002). This bacterial species alone is the most common cause of food-borne bacterial-derived gastroenteritis in the world and it is estimated that as much as 80% of all poultry carcasses in the European Union are contaminated with *C. jejuni* (European Food Safety Authority, 2010). Previously, it was thought that *C. jejuni* colonizes chickens without causing any physiological signs of illness (Hermans et al., 2012). However, a more recent study shows that *C. jejuni* colonization in broilers does provoke an inflammatory immune response, and that the level of inflammation stays elevated in certain breeds. This was seen by the elevated levels of the proinflammatory cytokines CXCLi1, CXCLi2, and IL-1 β . Additionally, the authors of this study found increased incidences of diarrhea in chickens colonized by *C. jejuni*, which may be linked to skin disorders found in these birds, such as pododermatitis, as well as damage to the intestinal mucosa (Humphrey et al., 2014).

Under harsh environmental conditions, such as in aquatic environments or on abiotic surfaces, *C. jejuni* is able to form biofilms (Maal-Bared et al., 2012). Aerobic and low nutrient environments promote the formation of these biofilms (Reeser et al., 2007; Reuter et al., 2010). Although the mechanism of *C. jejuni*

biofilm formation is unknown, studies have shown that this ability requires quorum sensing, functioning flagella and certain surface proteins (Asakura et al., 2007; Reeser et al., 2007). Studies have also shown that when in a biofilm, *C. jejuni* changes the regulation of certain genes. More specifically, it has been shown that iron uptake is upregulated, as well as proteins that are used for oxidative defense and membrane transport (Sampathkumar et al., 2006). *Campylobacter* biofilm formation may hint at a method of preventing *C. jejuni* infection in humans, as different strains of *C. jejuni* have been seen to have varying levels of ability to form biofilms due to mutations in either a putative flagellar protein or a phosphate acetyltransferase (Joshua et al., 2006).

C. coli

While *Campylobacter jejuni* is responsible for approximately 90% of all campylobacteriosis cases worldwide, *C. coli* accounts for approximately 10% of all cases (Gillespie et al., 2002). *C. jejuni* is typically the most abundant species of *Campylobacter* present in chickens and cattle (Thakur et al., 2006). However, *C. coli* has been shown to be more prevalent in broiler meat than *C. jejuni* in some countries (European Food Safety Authority, 2010; Torralbo et al., 2015). Additionally, *C. coli* is known to be more prevalent in pigs than *C. jejuni* (Thakur et al., 2006). Increasingly, incidences of *C. coli* are becoming more prevalent. One study reports that *C. coli* may be responsible for as high as 25% of all *Campylobacter*-related enterocolitis (Man, 2011). *C. jejuni* and *C. coli* share many of the same pathological symptoms. A recent case study has even shown

evidence for *C. coli*'s ability to cause the rare campylobacteriosis complication myopericarditis (Moffatt, Moloi, & Kennedy, 2017). Fortunately, serious campylobacteriosis complications such as myopericarditis rarely cause prolonged damage or death (Imazio et al., 2014). It is speculated that such complications are caused by excessive antibiotic treatment of campylobacteriosis, as antibiotics are commonly used as a treatment for *Campylobacter* infection, since there is no standard for how long antibiotics should be given or at what dosage (Hessulf et al., 2016).

Other Strains

Although *C. jejuni* and *C. coli* are the main causes of campylobacteriosis, other *Campylobacter* strains have been shown to cause disease (Meunier et al., 2016b). For instance, *C. concisus* is a strain of *Campylobacter* often found in human oral cavities of both diseased and healthy individuals (Zhang et al., 2010; Peterson et al., 2007). Although harmless to the oral cavity, *C. concisus* has been linked to triggering inflammatory bowel disease (Zhang et al., 2014). Similarly, *C. rectus* can be found in the human oral cavity. However, this *Campylobacter* species is known to cause periodontitis as well as gastrointestinal symptoms (Mahlen & Clarridge, 2009). *C. rectus* may also lead to complications such as Crohn's disease and ulcerative colitis (Mukhopadhyay et al., 2011; Man et al., 2010), and has been seen to cause severe acute otitis media under certain circumstances (Kakuta et al., 2016). *C. fetus*, a strain often found in cattle and sheep, produces the typical gastrointestinal symptoms associated with

campylobacteriosis in humans as well as systemic infections in rare cases (Wagenaar et al., 2014). Certain subspecies of *C. fetus* may also cause spontaneous abortion both in humans and in cattle (Fujihara et al., 2006; Mshelia et al., 2010). *C. curvus* is a relatively new *Campylobacter* species that is rarely found in humans, but when present may cause severe diarrhea (Abbott et al., 2005). Additionally, in a more recent study, *C. curvus* was present in a vaginal infection and may have contributed to premature birth (Mendz et al., 2014). *Campylobacter lari* is another species that is rarely isolated from humans, but is known to cause enteritis, bacteremia, and urinary tract infections (Bézian et al., 1990). *C. lari* has also been seen to infect prosthetics, and such an infection resulted in the death of an immunocompetent patient (Werno et al., 2002).

***Campylobacter* Mechanism of Infection**

Little is known about how *Campylobacter* is able to successfully infect humans and colonize chickens. *C. jejuni*, the most prevalent *Campylobacter* species, typically resides in the cecal mucosal crypts of colonized birds (Coward et al., 2008). *Campylobacter* counts as high as 10^8 CFU (colony forming units)/gram of cecal contents has been isolated from chicken cecal mucosa (Meade et al., 2009). Although it is generally accepted that this colonization has no effect on birds, evidence has recently suggested that *Campylobacter* does indeed promote an immune response in these animals, and that this response can be damaging to the intestines (Humphrey et al., 2014). Many efforts to

control or reduce *Campylobacter* loads in broiler farms have proven to be ineffective, perhaps in part due to the lack of knowledge of how this bacterium is spread to chickens. Several studies have demonstrated that *Campylobacter* is highly unlikely to be spread from breeder hens to eggs, either due to the difficulty of this pathogen infecting the chick or the lack of similarity in the genotypes of *C. jejuni* isolated from breeder hens compared to those later isolated from broilers (Jacobs-Reitsma, 1995; Sahin, Kobalka, & Zhang, 2003; O'Mahoney et al., 2011; Patriarchi et al., 2011). Instead, it has been shown that *Campylobacter* is able to survive outside of a host for an extended period of time, and that it is more likely that chicks are colonized when exposed to *C. jejuni* in the environment (Murphey, Carroll, & Jordan, 2006; Newell, 2002; Sahin, Morishita, & Zhang, 2002).

The most common mode of transmission of *Campylobacter* to humans in the developed world is through the consumption of undercooked poultry meat, which typically becomes contaminated during the slaughtering process when *C. jejuni* from the intestines comes into contact with the meat (Friedman et al., 2000; Humphrey, O'Brien, & Madsen, 2007). Once ingested, *Campylobacter* must invade the intestinal mucosa and adhere to intestinal epithelial cells. When the submucosa and nearby tissues are reached, bacteria are able to reach essential nutrients such as iron, and peristaltic forces no longer reach the bacteria, making it drastically easier to stay attached to the host. How *Campylobacter* is able to reach this area of the intestines is still a mystery, but it is known that flagellum, serine protease HtrA, and lipooligosaccharide play a role in *C. jejuni* invasion

(Backert et al., 2013). Once *Campylobacter* has managed to adhere to the intestinal epithelium, it induces diarrhea in its host. This increases its spread throughout the host intestines as well as to new hosts (Young, Davis, & Dirita, 2007; van Putten et al., 2009; Dasti et al., 2010).

Antibiotic Resistance in *Campylobacter*

Campylobacter antibiotic resistance is on the rise, with resistance to fluoroquinolone antimicrobials such as ciprofloxacin being reported most frequently in several countries (Engberg et al., 2001; Moore et al., 2006; Alfredson & Korolik, 2007). In the United States and Canada, ciprofloxacin resistance is estimated to be around 19-47% (Gaudreau & Gilbert, 2003; Gupta et al., 2004; Nachamkin, Ung, & Li, 2002), while in Europe ciprofloxacin resistance is widespread and may be as high as 99% in human and animal isolates (Alfredson & Korolik, 2007; Papavasileiou et al., 2007; Gallay et al., 2007; Hakanen et al., 2003; Krausse & Ullmann, 2003). A more recent study confirms the accuracy of these estimates, stating that ciprofloxacin resistance in human *Campylobacter* isolates is around 23% in Montreal, and does indeed appear to be rising (Gaudreau et al., 2014). Investigations in Germany, Ireland, and Italy have yielded similar results (Luber et al., 2003; Lucey et al., 2002; Pezzotti et al., 2003). In Africa and Asia, fluoroquinolone-resistant *Campylobacter* was not found before 1991, but is now highly prevalent, with resistance rates over 80% in some parts of Thailand and Hong Kong (Isenbarger et al., 2002; Putnam et al., 2003; Tjaniadi et al., 2003; Chu et al., 2004).

Fluoroquinolone resistance in Australia and New Zealand is much lower than in other countries, but is still present at low rates (around 3%). However, this is typically associated with *Campylobacter* infection acquired during travel. This lack of resistance is likely due to it being unlawful to distribute fluoroquinolones to food animals in Australia (Unicomb et al., 2003).

