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EVALUATION OF PROINFLAMMATORY CYTOKINES IN PIGS INFECTED WITH *CAMPYLOBACTER JEJUNI* AND *TRICHURIS SUIS*

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EVALUATION OF PROINFLAMMATORY CYTOKINES IN PIGS INFECTED WITH *CAMPYLOBACTER JEJUNI* AND *TRICHURIS SUIS*

By

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ABSTRACT

EVALUATION OF PROINFLAMMATORY CYTOKINES IN PIGS INFECTED WITH CAMPYLOBACTER JEJUNI AND TRICHURIS SUIS

By

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Campylobacter jejuni is a leading cause of gastroenteritis worldwide. In conventionally reared swine, *C. jejuni* is commonly found in the colon without pathology, but infection and pathology result when the swine whipworm, *T. suis*, is present. The central hypothesis of this dissertation was that *C. jejuni* induces natural host resistance via proinflammatory cytokine responses following primary oral infection, but this response is down-regulated in the presence of *T. suis* thus promoting *C. jejuni* infection. Specific aims: 1) Evaluate secreted pro- and anti-inflammatory cytokines (IL-8, IL-1-β, TNF-α, IL-4, IL-6, and IL-10) in the feces of pigs after single or dual challenge infections with *C. jejuni* and *T. suis*, 2) Assess IL-8, IL-1-β and TNF-α production from undifferentiated, crypt-like intestinal pig epithelial cells (IPEC-1 cells) following *C. jejuni* infection, and 3) Assess whether IL-8, TNF-α, or IL-1-β treatment of IPEC-1 cells have effects on *C. jejuni* invasion, adherence, and/or transepithelial electrical resistance (TER) across this mucosal membrane. In aim 1, weaned piglets were used in short-term (ST, 2 days) and long-term (LT, 23 days) challenge experiments. In the LT experiments, there were 5 groups with two groups given 2500 *T. suis* orally. At 21 days post-*T. suis* inoculation, some of the *T. suis*-infected pigs received *C. jejuni*, while the remaining pigs received either *C. jejuni* alone, *E. coli* DH5-α, or sterile milk. On day 23, all pigs were euthanized and gastrointestinal (GI) tissue samples taken to measure cytokine expression by real time PCR. *Campylobacter jejuni* stimulated increased proinflammatory cytokine

expression in the jejunum while *T. suis* down-regulated these responses here and in the proximal colon. *Trichuris suis* contributed significantly to diarrheal disease in both single and concurrent infections. Also, enzyme linked immunosorbent assay (ELISA) conducted on supernatants from feces indicated that *T. suis* significantly decreased expression of fecal IL-1-β during concurrent *C. jejuni* and *T. suis* infection. In the ST experiment where pigs were inoculated simultaneously with either *C. jejuni*, *T. suis*, *C. jejuni* and *T. suis*, *E. coli* DH5-α, or sterile milk none showed significant acute changes in fecal IL-8, IL-1-β, TNF-α, IL-4, IL-6, or IL-10 expression by ELISA. Here, cytokine responses were measurable using fecal ELISAs, but cytokine mRNA expression assays in tissues were more informative. Also, we discovered that secondary invasion of certain epsilon proteobacteria into GI tissues may affect cytokine responses. In aim 2, confluent undifferentiated IPEC-1 cells were infected with *C. jejuni* and culture supernatants analyzed for IL-8, TNF-α, or IL-1-β expression using ELISA. *Campylobacter jejuni* induced IPEC-1 cells to secrete TNF-α and IL-1-β, but IL-8 was constitutively secreted and not inducible by *C. jejuni* infection. These data show a pattern of cytokine responses indicative of an innate inflammatory response that we hypothesize to be responsible in part for eliminating these bacteria from the host. In aim 3, the TER of confluent IPEC cells was measured prior to and following treatment with recombinant swine IL-8, TNF-α, or IL-1-β. None of these cytokines had direct effects on *C. jejuni* interactions with undifferentiated, crypt-like intestinal epithelial cells. Collectively, these data provide evidence for *T. suis* contributing to cytokine dysregulation during concurrent infection. These immunomodulatory effects may promote *C. jejuni* infection of the intestinal mucosal epithelium by limiting the natural host resistance to bacterial infection.

DEDICATION

This dissertation is dedicated to my Lord and Savior, Jesus Christ;
my husband, Steven; my daughters, Jadyn and Kailyn and my mother, Carolyn Price.

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I. Campylobacter jejuni

Basic microbiology

Campylobacter jejuni (*C. jejuni*) is a gram negative bacillus that has a curved or spiral morphology. Its spiral shape and its polar flagellum at one or both ends of the cell mediates a high degree of darting corkscrew motility (81). It is 6.0 μm long and 0.2-0.5 μm wide (81). The generation time of *C. jejuni* is approximately 90 minutes (104). *C. jejuni* is nonsaccharolytic and requires enriched media and microaerophilic conditions (3-15% O_2, 3-5% CO_2) for adequate growth (81). Thermophilic conditions at 42°C facilitate the best growth of these organisms; however, they can also grow at 37°C. These organisms are sensitive to low pH ($\leq$ 5), organic acids, NaCl concentrations exceeding 2 %, and extended periods at temperatures between 10 and 30°C (104). In unfavorable growth conditions, *C. jejuni* will convert to a viable, coccoid, nonculturable state. It has been suggested that *C. jejuni* uses this 'dormant'-like state as an adaptation mechanism to survive in adverse environments(81). The estimated size of the genome is 1730 kb, based on the first two sequenced strains, and its DNA has a high AT content (81, 117).

Epidemiology

C. jejuni is one of the leading causes of acute gastroenteritis worldwide, exceeding diseases caused by *Salmonella* spp., *Shigella* spp., or *Escherichia coli* O157:H7 (6). It is estimated that there are 2.1 to 2.4 million cases of human campylobacteriosis occurring each year in the United States, and >99% of these reported

infections with *Campylobacter* are caused by *C. jejuni* (6, 7). In most cases, human campylobacteriosis is a sporadic illness (7). Fecal oral transmission in food animals can lead to contamination of meat. Documented sources for *C. jejuni* are improperly cooked poultry, beef, pork, or turkey. Contaminated water, contact with pets, and international travel are also sources of sporadic infection (7, 27, 81). Although the majority of cases are sporadic, there have been reports of outbreaks associated with the consumption of unpasteurized milk and contaminated water (1, 6, 27, 81, 131).

C. jejuni is considered to be a commensal in cattle, sheep, swine, fowl, dogs, cats, and rodents (7). It is now confirmed as a human enteric pathogen. The coupling factor relating *C. jejuni* to human disease is the transmission of *C. jejuni* to humans through the consumption of foods of animal origin. Most cases of human campylobacteriosis are associated with foodborne or waterborne transmission (20).

In developed/industrialized countries, *Campylobacter* gastroenteritis is most typically characterized clinically by diarrhea, fever, and abdominal cramps. The most common source of *Campylobacter* infections is the consumption and handling of chicken. Studies conducted in the United States, Europe, and Australia indicated that 50-70% of all *Campylobacter* infections have been attributed to the consumption of chicken (6). A study conducted in the retail market reported a 98% isolation rate of *C. jejuni* from chicken meat (7). Children (< 1 year of age) and young adults (age 15-44 years), particularly males, are most commonly infected. The reported seasonal occurrence of *C. jejuni* infection in the United States begins in May and peaks in August (6).

In developing countries studied, *Campylobacter* infections are hyperendemic. *Campylobacter* infections occur commonly in both children and adults resulting in

asymptomatic infection. Infection with disease is more prevalent among young children (<5 years of age) (6, 112). In developing countries the isolation rates of *Campylobacter* from patients range from 5 to 20%; these estimates of incidence are from laboratories that survey pathogens responsible for diarrhea. According to the World Health Organization (WHO), the estimated incidence rate of campylobacteriosis is 40,000 to 60,000/100,000 in children <5 years old (33). In developing countries, environmental contamination and contaminated foods are considered to be major sources of human infection (33). In many of these settings it is common to have animals living in close proximity to people, and this environment likely plays a significant role in the occurrence of campylobacteriosis.

In 2002, Coker et al. reported that the incidence of campylobacteriosis was increasing worldwide, and the WHO turned its attentions to this concern. The increased involvement of the WHO will stimulate public health awareness through strengthened diagnostic facilities for campylobacteriosis and will promote the establishment of national surveillance programs in countries where they do not exist (33). These efforts are expected to play a significant role in understanding the global epidemiology of human campylobacteriosis.

Disease

Symptoms of gastroenteritis associated with *Campylobacter* infection include diarrhea, fever, and abdominal cramps. These symptoms commence within 2-4 days following ingestion of *C. jejuni* (81). In most cases, illness is self-limiting, and the duration of the illness is usually 1 week or less. *C. jejuni*-induced diarrhea occurs following ingestion of as few as 800 organisms (25). In human clinical infection studies,

fresh blood, mucus, frank pus, and polymorphonuclear leukocytes were found in diarrheic stool samples (25). However, symptoms can be severe, and death can ensue. According to an estimated case/fatality ratio for all *C. jejuni* infections, there is one death per one thousand cases. Usually, *C. jejuni*-related deaths are associated with immunocompromised individuals (6, 7).

Clinical manifestations of disease due to *C. jejuni* vary between developed and developing countries. In developing countries, the clinical manifestations of *Campylobacter* enteritis are less severe than in developed countries. Watery, nonbloody, noninflammatory diarrhea is a common clinical characteristic of disease in developing countries. In many cases the patients are underweight and malnourished (33). In developed countries, such as the United States, disease is characterized by severe inflammatory diarrhea with bloody stool, fever, and abdominal pain (33).

The optimal drug for treatment of *Campylobacter* infection is erythromycin. *Campylobacter* continues to have quite a low rate of resistance to erythromycin despite its use over many years. Erythromycin is safe, low in cost, easy to administer, and has a narrow spectrum of activity (6). Macrolides such as azithromycin and clarithromycin, are also effective against *C. jejuni* infections, but they are more expensive than erythromycin and provide no clinical advantage (6).

Other disease manifestations produced by *C. jejuni* infections include bacteremia, meningitis, proctitis, septic arthritis, septic abortion, carditis, pancreatitis, urinary tract infection, and autoimmune disease(56, 124). In addition to the immediate effects caused by acute *Campylobacter* infection, there are long-term sequelae associated with campylobacteriosis. Studies have shown that reactive arthritis (ReA), Reiter's syndrome,

Guillain-Barré, and Miller-Fisher syndrome may accompany or follow *C. jejuni* enteritis (124). These post-infection sequelae result in serious health effects and have a significant economic impact (105).

Reactive arthritis (ReA) is a non-purulent joint inflammation which is commonly induced by enteric infections or urogenital tract infections (56). ReA is triggered by agents such as *Salmonella*, *Yersinia enterocolitica*, *Shigella flexneri*, *Chlamydia trachomatis* and *Campylobacter jejuni* (39). Locht et al. observed that ReA patients that had *C. jejuni* enteritis had more severe gastrointestinal symptoms and prolonged diarrhea than patients who had enterocolitis only (94). Reactive arthritis is usually self-limiting, but a 3-month course of Ciprofloxacin therapy decreases the symptoms for more severe cases (104). The annual incidence of ReA following *Campylobacter* infection is 4.3 per 100,000 cases (56).

Reiter's syndrome is another reactive arthropathy associated with *C. jejuni* infection. This syndrome is thought to be an autoimmune response stimulated by infection. It usually occurs 7-10 days after onset of diarrhea, and multiple joints can be affected, particularly the knee joint. This syndrome triggers pain and possible incapacitation which can last for months or become chronic (7). Reiter's syndrome is reported to occur in approximately 1% of patients with campylobacteriosis (7). The pathogenesis of Reiter's syndrome is not completely understood.

Guillain-Barré Syndrome (GBS) is another serious sequela of *Campylobacter* infection. It was the first of the postinfectious autoimmune diseases identified as occurring subsequent to *C. jejuni* colitis and is characterized by acute limb weakness and loss of tendon reflexes (acute flaccid paralysis) (33, 148). *C. jejuni* serotype O:19 are the

most prevalent strains associated with GBS worldwide (6, 33, 148). In the United States, the mean annual incidence rate of GBS is 1.3 cases per 100,000 population (148). Although *C. jejuni* infection can trigger GBS, the risk of developing GBS after *C. jejuni* infection is quite small (approximately 1 case of GBS out of 1000 *C. jejuni* infections) (6, 7, 148). Approximately 20% of the patients with GBS are left with some long term disability (7). GBS patients that suffer extensive axonal injury have a greater likelihood of need for mechanical ventilation and an increased risk of irreversible neurological damage (6). Approximately 5% of GBS patients die despite advances in supportive respiratory care (7). It is now recognized that the risk of development of GBS does not increase with the severity of *C. jejuni* infection. Additionally, many GBS-associated *C. jejuni* infections are asymptomatic. Neurological symptoms of GBS typically occur 1-3 weeks after the onset of diarrheal illness, and humoral immunopathogenic mechanisms are believed to be involved (6). Similar to Fisher Syndrome (FS), it is believed that molecular mimicry is a possible cause of GBS. Studies have identified the molecular mimicry between GBS-associated *C. jejuni* lipo-oligosaccharides and peripheral nerve glycolipids or myelin proteins, particularly the GM1 ganglioside, as a mechanism for anti-ganglioside antibody induction (6, 72, 159). It is strongly suggested that this association plays an important role in the pathogenesis of *Campylobacter*-induced GBS (6, 144). Li et al. demonstrated that *C. jejuni*-infected chickens spontaneously acquired paralytic neuropathy associated with GBS (93).

Likewise, Fisher syndrome (FS), also known as Miller Fisher syndrome, is an anatomically localized variant of Guillain-Barré syndrome (GBS) that is characterized by acute onset of ophthalmoplegia (paralysis of the eye muscles), ataxia, and areflexia (lack

of normal reflexes). It is considered a variant of GBS because some patients who present with FS progress to GBS (148). The estimated annual incidence rate of FS is 0.09 per 100,000 population. *C. jejuni* is the most frequently identified antecedent pathogen to FS/GBS. It is believed that molecular mimicry is also a possible cause of both FS and GBS. More specifically, studies have shown that anti-GQ1b IgG, an antiganglioside antibody, is associated with FS (82, 114, 166). This is thought to occur because GQ1b is enriched in the cranial nerves that innervate the extraocular muscles (166). Fisher syndrome is typically a benign, self-limiting illness in comparison to GBS, and can be treated successfully with plasmapheresis and intravenous immunoglobulin (114).

Model Systems

1). Animal models

There have been many attempts at establishing a suitable animal model system that would mimic human campylobacteriosis (4, 10, 11, 19, 44, 100, 116, 129, 154). However, the inability to fully reproduce clinical symptoms of *Campylobacter* enteritis has proven to be a hindrance in the progress of clarifying the biological significance of *C. jejuni* virulence factors. Human campylobacteriosis is characterized by watery diarrhea, abdominal pain, fever and the presence of blood and mucus in stools (6, 16, 33, 81, 95). Various experiments conducted have used animals that are not anatomically and physiologically similar to humans; therefore, requiring anatomical manipulation to acquire disease (4, 44, 73). Also, some animal model systems are quite expensive to maintain and/or have proven to yield inconsistent results (4, 73, 129). In the domestic ferret model it has been shown that some form of specific, aspect of immunity is

responsible for at least partial resistance to colonization and disease production although the mechanism has not been identified. Likewise, in this domestic ferret model study, humoral immunity did not appear to play a role in preventing colonization but did protect against enteric disease. Even though the use of the domestic ferret model proved to be informative regarding *C. jejuni* colonization and immunity, further immunological studies using this model are limited due to the lack of available immunologic reagents and genome sequence (19). Furthermore, other animal models have been used to specifically address certain sequelea associated with *C. jejuni* infection, such as, Guilain-Barré syndrome (GBS). *C. jejuni*-infected chickens have been shown to develop spontaneous paralytic neuropathy associated with GBS (93). Overall, an adequate animal model is needed to suitably assess the bacterial mechanisms and clinical manifestations associated with *C. jejuni* infection.

Despite the prior attempts and criticisms, it is known that the pig is anatomically and physiologically similar to the human, and studies have shown it to be a biologically relevant and important animal to use to observe *Campylobacter jejuni* virulence and host immune mechanisms. Newborn colostrum-deprived piglets that have been orally challenged with an invasive strain of *C. jejuni* have been successfully used in demonstrating the clinical manifestations associated with *campylobacteriosis*. The piglets developed clinical symptoms and histopathological lesions similar to that seen in human campylobacteriosis (10). Other swine model systems have proven to be of suitable use as well. For example, Mansfield et. al showed that a synergistic relationship between *C. jejuni* and *Trichuris suis* (*T. suis*) enhanced disease and pathology in gnotobiotic (germ-free) pigs (101). Likewise, additional swine disease model studies

demonstrated that the lyphoglandular complexes are important colonic sites for immunoglobulin A induction against *C. jejuni* (100). Together, these findings have provided significant support in using pigs as a suitable animal model for human campylobacteriosis, and investigators should now be able to use this system to further study virulence factors associated with this enteric disease.

To date, there are still unanswered questions although the swine disease model has provided a better understanding of some host immunomodulatory responses triggered by *C. jejuni* and *T. suis* infections. Therefore, a murine model system has been developed to continue the advancement in understanding the mechanisms involved in C. jejuni infection (99). Current studies have shown that C57BL/6 and congenic IL-10 deficient mice infected with *C. jejuni* 11168 are stably colonized and exhibit a robust Th1 directed antibody response, but only the congenic IL-10 deficient mice exhibit significant histopathological changes similar to responses seen in human when infected with *C. jejuni* 11168. These data suggest that colonization of the mouse gastrointestinal tract by *C. jejuni* 11168 is required but not adequate for enteritis development. Therefore, C57BL/6 and congenic IL-10 deficient mice can serve as suitable models of *C. jejuni* colonization and enteritis (99). Hence, the findings from these studies using the swine disease model and the existing mouse model will permit further study to characterize the immune responses that are possibly triggered by interactions between host factors of *C. jejuni* and/or *T. suis* which may have an effect on the development of enteric pathogenesis.

2). In vitro models

Extensive study of the mechanisms by which *Campylobacter jejuni* causes disease has not only been investigated *in vivo*, but various *in vitro* model systems have been developed to facilitate the understanding of the mechanisms involved in bacterial-host cell interaction (160). For example, Caco-2 cells is a cultured cell line that has been used in many *C. jejuni* experiments to specifically observe bacterial interaction with host cells that differentiate at confluency to mature enterocytes (137, 160). This cell line was derived from a human colon adenocarcinoma and has been used in experiments to mimic the intestinal epithelium. As these cells proliferate and differentiate in culture (maturation process of the intestinal epithelium), they form a polarized epithelial monolayer characterized by brush-border microvilli and tight junctions. When studying differentiation and the regulation of intestinal functions, the Caco-2 model is one of the most used models for *in vitro* study (64, 78). Likewise, INT 407 cells have been a widely used cell line in *C. jejuni*/host cell interaction studies. This cell line is derived from human small intestine and has been used experimentally to mimic the functions of human undifferentiated intestinal epithelium in response to bacterial exposure (160). In addition, primary swine intestinal cells have been used as a model for studying *C. jejuni*/host cell interaction. It has been shown that clinical *C. jejuni* isolates (*C. jejuni* F1474 and T13193) invade primary swine intestinal cells at a significantly higher frequency than an *Eshcherichia coli* control strain. Also, *C. jejuni* colonies recovered from these primary swine intestinal cells invade tissue cultured cells (INT 407) at a significantly higher frequency than the parental strain (*C. jejuni* T13192) suggesting that *C. jejuni* invasiveness may be an important *in vivo* virulence attribute (11). Hence, these

findings, in part, have influenced the use primary swine intestinal epithelial cells in ongoing *C. jejuni*/host cell interaction studies. For example, IPEC-1 cells (intestinal pig epithelial cells) are cells derived from neonatal pig intestinal epithelium. These cells have not been cloned, and this leads to some diversity of the population; however, these cells can be induced to differentiate by culturing them for 12-14 days. It has been shown that IPEC-1 cells secrete IL-6, IL-10 and IL-18 following *C. jejuni* exposure (Parathasarathy, dissertation, 2004 and Jones, dissertation, 2005).