In addition to fluoroquinolone resistance, some *Campylobacter* strains have acquired resistance to macrolides, but this appears to depend greatly on the strain as well as the animal from which it was isolated (Belanger & Shyrock, 2007). For instance, in North America erythromycin (the most commonly used macrolide against *Campylobacter*) resistance is normally around 10%. However, in *C. coli* found in turkeys and pigs in the same regions, erythromycin resistance was around 40% on average, with some instances of up to 80% (Luangtongkum et al., 2006; Thakur & Gebreyes, 2005). Similarly, throughout Europe macrolide resistance is low in *Campylobacter*, except for *C. coli* isolated from chickens and pigs, which has resistance rates up to 80% (Papavasileiou et al., 2007; McGill et al., 2006; Bardon et al., 2008; Gibreel & Taylor, 2006). In Asia and Australia, as in previously mentioned countries, macrolide resistance is mostly found in *C. coli* isolated from pigs (Miflin, Templeton, & Blackall 2007; Hong et al., 2007; Senok et al., 2007; Shin & Lee, 2007). In Africa, interestingly, erythromycin resistant *Campylobacter* is more commonly isolated from humans than from any food animal (Gibreel & Taylor, 2006; Samie et al., 2007; Kassa, Gebre-Selassie, & Asrat, 2007).

Although the major antibiotics used against *Campylobacter* are erythromycin and ciprofloxacin, it is important to note that many strains of *Campylobacter* have been found to be resistant to additional antibiotics that are less often used for this type of infection. Resistance to tetracycline in broilers is seen to change with age. One study reported that *Campylobacter* isolated from broilers 3-4 weeks old was not resistant to tetracycline, while 66.7% of isolates from 5-week-old chicks were resistant and 100% of isolates from chicks 6-7 weeks old were resistant. Resistance prevalence then decreases and levels off around 10-15% in mature chickens (Luangtongkum et al., 2008). This may be due to the initial time *Campylobacter* is attempting to colonize, followed by a spike, where the maximum amount of *Campylobacter* is present (and thus the maximum amount of gene transfer can occur), and then a leveling off. Tetracycline resistance is widespread and reported at relatively high numbers- 46% in Finland (Hakanen et al., 2003), 37.8% in Germany (Luber et al., 2003), and 43-68% in Canada (Gaudreau & Gilbert, 2003). Similar to erythromycin resistance, streptomycin resistance appears to be most prevalent in *C. coli* strains isolated from pigs, and is not as prevalent as other antimicrobial resistances (Pezzotti et al., 2003; McGill et al., 2006). Ampicillin resistances in *Campylobacter* isolates have also been reported in several countries, but prevalence appears to vary greatly from country to country-17% in Finland (Hakanen et al., 2003), approximately 50% in France (Gallay et al., 2007), and 31% in the United States (Luangtongkum et al., 2008). *C. jejuni* shows a higher

incidence of resistance than other *Campylobacter* strains (McGill et al., 2006).

Like most of the discussed antibiotic resistances, *Campylobacter* ampicillin resistance is rising, particularly in human isolates (Luber et al., 2003).

Mechanisms of Antimicrobial Resistance

In *Campylobacter*, antimicrobial resistance can be gained either through mutations or through horizontal gene transfer. Fluoroquinolone resistance in *Campylobacter* is acquired via specific point mutations in DNA gyrase A, in what is known as the “quinolone resistance-determining region” (Payot et al., 2006).

Other bacterial species such as *Salmonella* and *Escherichia coli* must have multiple point mutations in DNA gyrase A to gain fluoroquinolone resistance, but *Campylobacter* loses susceptibility to this antibiotic after just a single point mutation (Luo et al., 2003). Macrolide resistance is more difficult for *Campylobacter* to attain as it requires modification of a ribosomal target.

Resistance can occur via a point mutation in 23rRNA or in the L4 and L22 ribosomal proteins. Resistance can alternatively occur through enzyme-mediated methylation of these sites (Gibreel & Taylor, 2006; Payot et al., 2006). However, gaining macrolide resistance through ribosomal point mutations is far more common, and only *C. rectus* is known to gain resistance via methylation (Roe, Weinberg, & Roberts, 1995; Luangtongkum et al., 2009; Concoran et al., 2006).

Not all *Campylobacter* antibiotic resistance occurs via mutations. For example, tetracycline resistance is conferred through the gene *tet(O)*, which encodes a ribosomal protection protein (Taylor et al., 1985). This is the only gene

in *Campylobacter* that is known to provide tetracycline resistance. The *tet(O)* gene is common among various *Campylobacter* species and is present in isolates from many different animals (Moore et al., 2006). This gene is thought to have initially been acquired via horizontal gene transfer from either *Streptomyces*, *Streptococcus*, or *Enterococcus* (Batchelor et al., 2004; Taylor, Garner, & Allan, 1983). *Campylobacter* is highly resistant to beta-lactam antibiotics due to its ability to produce beta-lactamase (Zhang & Plummer, 2008), and also has intrinsic resistance to a variety of antibiotics such as bacitracin, trimethoprim, and vancomycin (Taylor & Courvalin, 1988; Corry et al., 1995).

Additional Complications of Antibiotic Resistance

Antibiotic resistance in *Campylobacter* is becoming increasingly common, making infections more difficult to treat. This resistance is probably due to overuse of antibiotic treatments during *Campylobacter* infection, with no standard dosage or length of treatment (Hessulf et al., 2016). Antibiotic resistance can spread easily, even between different *Campylobacter* strains, via horizontal gene transfer either in bacterial cultures (Jeon et al., 2008; Wilson et al., 2003) or in chicken intestines (Avrain, Vernozy-Rozand, & Kempf, 2004; Boer et al., 2002). *Campylobacter* effectively carries out conjugation at not only an intraspecies level (Pratt & Korolik, 2005; Avrain, Vernozy-Rozand, & Kempf, 2004), but at an interspecies and occasionally even an intergenous level (Gibreel, Skold, & Taylor, 2004; Nirdnoy, Mason & Guerry, 2005). Many of the plasmids that *Campylobacter* typically transfers via conjugation carry genes that confer

resistance to tetracyclines and aminoglycosides (Pratt & Korolik, 2005; Gibreel, Skold, & Taylor, 2004). Antibiotic resistance in bacteria is often associated with a fitness cost such as a reduced growth rate in antibiotic-free environments (Luangtongkum et al., 2009). This does appear to be the case with lower levels of erythromycin resistance in *Campylobacter* (Caldwell, Wang, & Lin, 2008). However, it has been demonstrated that fluoroquinolone resistance in *Campylobacter* is not only stable in the absence of antibiotics, but that fluoroquinolone-resistant mutants actually have enhanced fitness as they are able to effectively outcompete other strains in a chicken host (Luo et al., 2005).

CHAPTER TWO

LITERATURE REVIEW

S100 Proteins

The CDC and WHO have classified *Campylobacter* as a serious threat to public health due to the high number of reported infections each year, the seriousness of both the infection and post-infectious complications, and *Campylobacter's* resistance to the clinically-important antibiotics ciprofloxacin and azithromycin (Johnson, Shank, & Johnson, 2017). Because of this, it is important to understand how *Campylobacter* affects its host and it is necessary to consider non-antibiotic methods of treatment for this pathogen. One promising solution is S100 proteins, which are commonly used as markers of gastrointestinal inflammation (Leach et al., 2007). Some S100 proteins, such as S100A12 (calgranulin C) and S100A8/S100A9 (calprotectin), are known to have antimicrobial properties (Kehl-Fie & Skaar, 2010).

S100 proteins are regulators of several important cell processes in vertebrates including energy metabolism, growth, motility, cell cycle regulation, apoptosis, transcription, proliferation, differentiation, and inflammation. These proteins are absent in invertebrates. There are currently around 20 different known S100 proteins, all with the ability to bind calcium (Heizmann, Fritz, & Schäfer, 2002; Donato et al., 2013). S100 proteins are named for their solubility in 100% ammonium sulfate saturated solution at a neutral pH. They are small

(10-12 kilodaltons), acidic proteins that are classified by their two distinct EF-hand calcium-binding regions (Sedaghat & Notopoulos, 2008). Binding calcium allows them to regulate numerous intracellular and extracellular processes and signaling pathways. In this way, they are physiologically necessary to keep excess calcium out of certain pathways and to prevent the precipitation of calcium (Donato et al., 2013). S100 proteins are typically activated by a specific molecule such as a growth factor, cytokines, or toll-like receptor ligands. They can be secreted from the cell, where they act as damage-associated molecular pattern factors (DAMPs) or alarmins. Secreted S100 proteins help to regulate innate and adaptive immune responses as well as cancer cell locomotion and tissue repair under certain circumstances (Donato, 2007; Ehrchen et al., 2009; Hsu et al., 2009; Sorci et al., 2011).

Each S100 protein has its own set of intracellular functions that are upregulated and downregulated individually. These functions are often necessary for proper cell function, but are extremely unrelated (Table 1). For example, S100A1 is mainly expressed in skeletal muscle fibers, cardiomyocytes, and neurons (Donato, 2001) and is important for the contractile function of these muscles (Rohde et al., 2010), while S100A2 binds p53 proteins (proteins responsible for regulating cell growth and proliferation), and thus is recognized as a tumor-suppressing protein (van Dieck et al., 2009). S100 proteins have gained increasing amounts of attention due to many of them, like S100A2, being involved in cancer regulation. However, it is important to note that other S100

Table 1. Summary of S100 proteins. Each protein has an individual intracellular and extracellular function that may or may not be related to one another and to the functions of other S100 proteins (Donato et al., 2013).

Protein	Intracellular Function	Extracellular Function
S100A1	Muscle contraction, energy	Enhances Ca ²⁺ influx
S100A2	Tumor suppression	Chemotactic for eosinophils
S100A3	Epithelial cell differentiation	No known extracellular roles
S100A4	Cell survival, motility, and invasion	Cell migration, tumor growth
S100A5	No known intracellular roles	No known extracellular roles
S100A6	Cell proliferation	Regulates secretion
S100A7	Tumorigenesis	Innate immunity
S100A8	Immune response	Regulates inflammation
S100A9	Inhibits monocyte differentiation	Reduces inflammation
Calprotectin	Myeloid cell differentiation	Neutrophil chemotaxis
S100A10	Membrane protein trafficking	Macrophage recruitment
S100A11	Recombinational DNA damage	Chondrocyte differentiation
Calgranulin C	Vascular remodeling	Pro-inflammatory reactions
S100A13	Release of fibroblast growth factor	Secretion of FGF-1
S100A14	Cancer suppressor	Proliferation and apoptosis
S100A15	No known intracellular roles	Chemotactic for monocytes
S100A16	Adipogenesis	No known extracellular roles
S100B	Stimulate proliferation, inhibit	Protects neuronal cells
S100G	Cytosolic Ca ²⁺ buffer	No known extracellular roles
S100P	Transendothelial migration of tumor	Tumor growth and drug
S100Z	No known intracellular roles	No known extracellular roles

proteins carry out equally important functions, such as S100A11, which can repair damage to recombinant DNA and can stimulate cell growth (Murzik et al., 2008; Sakaguchi et al., 2008).