Also, several other *in vitro* studies have demonstrated the roles of cytokines in *C. jejuni* infection. Viable *C. jejuni* stimulates INT 407 cells to secrete IL-8, and this response has been shown to be mediated via multiple mechanisms such as *C. jejuni* adherence and/or invasion or *C. jejuni* cytolethal distending toxin (CDT) treatment (61, 63). Other proinflammatory chemokines, such as GROalpha, GROgamma, macrophage inflammatory protein 1 (MCP-1), and gamma IP-10, are secreted from INT 407 cells following exposure to viable *C. jejuni* and are believed to play a contributing role in the observed host inflammatory responses (70). Likewise, it has been shown that viable *C. jejuni* activates the mitogen-activated protein kinase pathways and the transcription factor, NF-κB, which regulates the transcription of inflammatory cytokines and chemokines (97). Viable *C. jejuni* also stimulates INT 407 cells to produce intracellular IFN-γ, IL-10, TNF-α, and IL-4 suggesting that viable *C. jejuni* is capable of generating a dissociated Th1 and Th2 immune response (5). Likewise, *C. jejuni*-infected human dendritic cells (DCs) trigger NF-κB activation, stimulates IL-1-β, IL-6, IL-8, TNF-α, IL-10, IL-12 and IFN-γ production, and induces DC maturation (69). Also, *C. jejuni* interaction with cultured human monocytic cells elicits IL-1-β, IL-6, IL-8, and TNF-α

induction and triggers NF-κB nuclear translocation (77). In addition, *C. jejuni* has been shown to invade avian macrophages and primary chick kidney cells and stimulate the production of nitric oxide (NO), IL-1-β, IL-6, IL-8 and K60 (143). Therefore, it is evident that *C. jejuni*-host cell interactions have been studied using multiple well established *in vitro* models.

Pathogenesis

Intestinal Colonization

Colonization is a key factor involved in bacterial survival in the different microenvironments of the gastrointestinal tract of humans and animals and is necessary but not sufficient for production of enteritis (146). Many commensal bacteria asymptomatically colonize the intestinal tracts of animals used as a food source (146, 164). Several studies have demonstrated the importance of *C. jejuni* colonization to its persistence (79, 98, 110, 149).

In order to survive as an intestinal commensal or foodborne pathogen, *C. jejuni* must be able to adapt to and survive in different microenvironments especially those of the gastrointestinal tract. This capability is likely controlled by a two-component regulatory system (TCR), which is important for the coordinate regulation of gene expression (28). It has been suggested that this novel TCR system plays an important role in the growth and colonization of *C. jejuni* . This RacR-RacS (reduced ability to colonize) system is believed to be involved in a temperature-dependent signaling pathway. A mutation of the response regulatory gene, *racR*, reduced the ability of *C. jejuni* to colonize the chicken intestinal tract and resulted in temperature-dependent

changes in protein profile and growth characteristics (28). It is suspected that other TCR systems may be involved in optimal *in vivo C. jejuni* colonization. For example, a dccRS two-component system (diminished capacity to colonize) was found to be important for *in vivo* colonization of immunodeficient limited flora (SCID-LF) mice and 1-day old chicks, but not relevant for *in vitro* growth or survival in epithelial cells (98). MacKichan et al. demonstrated that mutations in the dccR response regulon and the dccS sensor kinase reduced colonization in 1-day old chicks and SCID-LF mice models and had no significant effect on *C. jejuni* growth and/or survival in epithelial cells (98). In addition, several genes encoding putative periplasmic and membrane proteins that are regulated by this two-component system have been identified. After comprehensive analyses, the genes involved in the RacR-RacS and dccRS two-component systems (*racR* and *dccRS*) proved to be essential for optimal colonization (98). Likewise, it has been suggested that *C. jejuni* outer membrane glycoproteins may act as adhesins thereby promoting colonization (79). Mutation of the *pglH* gene involved in *C. jejuni N*-linked general protein glycosylation showed reduced *C. jejuni* adherence and invasion of human epithelial Caco-2 cells and significant colonization reduction in 1-day old chicks (79). In addition, chemotaxis and motility are believed to be significant virulence factors involved in controlling the mechanisms of *C. jejuni* colonization of the intestinal tract (These factors will be discussed in greater detail later in the literature review.) (149). Overall, these studies have made significant advances to the knowledge needed for the development of intervention strategies to control transmission of this foodborne pathogen. However, despite progress, further studies are needed to better understand the processes involved in *C. jejuni* colonization.

Virulence factors

The characterization of *C. jejuni* virulence factors has been intensively studied over the past few years because *C. jejuni* is known to be the most common cause of gastroenteritis worldwide (6, 33). Significant progress has been made in identifying and characterizing virulence factors of *C. jejuni*; however, the molecular mechanisms involved in pathogenesis remain unclear. Herein, I will discuss a few of the virulence properties that have been recognized and studied by researchers to elucidate the pathogenic mechanisms of *C. jejuni*.

Motility and Chemotaxis

As mentioned in the colonization discussion, one of the best-characterized virulence determinants of Campylobacters is flagella-mediated motility. *Campylobacter* spp. flagella production is necessary for motility (81). It is believed that the spiral-shaped cellular structure and the flagellum, in combination, give Campylobacters an uncommonly high level of motility in viscous environments (81, 91). *C. jejuni* is motile by the action of one or two polar flagella. It has two copies of the flagellin gene (*flaA* and *flaB*). Two unique promoters control flagellin gene expression, and at least one of the genes responds to environmental signals (147). It has been shown through targeted mutagenesis that the *flaA* gene is the main gene for flagellin production and motility (75, 162). For instance, disruption of *flaA* produces a short, truncated and nonfunctional flagellum that has greatly reduced motility, lacks the ability to invade INT 407 cells, and is unable to colonize the ceca of three-day-old chicks (110, 157, 162). However, when

there is a disruption of *flaB*, the flagellum length is normal, and there is normal flagella function, but slightly decreased motility as compared to the wild type strain (81).

Motility of *C. jejuni* is known to be phase variable, but the mechanism of this variation remains unclear. Specific genes identified as the motility accessory factor (maf) family of flagellin-associated proteins are related to flagellar biosynthesis and phase variation. It has been suggested that these *maf* genes may confer on *C. jejuni* flexibility in adapting to changing environmental conditions when flagellar expression and motility are undesirable (80). The *maf* genes may also be involved in reversible expression of flagella, which could be beneficial in evading the host immune response (111). The reversible expression of flagella in C. *jejuni* means that the genes involved in flagellum synthesis and mobility can to switched on and off depending on the changing environmental conditions (111). For instance, flagella genes may be initially switched on to allow the flagellum to act as an adhesin and acquire attachment to the host cell. However, once attached and colonization has been established, then flagella formation may be switched off (111).

It has also been suggested that the regulation of *C. jejuni* motility is associated with quorum sensing. *C. jejuni* produces functional autoinducer-2 (AI-2) cell-signaling activity, and this AI-2 production is regulated by the *luxS* gene. AI-2 production allows bacteria to assess the population density of other species of bacteria and communicate with these other species of bacteria (75). Studies have demonstrated that inactivation of the *luxS* gene causes *C. jejuni* to become less motile than the wild type (43). Conversely, quorum sensing has been observed to be related to *C. jejuni* motility. Quorum sensing in *C. jejuni* influences *flaA* transcription and autoagglutination, which is known to be

closely associated to the existence of functional flagella in *C. jejuni* (75). Additional evidence pertaining to the importance of the *fla* regulon has been acknowledged by the recognition of a FlgS/FlgR two-component signal transduction system. The FlgS sensor and the FlgR response regulator form this system, and it has been shown to be required for the initial stages of chicken cecal colonization, but not for survival and persistence. The signal that triggers this two-component system to turn on the *fla* regulon has not yet been identified (161).

Chemotaxis is also required for effective colonization and is an important virulence property. The ability Campylobacters have to detect chemical gradients along with motility functions enable the cell to move up or down the gradient (81). The importance of chemotaxis has been demonstrated *in vivo*; chemically mutagenized or non-chemotactic *C. jejuni* failed to colonize suckling mouse intestine (149). Two adjacent genes have been identified to control chemotaxis, a methyl-accepting chemotaxis protein (MCP) and a putative cytochrome *c* peroxidase. These were identified using signature-tagged transposon mutagenesis of *C. jejuni* 81-176 followed by testing for chick gastrointestinal tract colonization (59). It is suggested that MCP is required for proper chemotaxis to a specific environmental element, and the putative cytochrome c peroxidase may function to reduce periplasm hydrogen peroxide stress during *in vivo* growth. Loss of either gene function resulted in attenuation of growth throughout the gastrointestinal tract of chicks (60). This particular study was one of the first genetic screens involved in identifying the bacterial gene requirements necessary for establishing a commensalistic relationship with a natural host. Also, it is clear from

recent studies in murine models that although colonization is not sufficient for disease following oral *C. jejuni* infection, it is required for disease to be manifested (99).

Adherence

Bacterial adherence to target cells plays a critical initiating role in pathogenesis. Bacterial adhesins interact with the intestinal epithelium and help pathogenic bacteria resist peristalsis and the fluid flow of lumenal contents. Once bacteria are adhered to the intestinal epithelium, the bacteria can form colonies, release enzymes and toxins, invade cells, and/or alter intracellular function to their own advantage (68). Therefore, it is suggested that adherence is an important determinant of virulence for various pathogenic bacteria (50).

The interaction between adhesins and the host are believed to be a prerequisite for bacterial penetration of the mucosal surface. Several *Campylobacter* spp. adhesins have been identified and characterized since this genera was discovered. Various *in vitro* studies have shown that some *Campylobacter* spp. adhere to cultured cells (106, 127, 141). In 1986, flagella were identified as a potential *C. jejuni* adhesin by comparing adherence of flagellated and aflagellated variants to INT 407 cells (106). McSweegan et al. showed that removal of the flagella by shearing the bacterial cells reduced adhesion. Lipopolysaccharide (LPS) was also identified as another *C. jejuni* adhesin. I*n vitro C. jejuni* LPS specifically bound to epithelial cells and intestinal mucus gel (106). These findings support the importance of flagella and LPS in initiating mucosal surface adherence.

Other approaches have been used to determine the significance of campylobacter adherence. For example, Yao and colleagues used an insertional mutagenesis method for naturally transformable organisms to characterize non-adherent and non-invasive *C. jejuni* strains. They generated a series of *C. jejuni* strain 81-176 mutants with a kanamycin-resistant cassette placed in various regions of the open reading frame of the *flaA* gene encoding flagellin. By this means, they identified strains that had defects in motility, displayed reductions in invasion levels, and lacked the ability to adhere to the target cells. They suggested that flagellin can serve as a secondary adhesin, and other adhesins mediate a motility-dependent internalization process (162). In addition, these generated mutants were used to identify a *cheY* gene that was responsible for the non-adherent and non-invasive phenotypes observed in their experiments. Yao et al. also showed that the diploid *cheY* strain was able to colonize mice, but was attenuated in a ferret disease model (163). The results of the ferret disease model suggested that virulence was reduced due to the presence of excess CheY. This also implied that the 2 copies of *cheY* may affect subsequent *C. jejuni* interactions with target epithelial cells in the colon rather than affecting the ability of the organism to survive in the mucus lining of the small intestine (163).

As mentioned, cultured cells and animal models have been used extensively to demonstrate *C. jejuni* adherence to and invasion of epithelial cells (30, 168). Various studies have led to the identification and characterization of other adhesins (76, 84, 122, 141). For example, a Campylobacter adhesion to fibronectin (CadF), a *C. jejuni* 37 kDa protein, was identified as an adhesin. Various *in vitro* studies indicated that CadF was a conserved outer membrane protein that mediated the specific binding of *C. jejuni* to the

extracellular matrix component, fibronectin (84). Likewise, other experiments demonstrated the activity of another *C. jejuni* adhesin identified as a 43-45 kDa *C. jejuni* major outer membrane protein (MOMP). The interaction between *C. jejuni* MOMP and cultured INT 407 cell membranes showed significant binding activity, and this interaction suggested that the MOMP functioned as an adhesin and mediated bacterial-host tissue contact (141). JlpA, a *C. jejuni* specific novel surface-exposed lipoprotein, was another identified adhesin that mediated adherence to host epithelial cells. Insertion and deletion mutants of *jlpA* demonstrated that adherence to HEp-2 cells can be reduced compared to parental *C. jejuni* TGH9011. Likewise, anti-GST-JlpA inhibits *C. jejuni* adherence to HEp-2. Also, an immunoblotting assay showed that JlpA binds to HEp-2 cells, implying that JlpA is a *C. jejuni* adhesin. From these findings, it has been suggested that lipoproteins may play a role in bacterial infection because they induce tumor-necrosis factor and cause macrophages to release interleukin-6. It has been suggested that JlpA may stimulate host cytokine production because it is released into the culture medium. However, this function remains to be established and could have multiple effects on the immune system (76). Furthermore, *in vitro* and *in vivo* models were used to characterize PEB1 as an adhesin that enhances *C. jejuni* interactions with epithelial cells. To identify the role of PEB1 adhesion activity, *peb1A* was inactivated by allelic exchange, and used to challenge cultured cells. The inactivation of the *peb1A* locus significantly reduced *C. jejuni* adherence to HeLa cells, which suggested that PEB1 was an important *C. jejuni* adhesion (122). This study also provided information about the PEB1 adhesin function in *C. jejuni* invasion and colonization. *Peb1A* locus inactivation significantly reduced *C. jejuni* invasion of epithelial cells in culture and reduced the rate and duration of mouse

intestinal colonization as compared to the *C. jejuni* wild type isolate. Together, these data indicated that PEB1 plays an important role in bacterial-epithelial cell surface interactions (122).

In summary, the identification and characterization of these adhesins have proven to be beneficial in establishing a better understanding of *C. jejuni* host cell interactions. According to these studies, adhesins play important roles in mediating *C. jejuni* attachment to host epithelial cells. They also suggest that some adhesins may play a significant role in virulence and host resistance and immunity. Further studies will contribute to understanding the relationship between *C. jejuni* adhesion and infection.

Invasion

Campylobacter-host cell interactions have been described as intriguing and mechanistically unique (160). Over the years, researchers have been persistently trying to understand the mechanisms by which *Campylobacter* invade host cells. Both *in vitro* and *in vivo* models have been used to establish the invasive potential of *C. jejuni* (10, 21, 23, 29, 46, 57, 61).

In vivo studies have shown that *C. jejuni* invasiveness is clearly correlated with presentation of disease in the host (10, 21). In one particular study, newborn piglets were orally inoculated with a strain of *C. jejuni* isolated from a patient. The clinical and histopathological damage was similar to that observed in that patient and in other humans with campylobacteriosis. Here, internalized bacteria were only observed within the intestinal cells of these *C. jejuni*-infected piglets, and there was disruption of the microvilli in the infected area (10). In various studies, cultured mammalian intestinal

epithelial cells have been used to demonstrate the process of *C. jejuni* invasion (86, 87, 107, 135). For example, Konkel and colleagues identified a Campylobacter invasion antigen (CiaB) protein that affects *C. jejuni* invasion and shows similarities to type III secreted proteins (86). Further studies indicated that maximal invasion of eukaryotic cells and Cia protein secretion depended on intact flagellar synthesis genes, thus suggesting that Cia protein export occurs through the flagellar transport and/or assembly apparatus. Collectively, these findings suggested that the flagellar type III secretion pathway is required for Cia protein export (87). In addition, it has been suggested that the Cia proteins are secreted during colonization, and they may play a significant role in *C. jejuni* colonization (22). Konkel and colleagues also identified, CadF, a fibronectin-binding protein that is required for the binding of *C. jejuni* to intestinal epithelial cells. They also found that this interaction enhanced bacterial internalization (84). Monteville et al. found that maximal *C. jejuni* entry required the binding of CadF to fibronectin which thereby triggered host cell signaling events associated with bacterial uptake. In this study, INT 407 cells were infected with a *C. jejuni* wild-type isolate and at 30 and 45 minutes post infection, there was an increase in the level of tyrosine phosphorylated paxillin (a focal adhesion signaling molecule)(107). Likewise, Cjp29 an unknown glutamine-rich protein, was another protein required for *C. jejuni* invasion, and it was found inside INT 407 cells, suggesting that it may be secreted by a type IV secretion system (12).

Additional studies have been carried out in order to determine if invasion is a significant virulence factor in facilitating *C. jejuni* enteritis. Researchers have examined the possible roles of microfilaments and microtubules in the *C. jejuni* invasion process

(23, 71, 89, 113). Treatment of intestinal epithelial cells (INT 407 cells) with cytochalasin-D (a chemical that causes microfilament depolymerization) and colchicines and nocodazole (chemicals that cause microtubule depolymerization) inhibited *C. jejuni* invasion of INT 407 cells in a dose-dependent manner. There was a more pronounced inhibitory effect of microfilament depolymerization on *C. jejuni* invasion than that of microtubule depolymerization. Similar observations have been reported on *Klebsiella pneumoniae* enterohemorrhagic *E. coli*, enteropathogenic *E. coli* and *E. coli* associated with new born meningitis (23, 24). These overall findings suggest that the mechanisms involved in *C. jejuni* invasion is associated with the combined effect of microfilaments and microtubules of host cells (23).

C. jejuni vaccine strain 81-176 has two plasmids (pTet- an R factor plasmid encoding tetracycline resistance and the other, pVir, encoding the *cag* pathogenicity island usually found in *Helicobacter pylori*) that have been characterized and are believed to affect the virulence of *C. jejuni* by encoding a type IV secretion system. Specific mutations in some of the identified plasmid genes (*comB3* and *virB11*) have been shown to significantly reduce invasion of INT 407 cells (13). Other analyses indicated that there are four additional pVir genes (Cjp15, Cjp29, Cjp32, and Cjp49) which need further characterization using mutational analyses followed by testing in animal disease models, but they have been found to significantly affect *C. jejuni* invasion into intestinal epithelial cells. These four genes encode proteins that are envisaged to be soluble and once invasion occurs are located in the cytoplasm. Further studies are being done to elucidate the possible effector protein roles of this putative type IV secretion system (13).

The *C. jejuni* invasion process is multifactorial and influenced by many cellular processes (21). This invasion process appears to be *C. jejuni* strain dependent and host cell-type dependent. Freshly isolated clinical strains also appear to be more invasive (36). Invasion of the host cell is an important virulence mechanism of *C. jejuni*, and the molecular mechanisms mediating host cell invasion are gradually being elucidated.

Intracellular survival

Intracellular survival is definitely a key factor in the pathogenesis of *C. jejuni*; however, the mechanisms by which *C. jejuni* persist in the host cell have not yet been defined. Various studies have attempted to demonstrate the entry process, intracellular survival and location of *C. jejuni* (24, 35, 36, 85, 86). Using transmission electron microscopy and the gentamicin killing assay, DeMelo et al. observed cellular events and intracellular survival of *C. jejuni* in Hep-2 cells (36). At one hour post infection, DeMelo and colleagues noticed *C. jejuni* cell surface and microvilli interactions which possibly imply a phagocytic-like mechanism preceding *C. jejuni* internalization (36). Also at one hour post infection, there was notable evidence of host cytoskeletal rearrangement and the presence of *C. jejuni* enveloped within endocytic vacuoles in the cytoplasm of the host cell (23, 36, 83). Once internalized and about 9 hours postinfection, phagolysosome fusion occurred, and lysosomes assembled and co-localized in the cytoplasmic matrix. This lysosomal event could possibly explain the loss of *C. jejuni* viability and the change in the morphological structure from spiral to coccal form at this stage (83). Konkel et al. and Biswas et al. also did studies to further characterize *C. jejuni* internalization and intracellular survival. They concluded that *C. jejuni* internalization of INT 407 cells was

affected by both, host microfilament and microtubule depolymerization (23, 24, 83). In addition, Konkel et al. found that ammonium chloride and methylamine did not inhibit endosomal acidification and did not affect *C. jejuni* internalization by INT 407 cells (83), but Biswas et al. showed that monenesin significantly inhibited endosome acidification, thereby reducing the number of intracellular *C. jejuni* (24). Biswas et al. also showed that viable intracellular *C. jejuni* were reduced in number by treating INT 407 cells with G-strophanthin and monodansylcadaverine which indicated interference of receptor-mediated endocytosis (62).