S100 proteins can be secreted from the cell and are found in many human body fluids, such as semen, urine, saliva, and feces. Secreted S100 proteins are usually associated with active disease states, and some of these proteins can even be used as biomarkers for specific diseases (Mocellin, Zavagno, & Nitti, 2008; Foell, Wittkowski, & Roth, 2007; Gogas et al., 2009; Gazzolo & Michetti, 2010). S100 proteins have individual extracellular functions that may or may not relate to their intracellular functions, and for some of these proteins, extracellular function is determined by binding (Fritz et al., 2010). For example, S100A1 has relatively similar functions both inside and outside of the cell, as it is important intracellularly for muscle fibers and contraction, and extracellularly for calcium influx to cardiomyocytes (Rohde et al., 2010; Reppel et al., 2005). S100 proteins such as S100A2 has differing functions, as it is important for p53 binding intracellularly, but plays a role in chemotaxis extracellularly (van Dieck et al., 2009; Komada et al., 1996). Also, varying amounts of S100 proteins are required to activate their target receptors, depending on the function to be performed (Donato, 2007). Since they can be excreted under certain circumstances, some S100 proteins may be used as disease markers, particularly for inflammation. Such markers could be useful to indicate levels of neutrophils present and

amount of phagocyte activation, which could indicate presence of inflammation and possibly disease progression (Foell et al., 2004).

Calprotectin

Calprotectin is also known as the S100A8/S100A9 heterocomplex.

Intracellularly, S100A8 plays a role in the immune response that is not fully understood (Donato et al., 2013). Studies have shown that S100A8 is necessary for cell and/or immune function, as deleting this gene in mice is lethal as early as the embryonic stage. Mice embryos lacking the S100A8 protein were infiltrated with maternal cells prior to resorption (Passey et al., 1999). In some cells, S100A8 can be found in the nucleus, and this protein accounts for approximately 20% of all neutrophil cytoplasm (Grimbaldeston et al., 2003). Macrophages take up S100A8 when exposed to oxidative stress, and this process is increased when the anti-inflammatory cytokine interleukin (IL)-10 is present (Donato et al., 2013). Extracellularly, S100A8 helps to regulate inflammation, which it does by repelling neutrophils via oxidation sensitivity (Sroussi et al., 2006), and may play a role in maternal-fetal tolerance (Passey et al., 1999). Again, IL-10 upregulates S100A8 activity, as does corticosteroid (Hsu et al., 2005; Endoh et al., 2009). In contrast, S100A9 prevents reduction in telomerase activity that is caused by S100A8 intracellularly (Rosenberger et al., 2007). This protein is also able to inhibit dendritic cell and macrophage differentiation, and may contribute to tumor growth by producing intracellular reactive oxygen species (ROS) (Cheng et al., 2008). When S100A9 is depleted, neutrophils are unable to properly respond to

certain chemoattractants, which can result in transendothelial cell migration (Vogl et al., 2007; McNeill et al., 200). Like S100A8, S100A9 repels neutrophils once it has been secreted. In this way, it is able to reduce inflammatory responses as well as reducing neutrophil migration (Sroussi, Berline, & Palefsky, 2007). Some evidence suggests that S100A9 may even be able to mediate the immune response to a certain degree controlling the amount of B7 co-stimulatory molecules are expressed on antigen presenting cells (Shimizu et al., 2011).

Certain functions performed by S100A8 and S100A9 are still performed in the S100A8/S100A9 heterocomplex (calprotectin), while others are not. For instance, intracellularly, calprotectin may play a role in myeloid cell differentiation through kinase activity, which is similar to the function of S100A9 (Lagasse & Weissman, 1992; Murano, Collart, & Huberman, 1989; Averill et al., 2011). The S100A8/S100A9 heterocomplex also performs functions independent from either S100A8 or S100A9 alone, such as intracellular transportation of unsaturated fatty acids and arachidonic acid (Siegenthaler et al., 1997). Extracellularly, this complex has been shown to be chemotactic for neutrophils (Ryckman et al., 2003) and influential to the migration of several types of cells, such as myeloid-derived suppressor cells and some tumor cells (Sinha et al., 2008; Hermani et al., 2006). This indicates that unlike S100A9, the heterocomplex promotes a pro-inflammatory response. One study shows that calprotectin may also effect cell growth and induce certain types of apoptosis (Seeliger, 2003).

When S100A8 and S100A9 heterodimerize into calprotectin, both proteins are stabilized. This stabilization results in elongation of the S100A9 C-terminal α -helix, which causes tetramers to form. Formation of these tetramers causes two high affinity zinc-binding sites to be exposed at the interface of the S100A8/S100A9 subunits (Korndorfer, Brueckner, & Skerra, 2007). This conformational change allows for metal sequestration, which is responsible for calprotectin's antimicrobial properties (Kehl-Fie et al., 2011). Specifically, calprotectin has been shown to inhibit fungal species as well as *Staphylococcus aureus* by chelating zinc and manganese (Hsu et al, 2009; Corbin et al., 2008). It is also able to protect the skin from microbial invaders when produced by keratinocytes (Hsu et al., 2009).

Calgranulin C

S100A12, commonly referred to as calgranulin C, is an S100 protein with antimicrobial properties similar to calprotectin, but without a heterodimer form (Donato et al., 2013). S100A12 can typically be found in neutrophils, macrophages, and smooth muscle cells (Goyette & Geczy, 2010). Intracellularly, S100A12 functions to mediate between cytoskeletal elements and membranes (Vogl et al., 1999). Additionally, this protein binds calcium and appears to have calcium-dependent chaperone/anti-chaperone properties, which aid in the prevention of aldolase and GAPDH aggregation (Htakeyama et al., 2004). S100A12 expression in skin cells appears to halt growth (Hitomi et al., 1998). This is interesting, as S100A12 is also suspected to be involved in vascular

remodeling, and there is evidence that many vascular smooth muscle cell dysfunctions are associated with the overexpression of S100A12 (Hofmann Bowman et al., 2010). Additionally, S100A12 overexpression in the airways has the positive effect of reducing inflammation from allergies (Hofmann Bowman et al., 2011).

The extracellular functions of S100A12 are arguably more difficult to study than calprotectin, as rodents, the standard model for studying the functions of S100 proteins *in vivo*, do not have this protein in their genomes (Fuellen et al., 2004; Ravasi et al., 2004). It is known, however, that extracellular S100A12 is chemotactic for monocytes and mast cells at low concentrations (Yan et al., 2008), and that high levels of S100A12 can activate mast cells and IgE, stimulating pro-inflammatory cytokine production (Yang et al., 2007). S100A12 can be activated by $\text{TNF}\alpha$, IL-6, endotoxin, and LPS (Yang et al., 2001). In addition to promoting a pro-inflammatory immune reaction, this protein is important for fighting off parasites, as it is able to bind paramyosin which inhibits parasite growth and motility (Moroz et al., 2009). Like calprotectin, many of the functions of S100A12 are due to its ability to chelate zinc (Donato et al., 2013), but instead of also binding manganese, S100A12 is able to bind copper (Zackular, Chazin, & Skaar, 2015).

The Role of Zinc in *Campylobacter*

Zinc is an important nutrient for animals, as too little leads to defects in immune function and too much can be toxic (Prasad et al., 2007, Costello et al., 1997). Since the S100 proteins calprotectin and calgranulin C are known to bind zinc, and many microbes require zinc for growth, this zinc sequestration may be what is responsible for their antimicrobial properties (Donato et al., 2013). This is especially important in inhibiting *Campylobacter*, as this bacterium has its own zinc-binding protein (ZnuA) and has been shown to require zinc for colonization of the chicken gastrointestinal tract (Davis, Kakuda, & DiRita, 2009). In one study, the ability of a *C. jejuni* $\Delta znuA$ mutant strain to colonize chicks was observed and it was shown that colonization ability, but not replication, was greatly reduced in this mutant. Zinc-binding proteins isolated from the chicken ceca were found to be responsible for inhibiting the growth of these mutants (Giella & DiRita, 2012).

Aside from its importance for colonization and virulence, little is known about what specific functions zinc has in *Campylobacter*. In other microorganisms, zinc is known to act as a catalyst for multiple proteins by assisting in intramolecular interactions and protein folding (Si et al., 2017). Zinc has also been shown to stabilize protein structures and is thus likely involved in multiple cell functions (Berg & Shi, 1996). Not surprisingly, these roles make zinc important for microbial growth and morphology, with additional evidence

suggesting that zinc may also play a role in the regulation of certain transcriptional factors in these species (Moulin et al., 2016).

Ferrets as a Model for Gastrointestinal Illness

Ferrets (*Mustela putorius furo*) are an ideal animal model to use for *Campylobacter* infection because they are one of the few animals that produce clinical signs similar to symptoms in humans when infected, such as self-limiting diarrhea. Younger animals (approximately 6-7 weeks old) are ideal models as they produce more severe diarrhea upon infection (Bell & Manning, 1990). However, both young and adult ferrets will produce signs of disease (Burr et al., 2005). Commonly used rodents such as rabbits and germ-free mice can be used, but must be genetically manipulated prior to infection (Yrios & Balish, 1986; Caldwell et al., 1983). Additionally, ferrets are susceptible to post-infections complications from *Campylobacter*, such as inflammatory bowel disease (IBD) (Burgess, 2007). However, great care must be taken when studying these animals, as slight dietary changes may also produce IBD symptoms (Watson et al., 2016). Unfortunately, aside from descriptions of gastrointestinal symptoms, the literature available on *Campylobacter*-infected ferrets is lacking, and much of it is outdated, including information regarding the immune response to disease in these animals.

CHAPTER THREE

MATERIALS AND METHODS

Animals

Twelve weaning-aged ferrets (5.5-6 weeks of age), six males and six females, were obtained from Marshall BioResources (North Rose, N.Y.). Ferrets were individually housed at the Walters Life Sciences (WLS) Animal Facility at the University of Tennessee Knoxville. They were acclimated in the WLS animal facility for seven days to allow the animals to recover following transportation. Ferrets were fed Envigo Teklad global ferret diet and given water *ad libitum* throughout this study, except for restriction 2-3 hours prior to inoculation with *Campylobacter jejuni*.

Bacterial Cultures and Inoculation

The principal *C. jejuni* strain used in this study was 81-176. This strain was grown on *Campylobacter*-specific media and Gram stained to ensure purity of the culture. The pure culture was then streaked again on *Campylobacter*-specific media and incubated at 37°C under microaerophilic conditions for 24 hours. Six suspensions of *C. jejuni* 81-176 were made in 5mL phosphate-buffered saline (PBS) and diluted to OD₆₀₀ 2. Ferrets were anesthetized with isoflurane and inoculum was administered via orogastric tubing and 12mL syringe. Six ferrets were infected with 5mL *C. jejuni* 81-176 OD₆₀₀ 2 in PBS

combined with 5mL 5% sodium bicarbonate buffer. Six ferrets were mock infected with 5mL PBS combined with 5mL 5% sodium bicarbonate buffer. Morphine was administered to each ferret approximately one hour following inoculation.

Sample Collection and *Campylobacter* Enumeration

Ferrets were weighed and measured once per week post-infection. Fecal and serum samples were collected on days 1, 3, 6, 7, 9, 12, and 14 post-infection, followed by samples collection once per week. Three fecal samples were collected from each cage per time point in the morning. Approximately 200mg of feces from one sample from each animal was immediately weighed out and diluted 1:100 in PBS. The remaining samples were immediately frozen at -80°C. Diluted samples were further serially diluted in PBS to 10^{-8} and 100 μ L of each dilution was plated on *Campylobacter*-specific media. These plates were then incubated at 37°C in microaerophilic conditions for 48 hours before *Campylobacter* loads were determined.

Ferrets were individually anesthetized with isoflurane prior to blood collection. Blood was drawn from the vena cava and was limited to 0.2% of the animal's body weight for time points during days 1-14 post-infection, followed by 1.5% of the animal's body weight for blood draws once per week. After each collection, blood was allowed to clot in an upright position for at least 20 minutes. Samples were then centrifuged at 3500 rpm for 15 minutes. Serum was then

pipetted into clean 1.5mL Eppendorf tubes and frozen at -80°C. The remainder of the clotted sample was disposed of.

Serum Assay

Serum samples collected days 1 through 14 were analyzed at Vanderbilt University Medical Center (VUMC) using a Luminex mouse cytokine 1 panel. For this assay, a 96-well plate was washed prior to adding standards and samples. The appropriate matrix was then added to the wells, followed by antibody immobilized beads. The plate was then incubated overnight (16-18 hours) on a shaker at 4°C. After this incubation, plate contents were removed and the plate was washed three times with buffer. Detection antibody was then added to each well and the plate was shaken for one hour at room temperature. Following incubation, Streptavidin-Phycoerythrin was added to each well and the plate was incubated for 30 minutes at room temperature with shaking. The contents of the plate were removed and the plate was washed three times with buffer. After washing, drive fluid was added to each well and the plate was shaken at room temperature for five minutes. Finally, the plate was read on a MAGPIX using EXPONENT software and results were analyzed using Milliplex Analyst software. Using this method, levels of IL-1 β , IL-10, IL-12p40, and TNF α were compared in uninfected and infected ferret samples.

Fecal Assays

Several assays were performed on fecal samples from the first 7 time points (days 1 through 14 p.i.). An occult blood test was performed using a Beckman Coulter Hemoccult II test kit. This procedure involved smearing a thin layer of feces over the specified box, incubating 3-5 minutes, applying two drops of Hemoccult Developer solution, and reading the result within 60 seconds. Results were subjectively scored using “0” as a negative, “0.5” as a weak positive, and “1” as a strong or regular positive, which is shown as presence/absence of blue coloring. Positive and negative controls were included on the test strips for each sample box.

Levels of S100A12, calprotectin, and α 1-protease inhibitor were also analyzed in ferret fecal samples. To analyze S100A12, protease inhibitor tablets were dissolved in PBS (one tablet per 15mL PBS). Approximately 0.5g of each fecal sample was weighed out in a falcon tube and 5mL of the protease inhibitor mixture was added to each sample. Samples were then vortexed vigorously and 50 μ L of each sample was used in a LifeSpan BioSciences, Inc. human S100A12 ELISA kit. This same procedure was performed to measure S100A12 levels in *Campylobacter*-infected and uninfected human fecal samples as well. A similar procedure was used to measure levels of calprotectin in infected and uninfected ferret and human fecal samples, with the modification of weighing out 0.1g feces and adding 1mL protease inhibitor mixture. Additionally, we performed a 1:10 dilution with our samples (in sample diluent supplied in the LifeSpan

BioSciences, Inc. ELISA kit) to obtain results within the range of the kit (the upper detection limit was 2000pg/mL). Absorbance of the samples was determined using a plate reader immediately following the final step of the ELISA. ELISA plates were read at wavelength 450nm, with 620nm as the reference wavelength. To measure α 1-protease inhibitor levels in ferret fecal samples, a canine (Texas A&M) immunoassay and a human (Biovendor) ELISA kit were used.

***C. jejuni* Growth in the Presence of S100 Proteins in vitro**

Dose response assays were performed on *C. jejuni* wild-type strain DRH212 using S100A12 or calprotectin as a growth inhibitor. Concentrations of S100A12 and calprotectin started at 1000 μ g/mL and were serially diluted (1:1 in *Helicobacter pylori* media) to 15.625 μ g/mL to determine the minimal concentration necessary to inhibit *C. jejuni* growth. These assays were carried out in triplicate using *Helicobacter pylori* media (*Brucella* broth with fetal bovine serum and calprotectin buffer) as the growth medium. For each S100A12/calprotectin concentration, 100 μ L of diluted protein was added to 100 μ L of OD₆₀₀ 0.5 *C. jejuni* in a 96-well plate. These plates were read at 600nm following 24-hour and 48-hour incubation in a microaerophilic environment at 37°C.

Restoration of *C. jejuni* Growth Following Treatment with S100 Proteins

Once minimal inhibitory concentrations of S100A12 and calprotectin (500 μ g/mL) were determined, assays were carried out with the wild-type DRH212 *C. jejuni* strain and the minimal inhibitory concentration of S100A12 or calprotectin. Elements known to bind to or be sequestered by S100A12 or calprotectin were added to the wells at a concentration of 100 μ M. For S100A12, 100 μ L of 100 μ M CuCl₂, CaCl₂, and ZnCl₂ were added to a 100 μ L mixture of OD₆₀₀ 0.5 DRH212 with 500 μ L S100A12. Plates were incubated in a microaerophilic environment at 37°C and read at 600nm following 24-hour and 48-hour incubation to determine if any of these elements could restore *C. jejuni* growth *in vitro* following S100A12 treatment. The same procedure was used to examine elements that can restore *C. jejuni* growth *in vitro* following treatment with calprotectin. The only modification was MnCl₂ was used in place of CuCl₂, as per binding specificity of this protein. Knock-out calprotectin was also used in this assay to ensure that the solution itself was not responsible for the inhibition of *C. jejuni* growth.

RNAseq Analysis of S100A12-Treated *C. jejuni*

C. jejuni (strain DRH212) was incubated overnight at 37°C on two separate Mueller-Hinton agar plate containing 10 μ g/mL trimethoprim. Two more DRH212 cultures were incubated in identical conditions, but these had been

previously treated with 500 μ g/mL purified S100A12. Following this incubation, all four samples were suspended in 40mL Mueller-Hinton broth and each was diluted to OD₆₀₀ 1. These samples were then incubated at 37°C for approximately six hours. Samples were then centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1mL Trizol. Samples were frozen at -80°C until RNA extraction.

To RNA extract, the two wild-type DRH212 and the two S100A12-treated DRH212 samples were first thawed on ice until they were completely melted. They were then incubated at room temperature for five minutes. 100 μ L of chloroform was added to each sample and samples were vortexed. Samples were again incubated at room temperature for five minutes, followed by centrifugation at 15,000 rpm for five minutes at 4°C. 700 μ L of 100% isopropanol was added to fresh Eppendorf tubes. The aqueous phase was then removed from each centrifuged sample and added to the respective aliquots of isopropanol. These were vortexed briefly and centrifuged at 15,000 rpm for 10 minutes at 4°C. Supernatant was discarded, while the pellets were resuspended in 150 μ L of 70% isopropanol. Samples were centrifuged at 15,000 rpm for five minutes at 4°C. All supernatant was then removed and discarded and pellet was air dried for approximately 15 minutes. Finally, pellets were resuspended in 50 μ L deionized RNase-free water and frozen at -20°C.