It is known that *C. jejuni* has the ability to persist and multiply in epithelial cells and phagocytic host cells (35, 36, 85, 123). Konkel et al. showed that intracellular *C. jejuni* can elicit a cytotoxic effect on INT 407 cells causing deterioration of the cells (85). The weakening effects on the cell monolayers are believed to represent a pathogenic mechanism associated with *C. jejuni* enteritis (85). Likewise, Hickey et al. showed that *C. jejuni* strain 81-176 is capable of replicating extensively within human monocytic cell vacuoles. They also suggested that viable intracellular *C. jejuni* induce apoptotic death via secreted *C. jejuni* cytolethal distending toxin (62). Similar to the findings of Guerry et al., Siegesmund et al. showed that *C. jejuni* Cia-secreted proteins are required for maximal apoptosis induction of monocytic cells *in vitro* (142). It has been suggested that these secreted proteins are associated with a type III system of *C. jejuni*, and are responsible for intestinal epithelial cell invasion, and possibly play a role in *C. jejuni* enteritis (142). Siegesmund et al. also showed that the apoptotic death of *C. jejuni*-infected monocytic cells is caspase-1-independent and is not dependent on the concurrent

secretion of IL-1-β, which is caspase-1 dependent (142). However, it is unclear at this time as to how macrophage apoptosis is associated with *C. jejuni*-mediated enteritis.

There have been several studies which have given insight to the possible mechanisms of intracellular survival (35, 123). For instance, it has been demonstrated how *C. jejuni* iron superoxide dismutase (SOD) enzyme contributes to intraepithelial cell survival (123). This enzyme catalyzes the breakdown of superoxide radicals to hydrogen peroxide and dioxygen and acts against oxidative stress in the bacterial cell thereby contributing to the defense mechanisms of the bacterial cell (123). To date, only one *sod* gene has been identified in *C. jejuni*, which is similar to other reported FeSODs. In one particular study, Pesci et al. constructed isogenic *sod* mutants which proved to be significantly more sensitive to intracellular killing than wild-type *C. jejuni* strains thereby suggesting that SOD plays a role in *C. jejuni* intracellular survival (123). Likewise, *C. jejuni* expresses a single catalase enzyme identified as *katA* (35). It is believed that this catalase also plays a significant role in *C. jejuni* intracellular survival. Day et al. constructed *katA* mutants to address this hypothesis, and the findings of this study suggested that catalase plays a significant role in *C. jejuni* intramacrophage survival by providing resistance to hydrogen peroxide(35). Together, the findings of Pesci et al. and Day et al. suggest that organism persistence inside host cells is in part due to bacterial factors that combat reactive oxygen species and play a role in intracellular survival (35, 123) .

Lipopolysaccharide/Lipoolygosaccharide

In gram-negative bacteria, lipopolysaccharide (LPS) is a major constituent of the outer membrane, and it is composed of three distinct structural components: lipid A, which anchors the membrane; an oligosaccharide core; and the O antigen composed of one or more glycosyl residues that covalently attach to the core (88). LPS molecules that lack O-antigen units and have core oligosaccharides structures limited to 10 saccharide units are referred to as lipooligosaccharides (LOS) (130).

It has been suggested that *C. jejuni* LPS and LOS are excellent targets for the control of infectious diseases and for diagnostic purposes. *In vitro* and *in vivo* studies have demonstrated the functions of LPS and LOS in *C. jejuni* virulence (55, 108, 128, 167). It is believed that *C. jejuni* LPS and LOS mimic host ganglioside structures, specifically components of the perpherial nerve tissue (55). More specifically, LOS has been shown to play an important role in the pathogenesis of gastroenteritis due to *C. jejuni*. Phase variation of *C. jejuni* 81-176 LOS has been demonstrated *in vitro* and *in vivo*, and the ability of *C. jeju*ni to undergo phase variation in LOS biosynthesis genes may provide selective advantages in causing gastrointestinal disease (55). It has been suggested that phase variation of LOS biosynthesis allows a single bacterial strain to produce a repertoire of LOS molecules that differ in core length and carbohydrate content due to the addition of glycose groups at the nonreducing termini (128). *In vitro*, slip strand mismatch recombination of *cgtA*, a gene that encodes N-acetylgalactosaminyl (GalNAc) transferase, resulted in changes in the major core structure from GM_2 to GM_3 ganglioside mimicry. Also, site-specific insertional inactivation of the *cgtA* gene of *C. jejuni* LOS resulted in a mutant that mimics GM_3 and enhances internalization and

invasion of intestinal epithelial cells *in vitro* thereby suggesting that changes in LOS structure may directly affect the ability of *C. jejuni* to cause gastroenteritis (55). Also, variation in the sialylation of *C. jejuni* LOS was demonstrated via site-specific mutagenesis of the *neuC1* gene resulting in the loss of sialic acid in the LOS core, increased immunogenicity of the core, and decreased resistance to normal human serum (53, 55, 128, 167). Antigenic phase variation in ganglioside mimicry has also been demonstrated *in vivo* (128). In a volunteer experimental oral infection study, Prendergast et al. found that the LOS of *C. jejuni* isolates underwent antigenic phase variation in ganglioside mimicry during passage *in vivo*, and serological testing showed that antiganglioside antibodies were not present following experimental *C. jejuni* infection or following administration of a killed *C. jejuni* whole-cell vaccine (53, 108, 109, 167). They concluded that their findings were consistent with *in vitro* reports that have shown that *C. jejuni* possesses the genetic mechanisms for its LOS to undergo phase variation. They also believe that these LOS phase variation responses could cause difficulties in developing successful vaccines because *C. jejuni* can synthesize ganglioside mimics and has the ability to convert between different LOS structures (128). These observations also suggest that the ability of *C. jejuni* to undergo LOS phase variation may be advantageous to bacterial persistence in the host. Hence, the changes that can occur in the major core of *C. jejuni* due to bacterial phase variation suggest that *C. jejuni* LOS core structures are dynamic, and there are indications which imply that there may be differences in the mechanisms by which *C. jejuni* strains cause diarrheal disease (55). Further study is needed to elucidate *C. jejuni* phase variation and how these variations may play a role in *C. jejuni*-induced gastroenteritis.

Additionally, more studies have been conducted to elucidate the association between *C. jejuni* LOS and GBS (109). It has been demonstrated that molecular mimicry between human ganglioside and *C. jejuni* LOS play a relevant role in the pathogenesis of GBS (53, 109). Nachamkin et al. found that *cst-II* (α-2,3 and/or α-2,3/ α-2,8 sialyltransferase), *cgtA* (β1,4-*N*-aetylgalactosyltransferase) and *cgtB* (β1,3-galactosyltransferase)are LOS biosynthesis genes involved in *C. jejuni* LOS core extension and appear to be critical to *C. jejuni* ganglioside mimic expression associated with GBS-related *C. jejuni* isolates(21, 167). Also, based on LOS locus analysis and knockout mutants, Godschalk et al. suggested that specific classes of the LOS biosynthesis gene locus or specific types of genes involved in sialic acid biosynthesis and transfer may be crucial for the induction of neuropathogenic cross-reactive antibodies (53). Various studies have presented evidence implying that the development of auto-antibodies raised against distinct *C. jejuni* surface LPS and LOS is involved in the pathogenesis of Guillain-Barre syndrome(GBS)(55, 108, 166). In rabbits sensitized with *C. jejuni* LOS, anti-GM_1 IgG antibodies were detected and flaccid limb weakness developed, which resembles clinical signs of GBS in humans. The pathological studies of the sciatic nerve specimens of the paralyzed rabbits showed Wallerian-like degeneration identical to that seen in GBS patients (167). In addition, immune naïve mice injected with *C. jejuni* LOS generated monoclonal antibodies (mAb) that reacted with GM_1 and bound to human peripheral nerves. In general, these findings suggest that carbohydrate mimicry between *C. jejuni* LOS and human gangliosides are important for the development of GBS.

Overall, *C. jejuni* LPS and LOS appear to play an important role in the pathogenesis of *C. jejuni*-mediated enteritis and autoimmune diseases such as GBS, and further evaluation of these particular virulence determinants (LPS and LOS) may elucidate specific mechanisms involved in *C. jejuni*'s ability to persist in host cells causing pathology and disease.

Toxins

Campylobacter spp. that produce toxins have been isolated from various human and animal sources throughout the world. In attempts to study toxigenicity, researchers usually correlate toxin production with clinical presentation and *in vitro* analysis (156). Since *C. jejuni* is one of the leading causes of acute diarrhea in humans in developed countries and has been isolated from healthy and diseased young children in developing countries, I have limited the discussion to this species (156). There are two types of diarrhea induced by *C. jejuni*: 1) a noninflammatory, watery diarrhea without leukocytes or blood and 2) an inflammatory, bloody diarrhea containing leukocytes. In both of these clinical presentations, diarrheal disease caused by *C. jejuni* is believed to be associated with toxin production.

Enterotoxins and cytotoxins are two classes of proteinaceous toxins that differ in their primary mode of action. Enterotoxins are secreted proteins with the ability to bind to a cellular receptor, enter the cell, and elevate intracellular cyclic AMP (cAMP) levels. This elevation of cAMP causes changes in the cellular ion flux, which leads to excessive secretion of fluid, resulting in watery diarrhea (156). Cytotoxins kill target cells by inhibition of cellular protein synthesis and actin filament formation or by forming pores

in target cell membranes. Cytotoxins cause inhibition of cellular protein synthesis by inactivating ribosomes by depurination of a nucleotide in the 28S rRNA, and this inhibition leads to cell death. Also, these secreted proteins can damage the intestinal mucosa and cause diarrhea by covalently modifying Rho proteins resulting in the disruption of the actin cytoskeleton. Another cytotoxic mechanism can be directed specifically towards nucleated cells. For instance, the cytotoxin forms pores in the nucleated cell thereby inducing cytokine release, cytoskeleton dysfunction, secretion of granule constituents, and generation of lipid mediators. These profound local and distant effects in host tissues may lead to an overall diminution of the immune responses of the host (156).