Samples were DNase-treated to ensure that only RNA was present. To DNase-treat, 20 μ L DNase I 10x reaction buffer was added to 30 μ g of each

thawed RNA-extracted DRH212 sample. 8 μ L DNase was added to each mixture and they were each brought to a volume of 200 μ L by adding RNase-free deionized water. Samples were incubated in a 37°C heat block for 30 minutes, 8 μ L additional DNase was added, and the incubation was repeated. Samples were cleaned using a Zymo RNA clean and concentrate kit. Samples were then PCR'd to check for the presence of DNA (indicated by bands). DNA was still present, so 1 μ L DNase and 1 μ L DNase I 10x reaction buffer was added to each sample. Samples were incubated at 37°C for 30 minutes, 1 μ L additional DNase was added to each, and the incubation was repeated. Samples were then RNA cleaned and PCR'd once again to check for presence of DNA. The DNase-treatment, RNA clean, and PCR were repeated once more before samples could be confirmed to be purely RNA.

The four DNA-free samples were treated with an Illumina Ribo-Zero Magnetic Kit to remove rRNA. DNA libraries were then made from each RNA sample using a MiSeq Reagent Kit v3 and following the provided protocol. Samples were checked for good quality using a bioanalyzer. DNA libraries were labelled with individual indexes, run through a MiSeq, and results were analyzed for changes in gene regulation between wild-type DRH212 and S100A12-treated DRH212.

CHAPTER FOUR

RESULTS AND DISCUSSION

Sample Collection and *Campylobacter* Enumeration

Campylobacter-infected ferrets did not significantly differ from uninfected ferrets in length or weight (Figures 1A and 1B). As expected, males were significantly larger and heavier than females, and lengths and weights of these animals increased steadily over the period of the experiment. Following oral challenge with *C. jejuni*, *Campylobacter* loads in infected ferrets peaked at day 3 post infection (p.i.). *C. jejuni* could no longer be detected by day 12 p.i. and there were no clinical signs of infection by this time (Figure 2). This peak and duration of infection is consistent with previous studies using ferrets as a disease model for *Campylobacter* infection (Bell & Manning, 1990; Nemelka et al., 2009). Using our screening methods, the limit-of-detection for *Campylobacter* was at 2.0×10^3 CFU/g of feces. *C. jejuni* could not be detected in uninfected ferret feces at any time point and thus are not shown. Ferrets did not have frank blood present in their stools at any time point. Some infected ferrets displayed mildly watery stool early in infection, but no severe diarrhea was observed throughout the experiment.

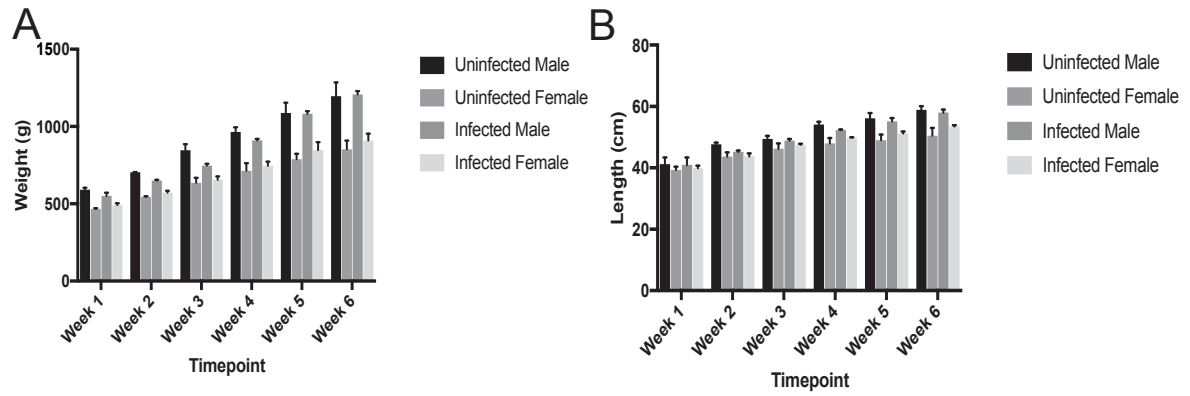


Figure 1. Effect of *C. jejuni* infection on growth and development of ferrets. Comparison of ferret weight (A) and length (B). Data was analyzed with unpaired student's t-tests using the Holm-Sidak method with $\alpha=0.05$ ($n=3$).

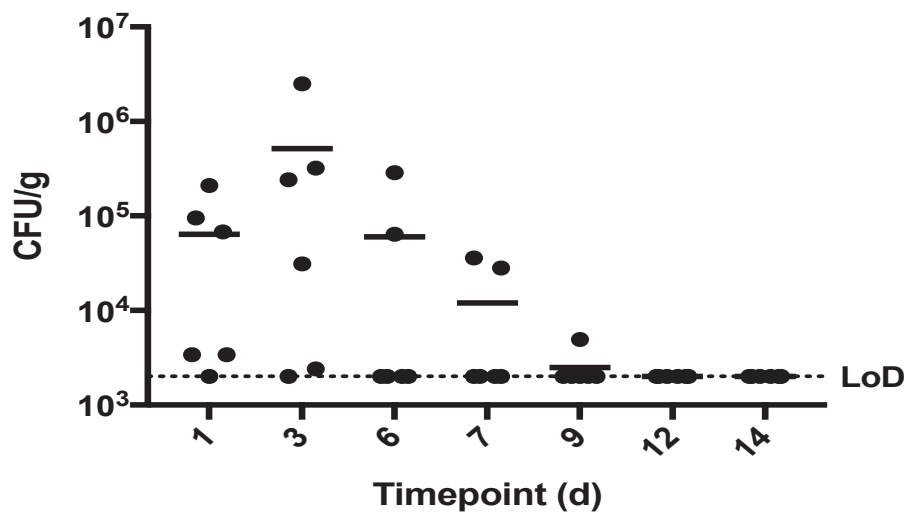


Figure 2. *C. jejuni* loads in feces of infected ferrets. Immediately after collection, fecal samples were serially diluted (1:10) in PBS. Dilutions were then plated on *Campylobacter*-specific media and incubated 48 hours at 37°C. The horizontal bars represent the average *C. jejuni* loads found in feces of infected ferrets.

Serum Assay

We initially analyzed serum samples from days 6 and 7 (the height of infection) for the levels of 7 cytokines (interferon- γ , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, and TNF α) using a Luminex mouse cytokine panel. We chose our samples based off a previous study looking at cytokine production during *C. jejuni* infection in human dendritic cells (Hu et al., 2006). We determined that IL-1 β , IL-10, IL-12p40, and TNF α were the most elevated from baseline, so we analyzed the levels of these four cytokines in serum samples from days 1-14. When we compared all seven sets of sera from the initial time points, we saw a significant increase in levels of IL-10 (an anti-inflammatory cytokine) at days 1, 3, 9, and 12 in infected ferrets. We saw a significant increase in TNF α (a pro-inflammatory cytokine) at days 1, 3, 6, and 7 in infected ferrets (Figure 3). There was no significant difference in levels of IL-1 β or IL-12p40 in infected versus uninfected ferrets at any time point.

Infected ferrets showed a significant increase in the levels of both IL-10 and TNF α present in serum. A large increase in TNF α production during early *Campylobacter* infection was also seen in a previous study (Hu et al., 2006). In this same study, IL-10 was also shown to increase significantly, but only at the latest time point (48 hour). Initially, we observed that significantly higher levels of TNF α were produced in *C. jejuni* infected ferrets (days 1, 3, 6, and 7). This signifies an increase in intestinal inflammation early in infection, since TNF α is a pro-inflammatory cytokine secreted by macrophages to induce an inflammatory

state. In addition to its upregulation during enteric pathogen infection (such as *Campylobacter* and *Salmonella*), $\text{TNF}\alpha$ is seen to be elevated in chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis (van der Veek et al., 2005). IL-10, an anti-inflammatory cytokine, has a relatively unknown mechanism of action, but has been seen to successfully inhibit inflammatory responses. Additionally, IL-10 is thought to play a role in the prevention of inflammatory bowel disease, as both humans and mice with deficient IL-10 display severe intestinal inflammation (Ip et al., 2017). IL-10 levels were significantly elevated in infected ferrets towards the very beginning and at the end of infection (days 1, 3, 9, and 12). This indicates that IL-10 is initially elevated to keep the $\text{TNF}\alpha$ levels regulated and inflammation in control. IL-10 is also elevated late in infection in order to reduce the immune response back to baseline once most of the infection has cleared.

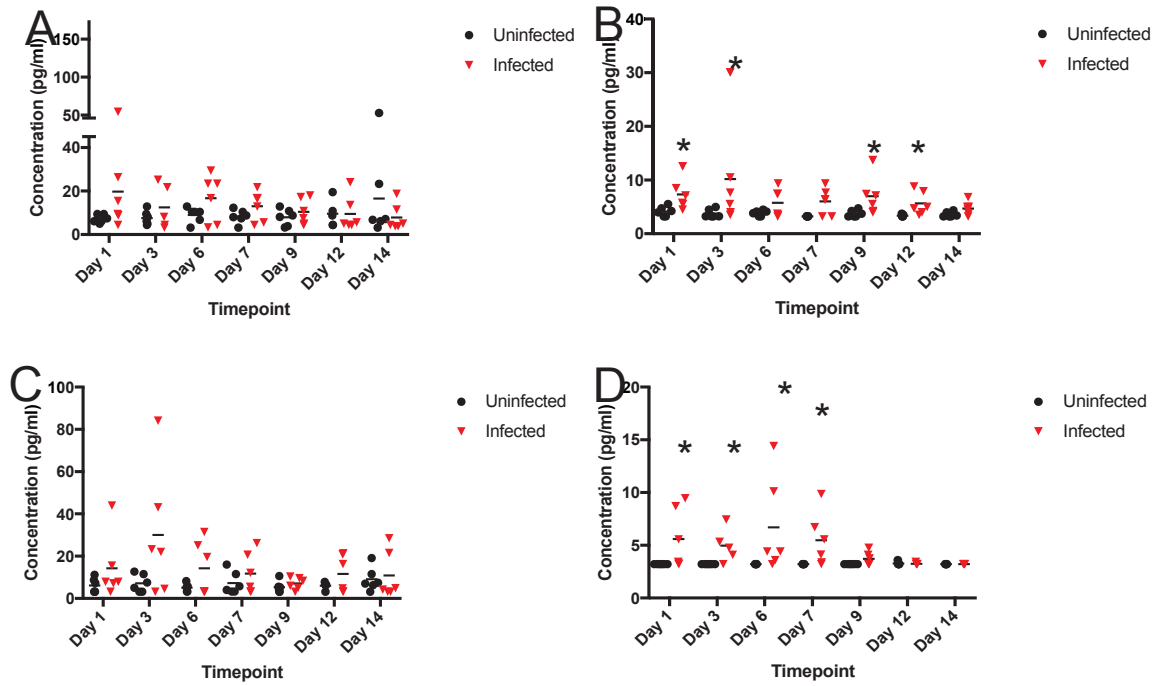


Figure 3. Cytokine levels in ferret serum. (A) IL-1 β ; (B) IL-10; (C) IL-12p40; (D) TNF α . Cytokine levels were measured at Vanderbilt University Medical Center using the Luminex platform and mouse cytokine panel 1. *p<0.05, Mann-Whitney U test, n=6.

Fecal Assays

Occult blood is blood in stool that is not visible to the naked eye. Occult blood tests were performed on all ferret fecal samples from days 1-14 using a Beckman Coulter Hemoccult II test kit. Tests were scored based on the presence of blue coloring, with “0” meaning negative (no blue present), “0.5” meaning a weak positive (some blue, usually more towards the edge of the sample), and “1” being a normal or strong positive (deep blue coloring, typically present throughout the entire sample spot). Although there was no significant difference in occult blood scores at any time point for infected versus uninfected ferrets, infected samples from days 1, 12, and 14 appeared to have a higher incidence of occult blood (Figure 4). We suspect that uninfected ferrets may have had occult blood in their fecal samples due to stressors and lack of dietary restriction prior to testing, which have been seen to play a role in the motility and amount of secretion of the ferret gastrointestinal tract (Johnson-Delaney, 2006).

Neither a canine immunoassay nor a human ELISA for α 1-protease inhibitor was able to find any detectable levels of this protein in any of the ferret fecal samples at any time point (data not shown). Amount of α 1-protease inhibitor in human feces was not determined. Our inability to detect an elevated amount of α 1-protease inhibitor in any of the ferret samples using human or canine antigens may be a binding specification error. However, we consider this to indicate that α 1-protease inhibitor levels during *C. jejuni* infection in humans cannot be accurately modeled in ferrets.

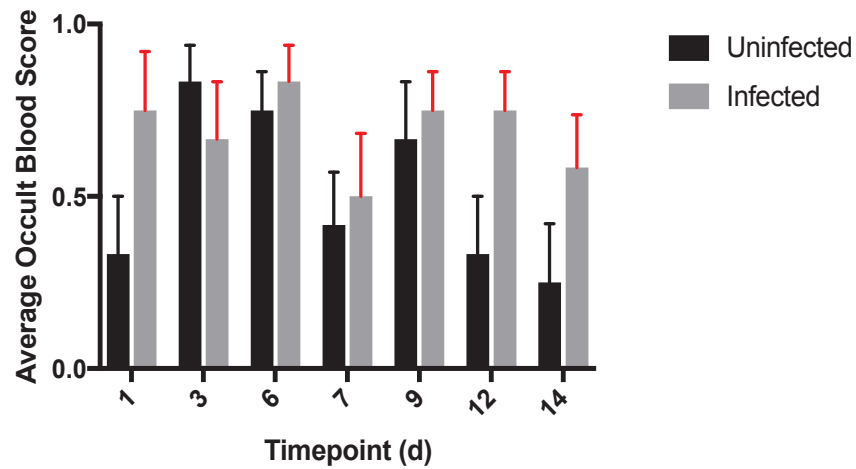


Figure 4. Occult blood found in ferret feces via Hemoccult II test. A score of 1 was positive, 0.5 was a weak positive, and 0 was a negative. There were six ferrets per group per time point. Data was compared using a Mann-Whitney U test with $\alpha=0.05$ ($n=6$).

To evaluate levels of calprotectin in ferret and human fecal samples, a LifeSpan BioSciences, Inc. ELISA kits for calprotectin was used. Using a Mann-Whitney U test ($p < 0.05$), neither *C. jejuni* infected ferret fecal samples at any time point nor *C. jejuni* infected human fecal samples were found to have significantly higher levels of calprotectin when compared to the uninfected samples (Figures 5A and 5B). Almost all ferret fecal samples had undetectable levels of calprotectin, while nearly all human fecal samples appeared to have normal levels of calprotectin ($< 50 \mu\text{g/g}$). This was likely due to a lack of binding specificity between the ferret antigens in the fecal samples and the human antibodies in the ELISA kit. The lack of elevation of calprotectin in infected human samples could be due to the use of an ELISA kit with improper detection levels. However, calprotectin is known to be an inaccurate biomarker when low levels of inflammation are present, and may also be drastically effected by the use of NSAIDS (Siddiqui, Majid, & Abid, 2017). Using our methods, we cannot accurately model calprotectin levels of *C. jejuni* infected humans in ferrets.

S100A12 levels in ferret and human fecal samples were evaluated using a LifeSpan BioSciences, Inc. ELISA kit for S100A12. At day 7 post-infection, infected ferret fecal samples were shown to have a 2-fold increase of S100A12 present compared to uninfected ferret fecal samples (Figure 6A). This result was found to be significant using an unpaired two-tailed Student's t-test with Welch's correction ($p < 0.05$). No infected ferret fecal samples from any other time points had significantly elevated S100A12 levels compared to uninfected fecal samples.

This makes sense with the *Campylobacter* loads we saw, as this time point would be just after peak infection. Using these same methods, human fecal samples were also found to have a statistically significant ($p < 0.05$), approximately 2-fold, increase in S100A12 levels when infected with *Campylobacter* versus those uninfected (Figure 6B). Since S100A12 levels in ferrets reflect the 2-fold increase in infected compared to uninfected samples that is shown in humans, we can conclude that ferrets can be used to model the effects of S100A12 during *C. jejuni* infection in humans. This is useful as S100A12 is as accurate as calprotectin in identifying intestinal inflammation, but with greater specificity, and therefore less false-positives (Sidler, Leach, & Day, 2008).

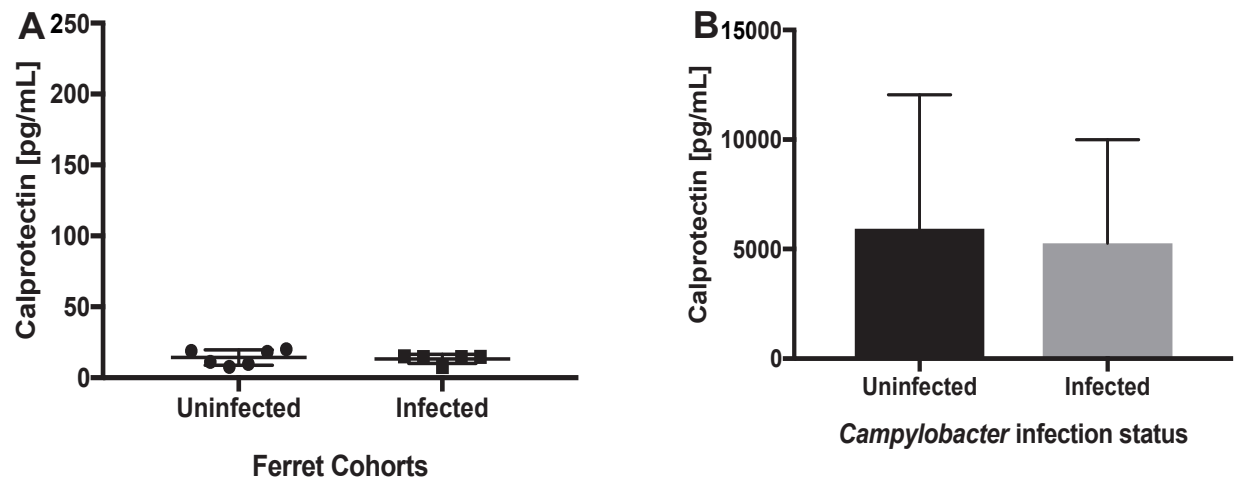


Figure 5. Calprotectin concentrations in fecal samples. (A) Ferrets at day 7 post-infection (n=6). (B) Human feces submitted to the University of Nebraska Clinical Microbiology Laboratory (UTK IRB-17-03795-XM) (n=7). Data was compared using a Mann-Whitney U test with $\alpha=0.05$.

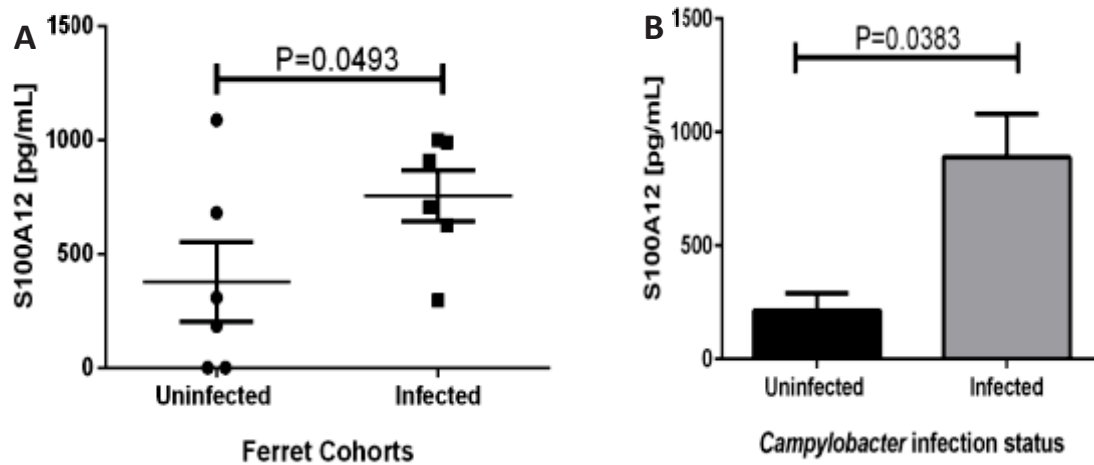


Figure 6. S100A12 concentrations in fecal samples. (A) Ferrets at day 7 post-infection (n=6). (B) Human feces submitted to the University of Nebraska Clinical Microbiology Laboratory (UTK IRB-17-03795-XM). Data was compared using an unpaired two-tailed Student's t-test with Welch's correction with $\alpha=0.05$.