C. jejuni enterotoxin activity has been demonstrated in cell culture by the elongation of Chinese hamster ovary (CHO) cells, rounding of mouse adrenal tumor cells (Y-1), or by a measurable increase in intracellular cAMP in exposed cells (156). Likewise, Ruiz-Palacios et al. showed that supernatant of a prototype virulent *C. jejuni* strain causes the induction of fluid secretion in the rat ileal loop test (RILT), but not in the rabbit ileal loop or the infant mouse assay (136, 156). It has been suggested that enterotoxin production is responsible for the watery diarrhea form of campylobacteriosis, as opposed to the other form with inflammatory, bloody diarrhea (156). However, controversy surrounds *C. jejuni* enterotoxin activity because, to date, no gene encoding this product has been found. Also, the inability to consistently reproduce some of the *in vitro* and *in vivo* assays used to detect enterotoxin activity continues to fuel the controversy. These concerns will not be resolved until the enterotoxin structural gene has been cloned, sequenced, and the functional experiments are proven to be reproducible.

C. jejuni cytotoxins are also not well characterized. Several studies have evaluated the correlation between cytotoxin production and clinical manifestations. It has been suggested that cytotoxin-producing *Campylobacter* strains are more commonly isolated from patients with inflammatory diarrhea than other strains (156). Various cell lines, bacterial culture conditions, and bacterial strains have been used to study *Campylobacter* spp. cytotoxin production (156). Although diverse methodologies have been used to classify *Campylobacter* spp. cytotoxin production, to date, some of the research has not been reproducible (125).

However, to date, the cytolethal distending toxin (CDT) is the best characterized *C. jejuni* toxin. In 1996, the *cdt* genes from *C. jejuni* strain 81-176 were cloned and sequenced (125). Lara-Tejero et al. discovered that the *cdt* genes are required to form a tripartite holotoxin that promotes CDT activity (90). They found that 3 subunits, CdtA, CdtB, and CdtC, make up the *C. jejuni* CDT holotoxin, and all subunits are required for complete toxic activity (90). It has been observed that CdtA and CdtC bind the host cell and comprise CdtB. The CdtB subunit has been shown to be the active enzymatic subunit which is imported into the nucleus and functions similar to a DNAse 1 enzyme (32, 88, 92). It is proposed that the CdtB subunit damages the DNA and inhibits the dephosphorylation of Cdc2, a key kinase regulator of cell cycle progression (90). *C. jejuni* CDT causes elevated intracellular cAMP levels, blocks the G_2/M phase of eukaryotic cells prior to cell division, induces cytoplasm distension, and eventually leads to cell death (32). This cell cycle arrest activity has been demonstrated in experiments using a yeast model system that showed that CdtB induced G_2 cell cycle arrest accompanied by degradation of the host cell chromosomal DNA (58).

In addition to these findings, it has been suggested that *C. jejuni* CDT plays a role in host cell immunomodulation. *In vitro*, CDT stimulates cultured human intestinal epithelial cells to produce IL-8 (63). In an *in vivo* challenge experiment, mutant *C. jejuni* lacking the *cdtB* gene successfully colonized NFκB deficient mice producing gastritis but caused much less severe disease than the isogenic parent wild type *C. jejuni*. This finding suggests that CDT has a proinflamatory effect similar to CDT effects reported *in vitro* (51). Also, IgG2a levels were lower in mice infected with the CDT mutant than the mice infected with the wild type strain (51). These findings suggest that CDT plays a significant role in stimulating an immune response and in developing disease.

Toxin production by *Campylobacter* spp. has been a very complex virulence property to classify, and the difficulties with *Campylobacter* spp. toxin classification may reflect significant strain variation in this species. There has been a continuous effort towards developing *in vitro* and *in vivo* methodologies to determine the modes of action of putative toxins (165). Although there have been several reports of toxin producing *Campylobacter* spp., the correlation with disease and the mechanisms of action require further study.

Immune responses

The mechanisms by which *C. jejuni* elicit a host immune response are not well understood. In developing countries, it is believed that healthy infants, children and adults are constantly exposed to *Campylobacter* antigens in the environment, and consequently, there is early development of serum antibodies to *Campylobacter* species (33). However, most cases in developed countries occur later in life and are usually

primary, sporadic infections with more severe and prolonged clinical manifestations, and the immune response related to disease is not clear (33). Therefore, the host immune responses to campylobacteriosis differ among people in developing and developed countries, and various studies have been conducted to determine the importance of *C. jejuni* immune responses and host factors that affect the outcome of disease.

Humoral immunity is necessary to resolve *C. jejuni* infection. During *C. jejuni* infection, the levels of all immunoglobulin classes rise. However, IgA is one of the most important isotypes because it can cross the gut wall and immobilize organisms in the gut (155). In a swine disease model, a significant efflux of immunoglobulin A (IgA) was detected in the enlarged germinal centers of the lymphoglandular complexes (LGCs) of *C. jejuni*-infected pigs (100). This immune response suggests that there are important colonic sites worthy of further immunological consideration to elucidate mechanisms of resistance or susceptibility to disease. In addition, Cawthraw et al. detected significant IgA antibody levels in the postinfection serum, saliva and urine of patients with *C. jejuni* infections (31). Also, serum and salivary antibodies against flagellin were detected suggesting that flagellin is an important surface antigen against which immunity is directed (19). In a domestic ferret model system, humoral immunity does not appear to play a role in preventing colonization but does protect against enteric disease. Taken together, these findings suggest that a Th2-type immune response can in part play a protective role during *C. jejuni* infection.

T-cell-mediated responses to *C. jejuni* infection appear to be of relevance as well. Rhijn et al. demonstrated peripheral blood γδ T cell expansion following the exposure of patients to crude sonicates of five different *C. jejuni* serotypes (153). This expansion was

dependent on the presence of $CD4^-/\alpha\beta^+$ T cells in cultures or the addition of exogenous IL-2 or IL-15, and γδ TCR mediated the *C. jejuni*-specific stimulation (153).

Also, several *in vitro* studies have demonstrated the roles of cytokines in *C. jejuni* infection. Viable *C. jejuni* stimulates the secretion of IL-8 by INT 407 cells (human intestinal epithelial cells), and this response is mediated via multiple mechanisms, 1) *C. jejuni* adherence and/or invasion or 2) *C. jejuni* cytolethal distending toxin (CDT) treatment (61, 63). Other proinflammatory chemokines, such as GROalpha, GROgamma, macrophage inflammatory protein 1, MCP-1, and gamma IP-10, are secreted by INT 407 cells due to exposure to viable *C. jejuni* and are believed to play a contributing role in the observed host inflammatory responses (70). Furthermore, it has been shown that viable *C. jejuni* activates the mitogen-activated protein kinase pathways and the transcription factor NF-κB which regulates the transcription of inflammatory cytokines and chemokines (97). Viable *C. jejuni* also stimulates INT 407 cells to produce intracellular IFN-γ, IL-10, TNF-α, and IL-4 suggesting that viable *C. jejuni* is capable of generating a dissociated Th1 and Th2 immune response (5). Likewise, *C. jejuni*-infected human dendritic cells (DCs) trigger NFκB activation, stimulates IL-1-β, IL-6, IL-8, TNF-α, IL-10, IL-12 and IFN- γ production, and induces DC maturation (69). Also, *C. jejuni* interaction with cultured human monocytic cells elicits IL-1-β, IL-6, IL-8, and TNF-α induction and triggers NFκB nuclear translocation (77). In addition, cultured swine intestinal epithelial cells secrete IL-6, IL-10 and IL-18 following *C. jejuni* exposure (Parathasarathy, dissertation, 2004 and Jones, dissertation, 2005). Similar to mammalian cells, avian primary chick kidney cells and avian macrophages express the production of nitric oxide (NO), IL-1-β, IL-6, IL-8 and K60 due to *C. jejuni* invasion

(143). *In vivo*, *C. jejuni* also induces peritoneal phagocyte oxidative activity, increased polymorphonuclear (PMN) cells from the spleen, and IL-2 production in infected BALB/c mice (115, 116). Altogether, these findings suggest that cytokines likely play significant roles in *C. jejuni*-mediated infection.

Overall, the findings of these studies appear to suggest the cooperation of both innate and adaptive host immune responses to resolve *C. jejuni* infection *in vivo*. It is suggestive that the inflammatory immune responses induced by viable *C. jejuni* play an important contributing role in directing host immune responses towards generating and/or modulating protective immunity for the benefit of the host. However, in some cases depending on the susceptibility of the host, inflammatory responses can benefit bacterial survival during immune assault. Therefore, it is imperative that further study is done to elucidate the dissociated immune responses induced by viable *C. jejuni* to determine how the responses play a role in immunopathogenetic events associated with inflammatory diarrhea due to *C. jejuni* infections.

II. ***Trichuris suis***

Basic parasitology

Trichuris suis (T. suis) is a member of the nematode superfamily Trichuroidea. A distinguishing characteristic of this phylum is the stichosome esophagus which consists of stichocytes that have been shown to be involved in feeding and attachment(17, 74, 151). There are five genera associated with this superfamily: *Trichinella*, *Trichuris*, *Capillaria*, *Trichosomoides*, and *Anatrichosoma*. There are also related species of interest: *Trichuris ovis* (*T. ovis*)-cattle, sheep and goat; *Trichuris discolor* (*T. discolor*)-cattle; *Trichuris suis* (*T. suis*)-swine; *Trichuris vulpis* (*T. vulpis*)-dog; *Trichuris felis* (*T.* felis)-cat; *Trichuris muris* (*T. muris*)-mouse; *Trichuris trichiura* (*T. trichiura*)-humans and non-human primates; *Trichuris leporis* (*T. leporis*)-rabbits. These parasites are known as whipworms and are normally found in the host intestine (126).

Morphology

T. suis is a swine whipworm helminth that has a thread-like appearance featuring a slender anterior end which attaches to the mucosa of the pig's cecum and has a thick posterior end which protrudes freely into the lumen. This whipworm is about 5-8 cm long and is dioecious (exists as separate sexes). The male nematode is smaller than the female, and the male has a distinct curved spicules at its posterior end. *T. suis* eggs are barrel shaped with a thick shell that consists of three outer layers and one thin inner vitelline membrane with bipolar plugs. The external surface and the body cavity lining of *T. suis* are covered by the cuticle. It serves as a tough, flexible, protective covering

that is resistant to most digestive enzymes. Beneath the cuticle is the hypodermis layer which aids in regulating the body wall permeability. This hypodermis layer gives rise to the bacillary band which displays glandular activity. Directly underneath the hypodermis lies muscle cells consisting of a large vacuolar cytoplasmic body called the stichosome, which enfolds a narrow capillary-like esophagus. The alimentary tract of the parasite is complete with a mouth, buccal capsule (not always present), and an esophagus that empties into the intestine and onto the anus. The nervous system consists of dorsal and ventral nerve cords that surround the esophagus. There have been no findings of a circulatory or respiratory system. However, it is believed the bacillary band is utilized for oxygen exchange between the host's tissues and the nematode's internal organs via diffusion through pseudocoelomic fluid. The male reproductive system consists of testis, vas deferens, ejaculatory duct, spicules, and spicular muscles. The female reproductive system consists of the vagina, uterus, oviduct, and ovary. The life span of *T. suis* usually does not exceed 4-5 months (17, 74).

T. suis life cycle

There are five larval stages in the life cycle of *T. suis* which accounts for the development of the egg into a mature adult. The prepatent period is 41-45 days, and the life span of this whipworm usually does not exceed 4 to 5 months (17, 74, 151). *T. suis* female reproduces 3000 to 5000 eggs per day following copulation. The eggs are unsegmented, barrel shaped with a thick shell and are operculate at each end. The female lays the unfertilized eggs, and they are passed out of the pig via the feces, and the infective L1 larvae began to develop inside the egg. The development of the egg is

influenced by temperature, and studies have shown that the optimal temperature for the development of the infective L1 larvae is 34°C. By day 19, infective larvae are formed, and a characteristic feature of infective L1 larvae is the presence of an oral spear (stylet). The infective L1 larvae are ingested by the pig, and continued development of the larvae to the adult stage, which occurs in the mucosa of the cecum and colon. The hatching of the L1 larva began as early as 9 hours post-infection, and there is continued hatching for up to 3 days in the small intestine, cecum, and colon. The oral stylet is used by the L1 larvae to pierce the surrounding membrane and emerge from the shell. The L1 larvae burrow into the mucosa via the Crypts of Lieberkühn and penetrate the epithelial and goblet cells lining the crypts. As the larvae continue to grow, by day 7, they begin to invade the lamina propria cells. On day 10, the first molting (shedding of the cuticle form the body) occurs, and the L1 develops to the L2 larvae stage. The L2 larvae continue to grow and body organs differentiate as the L2 larvae migrate deeper towards the mucosal epithelium that begins the histotrophic phase of the *T. suis* life cycle. This molting process continues throughout the developing stages. Around day 16, the L2 larvae undergo another molt to become L3 larvae. At this stage, the most posterior end of the L3 larvae protrude into the gut lumen, and the body length is increased significantly. The reproductive system is also developing at this time and further differentiation of body organs is occurring. On day 20, molting occurs again, and the L3 larvae develop to the L4 stage, and the reproductive tract can be distinguished in some larvae. The entire posterior end of the larvae is free in the lumen while the anterior end is embedded in the mucosa. By day 28, the larvae lay in a shallow tunnel on the mucosal surface covered by mucosal epithelial cells. The last molting occurs around days 32 and 37; at this time, the

L4 larvae develop into the adult stage L5 larvae. The sexual organs are completely developed, and the females appear larger than the males. By day 41, eggs are seen in the uterus, vagina, and feces of the female permitting the life cycle to start again (26).

Epidemiology

T. suis are most prevalent throughout the world in places with warm, humid climates (52). These whipworm nematodes cause severe disease, and a 1991 study reported that approximately 45% of swine farms nationwide were infected with *T. suis*. Moderate to heavy infection can cause serious economic losses due to scours, which results in diarrhea, reduced carcass weight, and even death. A controlled trial experiment showed that the specific economic losses were 26 lbs. of pork and 2 dead pigs out of 12 animals (26). Severe *T. suis* infection also affects the swine industry by forcing an extension in the pig's growth and processing time by fifteen extra days before being able to sell at the market (26). The incidence of *T. suis* in swine herds is of great economical relevance, and studies have been conducted to inform the swine industry and the general public of the consequences of *T. suis* infection. A study conducted to determine the health status of a feral swine population in Kansas, USA indicated that 10% of the pigs were infected with *T. suis* (52). Also, a survey of gastrointestinal pig parasites on free-range, organic and conventional pig farms in the Netherlands indicated that *T. suis* is quite prevalent on various farms. Results showed that *T. suis* was found on 37.5% of the free-range farms, 36.4% of the organic farms, and 11.1% of the conventional farms. This study suggested that *T. suis* infection in pigs on farms with outdoor facilities is higher than pigs on conventional farms. The infection was most commonly seen in the sows on

the free range farms (50%) and organic farms (30%) (40). Likewise, on intensive farms in Guangdong Province, People's Republic of China, of the 3636 pigs sampled, 189 (5.2%) were infected with *T. suis*, and *T. suis* was the most common nematode causing infection in the breeding, young and adult pigs. There was no strategic anti-parasite treatment regime implemented on these farms (158). Further studies are needed to better address preventive strategies associated with *T. suis* infection, economical loss, and public health awareness.

Pathology

Human Pathology. Gastrointestinal nematode infections affect people throughout the world and tend to be chronic with high reinfection rates (49). *Trichuris* infections in humans are symptomatic in developing countries, and most patients in developed countries are asymptomatic unless exposed to heavy infection. Heavy infections in humans cause chronic diarrhea with bloody stool. Other symptoms include abdominal pain and distention, nausea, vomiting, flatulence, headache, weight loss, malnutrition and anemia. Untreated infections in some children can lead to clubbing of the fingers and growth retardation (151).

Animal Pathology. *Trichuris* infections also affect livestock production worldwide and cause economic losses for farmers (26). The economic loss in the swine industry is due to serious *T. suis* infections that cause 'scours' (severe mucohemorrhagic diarrhea) which is associated with increased weight loss, unthriftiness, anemia, dehydration and death in heavy infections (26).

T. suis is found in the cecum and colon of swine (18). Usually it is the younger, weaned pigs that acquire catarrhal enteritis with serious pathology (121). Trichuriasis is commonly called '21-day scours' by swine producers because of the severe, bloody diarrhea associated with the infection that usually occurs 21 days after pigs are exposed to a *Trichuris* contaminated lot (17, 26, 74). The histotrophic phase of *Trichuris* infection lasts for thirteen days in the mucosa, and onset of clinical signs are associated with the reemergence of third-stage larvae into the cecal lumen (17, 74). Much of the pathology associated with this helminth infection is due to the burrowing activities of the larval and adult stages in host colonic tissues and secretion of proteolytic enzymes and proteases (17, 74). However, following the initial mucosal damage by the parasite, secondary invasion by opportunistic gut microflora may result; thereby, amplifying the severity of the infection (17, 74, 101, 103, 138). The parasite maintains a syncytial environment (fuses with the cells) in the cecal epithelium with its anterior end within the host epithelium and its posterior portion free in the cecal lumen (17, 74). Beer et al. suspected that these syncytial tunnels created by *T. suis* burrowing contributed to the secondary invasion by gut microflora, which contributed to the overall pathology (17, 138).

Likewise, Mansfield and Urban also conducted an *in vivo* experiment describing a porcine model of mucohemorrhagic diarrhea that demonstrated the overgrowth of opportunistic bacteria during subclinical *T. suis* infection (103). The findings of this study suggested that secondary bacterial infection likely plays a contributing role in inducing pathology during *T. suis* infection. Results also indicated that antibiotic therapy relieved the pathology observed (103). In this study, pigs infected with *T. suis* only and

receiving no antibiotics exhibited mucopurulent lesions in the distal colon surrounding the lymphoglandular complexes. This observation led to the isolation of 3 opportunistic bacteria, including a tissue invasive bacterium, *Campylobacter jejuni* (103). The pathology observed supported the early study of Rutter and Beer who observed secondary bacterial invasion while studying the synergistic relationship between *T. suis* and the microbial flora of the large intestine. They believed this synergistic relationship caused swine dysentery (138). With this in mind, Mansfield et al. conducted another study using gnotobiotic (germ-free) pigs to examine the hypothesis that disease and pathology is induced by the synergistic effect of *T. suis* and *C. jejuni* in the colon. The pigs infected with both *T. suis* and *C. jejuni*, showed severe clinical signs and pathology; whereas, the *T. suis* only and *C. jejuni* only-infected pigs showed no clinical signs or pathology (101). This confirmed that secondary invasion by bacteria can contribute to the clinical syndrome seen during this helminth infection.

While studying the pathogenesis caused by the interaction between *T. suis* and *C. jejuni* (the secondary opportunistic bacterium), Mansfield et al. also detected the presence of the histopathologic lesions in the colon of infected pigs (100, 101, 103). Lesions were seen in the proximal and distal colon. At the site of worm attachment in the proximal colon, there was thickening of the intestinal layers and crypt and absorptive cell destruction. There was also an infiltration of inflammatory cells in the lamina propria that included lymphocytes, macrophages, neutrophils, eosinophils and plasma cells. In the distal colon, the lymphoglandular complexes (LGCs) in the submucosa were enlarged mainly due to enlarged B cell-dependent germinal centers, and some lymphocytes, macrophages and a few eosinophils were present (100, 101, 103).

T. suis Excretory/Secretory Products (ESP) and antimicrobial activity

There have been several *T. suis* ESP identified and characterized, and it is believed that these *T. suis* products may be involved in nematode tissue invasion, migration and feeding of the larvae and adults. It has also been suggested that ESP could possibly be facilitating or directly inducing the pathology seen during infection. *T. suis* ESP was collected while isolating and maintaining *T. suis* in culture. These ESP components will be discussed in detail in the following section:

1. Zinc metalloprotease

Hill et al. collected large quantities of a *T. suis* 45 kDa zinc metalloprotease from the culture fluids of adult worms. This protease functions at an optimal pH of 7 and has been localized to the parasite stichosome. It degrades fibrinogen and elastin, which is indicative of its possible role in feeding and/or tissue penetration. The secreted protease may also be associated with submucosal connective tissue migration in the cecum and colon of pigs, but further studies are needed to define its function (65).