C. jejuni* Growth in the Presence of S100 Proteins *in vitro

Both calprotectin and calgranulin c (S100A12) were able to significantly inhibit *C. jejuni* growth *in vitro* (Figure 7). A knock-out calprotectin mutant created by Dr. Steve Damo at Fisk University was used to ensure that *C. jejuni* growth was not inhibited by factors other than metal sequestration. At the highest concentration (1000µg/mL), the knock-out mutant was able to significantly reduce growth, suggesting that the suspension media may have played a role in inhibition. However, the knock-out mutant did not inhibit growth at any other concentration, while both calprotectin and calgranulin c were able to inhibit growth at 500µg/mL. Additionally, calprotectin could significantly inhibit *C. jejuni* growth at concentrations as low as 250µg/mL.

Calprotectin and calgranulin c (S100A12) inhibited *C. jejuni* growth *in vitro* at concentrations as low as 250µg/mL and 500µg/mL, respectively. The resuspension media that these proteins were stored in (PBS) may have slightly inhibited growth, as indicated by *C. jejuni* growth inhibition by a knock-out calprotectin mutant at 1000µg/mL. However, at concentrations lower than 1000µg/mL, no inhibition was seen in *C. jejuni* that was treated with the knock-out mutant. This confirms that we are indeed seeing growth inhibition caused by the S100 protein treatments. Calprotectin and calgranulin c are known to have antimicrobial properties, possibly due to their ability to sequester necessary elements such as manganese, copper, and zinc (Kehl-Fie & Skaar, 2010). This

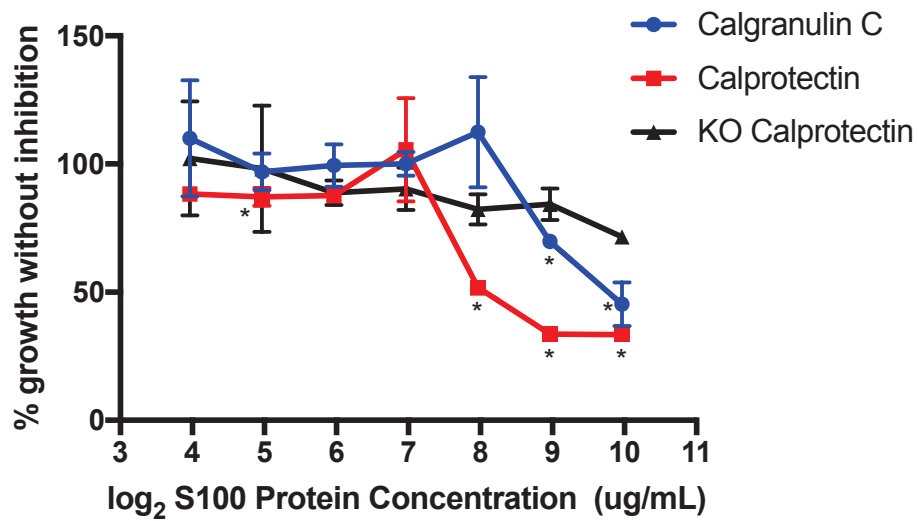


Figure 7. S100 protein inhibition of *C. jejuni* growth *in vitro*. Dose response analysis using purified S100A12 (calgranulin C), calprotectin, or a knock-out calprotectin mutant. *C. jejuni* cultures were treated with one of the three compounds and growth was compared to that of untreated *C. jejuni* cultures (* $p < 0.01$, Mann-Whitney U test, $n = 3$).

implies that the *C. jejuni* growth inhibition we have seen is due to the necessity of these elements in *Campylobacter* survival.

Restoration of *C. jejuni* Growth Following Treatment with S100 Proteins

C. jejuni isolates were treated with 500 μ g/mL of either calprotectin or calgranulin c. *C. jejuni* was then supplemented with 100 μ M calcium, zinc, and either manganese (calprotectin-treated) or copper (calgranulin c-treated) to see if these compounds could restore growth (Figures 8A and 8B). Calprotectin-treated *C. jejuni* grew significantly less than *C. jejuni* treated with a control (PBS). Calcium failed to restore growth in *C. jejuni*. Manganese was able to restore growth enough to not be significantly different from the control, while *C. jejuni* treated with zinc grew significantly better than the *C. jejuni* treated with the control. Likewise, calgranulin c-treated *C. jejuni* grew significantly less than *C. jejuni* treated with PBS. Neither copper nor calcium was able to significantly restore growth. Cultures treated with zinc again grew significantly better than those treated with the control. These results hint at the mechanism of action for these S100 proteins and indicate that either calcium and copper are not necessary for *C. jejuni* growth, or that S100 proteins are not as successful at sequestering these elements as previously thought.

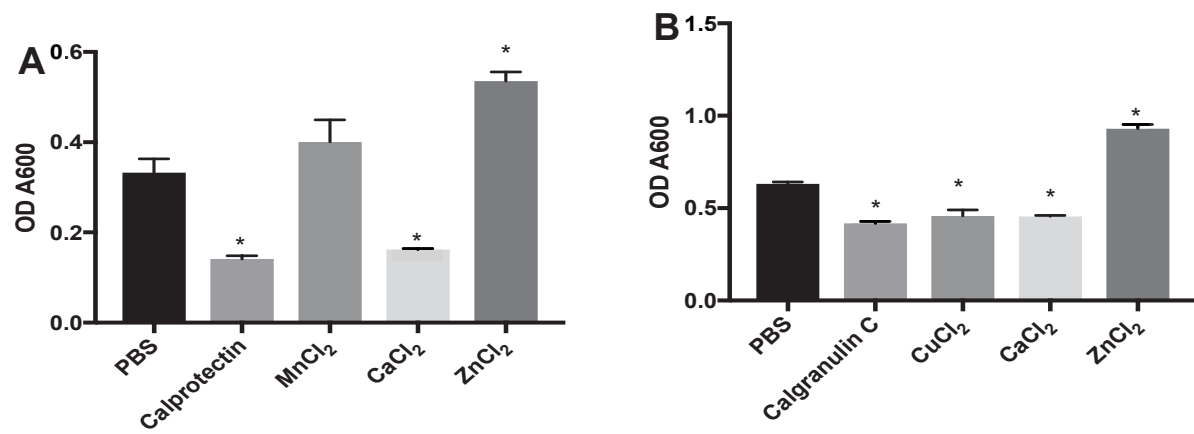


Figure 8. Supplementation of media for *C. jejuni* growth restoration following S100 protein treatment. (A) Supplementation of calprotectin-treated media (500 μ g/mL) with 100 μ M MnCl₂, CaCl₂, and ZnCl₂. (B) Supplementation of S100A12-treated media (500 μ g/mL) with 100 μ M CuCl₂, CaCl₂, and ZnCl₂. (*indicates growth significantly different from that of *C. jejuni* growth in PBS; $p < 0.01$, unpaired two-tailed Student's t-test with Welch's correction, $n=3$).

RNAseq Analysis of S100A12-Treated *C. jejuni*

Using RNAseq, S100A12-treated and untreated *C. jejuni* (strain DRH212) cultures were analyzed for differential gene expression. Surprisingly, we did not see any upregulation in zinc transportation systems when comparing S100A12-treated cultures to untreated cultures. We expected the S100A12 treatment to cause an increase in these systems from our hypothesis that S100A12 blocks zinc uptake, which was formulated from our *in vitro* analysis of S100A12-treated *C. jejuni* cultures. We saw 15 different upregulated systems in S100A12-treated cultures, with energy production and conversion most frequently upregulated (Figure 9A). Four different systems were downregulated in the S100A12-treated cultures, with translation and ribosomal structure and biogenesis accounting for over half of all downregulated genes (Figure 9B). However, this system also appears to be upregulated in treated cultures, which suggests multiple genes are responsible for these functions in *Campylobacter*, and not all of these gene pathways are inhibited by S100A12. Likewise, cell wall/membrane/envelope biogenesis was seen to be both upregulated and downregulated. This unexpected outcome may be due to the short treatment time of DRH212 with S100A12 (six hours). A longer treatment time as well as a higher S100A12 dosage (we used the minimal inhibitory concentration of 500 μ g/mL) may help us to more accurately predict changes in gene regulation of *C. jejuni* that has been treated with S100A12. Alternatively, zinc may not be necessary throughout different *Campylobacter* growth phases. To test for this, the S100A12 treatment

should be repeated and gene transcription should be monitored at different time points across multiple different growth phases. RNA seq may not be a viable method to determine this, but quantitative real-time PCR could be a favorable alternative. Zinc transporter upregulation may be happening at a translational, not transcriptional, level instead. If this is the case, it would be useful to observe Western blots and reverse transcription PCR of S100A12-treated and untreated *C. jejuni* cultures.

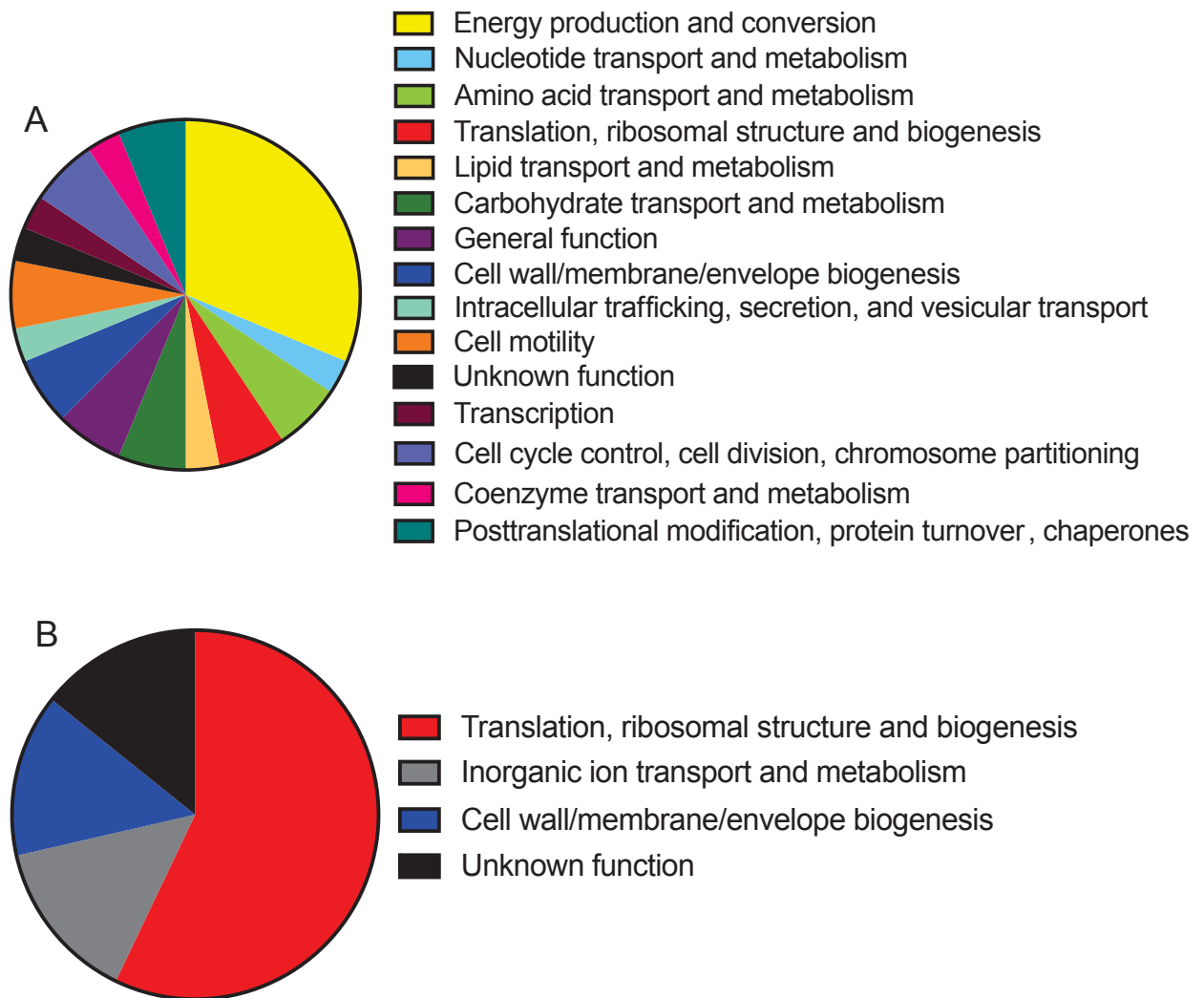


Figure 9. Differences in gene regulation of S100A12-treated *C. jejuni* and untreated *C. jejuni*, analyzed using RNA seq. Levels of gene transcription were compared to levels in untreated *C. jejuni* cultures. (A) Upregulated genes (n=32). (B) Downregulated genes (n=7).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

Campylobacter is a leading cause of bacterial-derived gastroenteritis worldwide, costing approximately \$1.7 billion annually in the United States alone (Johnson, Shank, & Johnson, 2017). In the developed world, campylobacteriosis is most commonly acquired through the consumption of contaminated poultry meat (Johnson et al., 2015). Little is known about how *Campylobacter* is able to successfully invade and colonize its hosts, though it typically resides in cecal mucosal crypts (Coward et al., 2008). Campylobacteriosis is a serious concern, as it can lead to several severe post-infectious complications such as Guillain-Barré syndrome, septicemia, reactive arthritis, and inflammatory bowel diseases (Goldstein et al., 2016; Kaakoush et al., 2014). Additionally, antibiotic-resistant *Campylobacter* prevalence has been steadily increasing around the world (Engberg et al., 2001; Moore et al., 2006; Alfredson & Korolik, 2007), which is likely due to the overuse of antibiotics (Hessulf et al., 2016).

A possible non-antibiotic *Campylobacter* treatment involves the use of S100 proteins, particularly calprotectin (S100A8/S100A9) and calgranulin C (S100A12). These proteins are used as biomarkers to detect the presence of inflammation, as they are commonly secreted from neutrophils (Leach et al., 2007). Additionally, these proteins are known to have antimicrobial properties (Kehl-Fie & Skaar, 2010). This may be due to their ability to bind calcium and to sequester metals such as zinc, manganese, and copper (Zackular, Chazin, & Skaar, 2015).

We decided to observe levels of S100 proteins both *in vitro* and *in vivo* to determine their possible effects on *Campylobacter jejuni*. For our *in vivo* studies we used ferrets as a model for human infection due to their being one of the only animals to produce similar clinical signs to humans following infection with *Campylobacter* (Bell & Manning, 1990). We did not observe any noticeable differences in the sizes of infected versus uninfected ferrets. We observed peak *Campylobacter* loads at three days post-infection. We saw a significant increase in the levels of IL-10 at the beginning and end of infection, and in $\text{TNF}\alpha$ at the initial time points following infection. This indicates a strong pro-inflammatory immune response caused by $\text{TNF}\alpha$, while IL-10, an anti-inflammatory cytokine, was likely regulating the immune reaction. There was no significant increase in occult blood between infected versus uninfected ferrets, but this could be due to the sensitivity of the ferret digestive tract (Watson et al., 2016). Finally, we did not observe a significant increase in calprotectin, but we did see significantly elevated levels of calgranulin C in infected ferrets at peak infection. This indicates that ferrets are a viable animal model for human campylobacteriosis due to their reflection of calgranulin C levels in humans during infection.

Our *in vitro* observations of the effects of calprotectin and calgranulin C on *Campylobacter jejuni* show that each of these proteins can effectively inhibit growth. Calgranulin C inhibits growth at concentrations as low as $500\mu\text{g/mL}$, while calprotectin can inhibit growth with as little as $250\mu\text{g/mL}$. Following this experiment, we supplemented back metals known to be sequestered by these

proteins to help determine a possible mechanism of action for *Campylobacter* inhibition. Following treatment with 500µg/mL of calgranulin C, we saw that 100µM of zinc was able to significantly restore *C. jejuni* growth, while 100µM of calcium or copper could not. Similarly, when *C. jejuni* was treated with 500µg/mL calprotectin, 100µM of zinc was able to significantly restore growth. Additionally, 100µM of manganese was able to restore calprotectin-treated *C. jejuni* growth, while 100µM of calcium was not. MiSeq was used to determine which genes were being upregulated and downregulated in response to calgranulin C treatment, but we did not get the results we expected, which would have shown us upregulation of a zinc transporter or related gene. Instead, we observed that the most commonly upregulated genes in calgranulin C-treated *C. jejuni* were genes involved in energy production and conversion. The most commonly downregulated genes were those involved in translation and ribosomal structure and biogenesis. A longer calgranulin C treatment time (we only incubated for 6 hours) or a higher calgranulin C dosage (we used the minimal inhibitory concentration of 500µg/mL) may have produced clearer results. Alternatively, it would be useful to look into *Campylobacter* zinc regulation across multiple growth phases and at translational and post-translation zinc regulation.

In the future, it would be interesting to observe the effects of *Campylobacter* on a malnourished host and to compare the effects to those of a healthy host. This would be important to look at as source of infection, age groups commonly infected, and post-infectious complications from

Campylobacter differs greatly between first and third world countries.

Campylobacter infection in third world countries is common in children five years old and younger and is typically acquired from contaminated drinking water (Johnson, Shank, & Johnson, 2017). Persistence and stunting following *Campylobacter* infection are more commonly seen in these areas of the world as well (Amour et al., 2016). This implies that diet plays a key role in infection and immune response to campylobacteriosis. We suspect that stunting may be caused by a lack of zinc in the host, as some S100 proteins are seen to be upregulated during infection and to bind zinc (Donato et al., 2013), and *Campylobacter* is also known to scavenge zinc from its host environment (Davis, Kakuda, & DiRita, 2009). Zinc deficiencies have been shown to lead to imbalances in the immune system, particularly in children with diarrhea (Giella & DiRita, 2012).

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VITA

Janette M. Shank was born in York, Maine on September 1, 1994 to Gregory and Victoria Shank. Her family moved to Groton, Connecticut soon after she was born. She lived in Groton for nine years before moving to Ledyard, Connecticut, where she went to Ledyard Middle School. She then moved to Patton, Pennsylvania, where she attended Cambria Heights High School and participated in the Upward Bound program in Loretto, Pennsylvania. She entered college at Indiana University South Bend (IUSB) in the fall of 2012. There she took courses in molecular biology, microbiology, genetics, and chemistry. After graduating from IUSB, she moved to Knoxville, Tennessee to attend the University of Tennessee. She was accepted by the Department of Microbiology in 2016 and started her graduate studies in the Johnson lab.

Janette worked as a graduate teaching assistant from the fall of 2016 to the summer of 2017 for Biology 159. She then became a graduate research assistant for the fall 2017 semester. During this time, she studied the effects of *Campylobacter jejuni* infection *in vivo*, using ferrets as a model for human infection.