2. Phenol oxidase

The association of phenol oxidase production with *T. suis* ESP was established during the adult worm culturing process. A brown pigment was observed in female *T. suis* after 1 day of culturing under aerobic conditions. Early work showed that this enzyme is associated with the tanning (oxidative process) of the eggshell proteins or with

eggshell synthesis. This observation was mainly noted in the female worms, and the function of this enzyme suggested that it may be needed to enhance persistence of ova in the environment. Phenol oxidase was found to be localized anteriorly in the parasite in the proximal part of the uterus posterior to the stichosome. It was clearly associated with the female reproductive tract, and the enzyme activity was also triggered when the female worm was removed from an anaerobic environment in the cecum into an aerobic environment during the culturing process. Egg shells of the parasite also had evidence of phenol oxidase activity and the authors postulated that this enzyme plays a crucial role in parasite survival in the environment. They also suggested that the phenol oxidase could be a target for control measures to reduce the risk of *T. suis* infection. Inhibition of phenol oxidase activity could increase the sensitivity of the *T. suis* eggs and allow unfavorable environmental conditions to prevent normal egg shell hardening thereby positively effecting swine production facilities (47, 48).

3. Pore-forming protein

A major 47 kDa pore-forming protein (also referred to as TT47) has been identified in *T. trichiura* ESP, the human whipworm. Likewise, its counterpart, TM43, has been described in the *T. muris* mouse model. Studies have shown that the protein induces ion-conducting pores in phospholipid bilayers. Therefore, it is hypothesized that these proteins facilitate invasion of the host gut by *Trichuris* thereby enabling the worm to form a syncytial tunnel in the epithelium thus promoting survival in the host. Although this pore-forming protein has not been identified in *T. suis* ESP, it is still noteworthy to recognize the possible role it plays in *Trichuris* infection (37, 38).

4. Thiol protease

A thiol protease was identified in the cultured fluids of *T.* suis. The optimal pH of this protease is between 5.5 and 8.5, and its function at this time is unknown. The secreted protease was also detected in the extracts of adult worm gut tissue implying that the protease may be involved in nutrient digestion and absorption (67).

5. Specific diagnostic antigen

Diagnosis of *T. suis* infection in swine has been problematic because the symptoms associated with trichuriasis are quite similar to other common swine gastrointestinal disorders. However, a 20 kDa ES glycoprotein has been identified in the cultured fluids of adult *T. suis*, and it is specifically diagnostic for *T. suis* infection in pigs. Western blots or enzyme-linked immunoabsorbent assays (ELISA) conducted using this antigen had no crossreactivity when conducted with sera from pigs infected with *Ascaris suum*, *Trichinella spiralis*, *Oesophagostomum dentatum*, or *Toxoplasma gondii*. Interestingly, the antigen was also diagnostic for *T. vulpis* infection in dogs, but did not crossreact with sera from dogs infected with *Toxocara canis*, *Ancylostoma caninum*, or *Strongyloides stercoralis* (66).

6. Secretory serine protease inhibitor

A 6.6 kDa secreted protease inhibitor was identified in *T. suis* extracts and culture fluids. Studies indicated that it has specificity for trypsin and chymotrypsin. It has been

suggested that the inhibitor may function in immunomodulation of host effector mechanisms by inactivating immune cell proteases involved in the inflammatory process (133).

7. Secretory chymotrypsin/elastase inhibitor

A 6.437 kDa protease inhibitor was purified from adult *T. suis* with specificity for chymotrypsin, pancreatic and neutrophil elastase, chymase and cathepsin G. It too has been suggested to function in immunomodulation of host effector mechanisms (134).

8. ESP antimicrobial activity

Cultured *T. suis* ESP from adult parasites demonstrated antimicrobial activity. Bacteria including *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli* and *Staphylococcus aureus* were sensitive to this activity in a dose-dependent manner. The activity was heat stable and showed resistance to pronase E and trypsin digestion. It is believed that the antimicrobial activity may play a role in *T. suis* survival during infection in the bacteria rich environment of the colon (3). In addition to characterizing this activity, Abner et al. demonstrated a dose-dependent cytotoxic response in ESP-treated cultured intestinal epithelial cells. Intestinal epithelial cells were exposed apically and basolaterally to *T. suis* ESP. Both treatment routes caused the transepithelial electrical resistance to be significantly reduced. This response suggested that *T. suis* ESP can induce mechanical damage at the site of worm attachment. Also, when swine intestinal epithelial cells were pretreated with ESP and then exposed to *C. jejuni* to examine the effects of ESP on bacterial invasion, it was found that intracellular *C. jejuni* were

significantly reduced. The antimicrobial activity was believed to be partially responsible for this response (2). Other studies also showed that *T. suis* ESP stimulated the production of IL-6 and Il-10 in undifferentiated and differentiated cultured swine intestinal epithelial cells (118). These findings suggest that *T. suis* ESP may play a role in immunomodulation and development of enteropathy in the live host.

Immune Response

Recently, gastrointestinal nematode infections and the host factors involved in immunity to nematodes have been studied more thoroughly. These studies are becoming especially relevant since certain nematode eggs (specifically *T. suis* ova) are being tested for possible use in treatment of Crohn's disease, and clinical studies have shown that exposure to *T. suis* reduces disease activity in patients with Inflammatory Bowel Disease (IBD) (41, 102, 145). There are few reports specifically pertaining to immune responses to *T. suis*. However, this area of study is evolving and more research is being conducted using *T. suis*-infected swine as a model system for human trichuriasis because of the similarities in human and pig digestive anatomy and physiology and parasite species (119). Studies have shown that swine infected experimentally and naturally with *T. suis* stimulate cytokine mRNA gene expression in local tissues of the proximal colon and in mesenteric lymph node (MLN) cells. At 35 days post-infection, IL-10 mRNA was significantly expressed in the MLN of the experimentally infected pigs as compared to the control pigs, and IL-12 gene expression was undetectable. However, IL-10 and IL-12 mRNA was significantly expressed in the MLN of pigs naturally infected with *T. suis* contaminated soil. This study demonstrated the ability to quantitate local cytokine gene

expression in mesenteric tissue using a competitive polymerase reaction (cPCR) assay (102). Studies have also been conducted to demonstrate the development of acquired immunity to *T. suis* infection in pigs. In one study, pigs were given a single challenge infection with *T. suis* and groups were killed each week for 7 weeks in a time course design. Pigs developed Th2 predominant adaptive responses as measured by mRNA expression of cytokines in the proximal colon that resulted in expulsion of the adult worms by 49 days after infection. These pigs also had a significant decrease in Th1 associated cytokines in these tissues. In another study, trickle inoculation was carried out by exposing pigs to multiple small doses of *T. suis* eggs over an eight week period (administering 250 infective eggs twice weekly), and during this time of observation, acquired immunity to *T. suis* developed in the infected pigs. The pigs given trickle infections in this study also displayed a noticeable decrease in worm fecundity and reduced weight gains along with the development of a more feeble resistance level than in pigs given a single challenge infection (119). Overall, these findings are evident of the significant progress being made in the advancement of *T. suis* immunological studies in spite of the limited reagents for investigating swine immune responses.

Swine experiments are quite expensive to conduct in laboratories and the availability of porcine reagents are limited. Therefore, most immunological studies have thus far been conducted in mice where *T. muris* is the infectious agent. The well-defined *T. muris*-mouse model system has been extensively used to study *trichuriasis* and host immune responses. Various studies have demonstrated that the immune responses elicited by *T. muris* infection are driven by a pattern of cytokines derived from two distinct $CD4^+$ helper T cell subsets (42, 49, 54, 152). Generally during chronic

gastrointestinal helminth infections, $CD4^+$ helper T cells are driven towards a polarized Th2 response that results in worm expulsion. The Th2 response is associated with the secretion of IL-4, IL-5, IL-9, IL-10, and IL-13 and the promotion of eosinophilia, mastocytosis, and the production of IgE and IgG1 which promotes resistance to nematode infection thereby triggering worm expulsion (54). Conversely, a Th1 response has also been shown to inhibit the protective immunity to *T. muris* infection, and induction of IFN-γ, IL-2, IL-12, lymphotoxin, and the production of IgG2a are characteristics of this Th1 response that promotes worm survival. There are various factors which influence the polarized $CD4^+$ T cell response and the course of an infection. Studies have shown that antigen dose, trickle infections, and co-infections play a role in determining the dominant immune response to intestinal nematodes (15, 103, 119, 120). These findings are also supported by the *in vivo* studies of Else et al. Here, Else et al. demonstrated parasite expulsion in a mouse strain that was normally susceptible to T. muris by the deletion of a key cytokine gene of the Th1 pathway. They experimentally infected interferon gamma (IFN-γ) deficient AKR mice, which are normally susceptible, with *T. muris* and showed that these mice expelled the parasite proving a role for IFN-γ in the establishment of chronic helminth infection. In addition, they blocked IL-4 function in normally resistant mice (BALB/K) and inhibited IL-4 dependent-*T. muris* resistance to infection. However, when IL-4 was conversely administered to the susceptible AKR mice late in the infection, the adult worms were expelled (42). This study shows that the use of mouse strains with different genetic backgrounds has led to the conclusion that genetic variability plays a role in influencing the $CD4^+$ T cell-directed immune response (42). Furthermore, there have been similar *in vivo* helminth model experiments conducted which included

Heligmosomoides polygyrus and *Nippostrongylus brasiliensis*, and similar findings were identified: 1. Prolonged parasite survival is associated with Th1 responses and 2. Host protection is associated with Th2 responses (49, 152).

TNF-α is also involved in the mechanisms of protective immunity to *T. muris* infection. Studies have shown that administering or blocking TNF-α does effect the outcome of host protection, but has no effect on the magnitude of the Th2 responses, thereby suggesting that TNF-α may play a role in regulating Th2 effector function (8). Likewise, BALB/c IL-4 knockout (KO) mice were treated with anti-TNF-α mAb or rat IgG intraperitoneally, and worm expulsion increased considerably by day 35 with 43% worm clearance. Th2 responses were not altered (IL-5, IL-9, IL-10 and IL-13 production remained). Prior results had indicated that blocking IL-13 with a soluble IL-13R fusion protein in the absence of IL-4 completely abrogated host protection (14). Therefore these additional findings confirmed that IL-13 plays a role in worm expulsion in the absence of IL-4 and TNF-α. This study suggests that IL-4 is not independently promoting *T. muris* resistance, but IL-13 and TNFα are required as well (8).

Further immunological studies provided the evidence that support the role of IL-18 in susceptibility to gastrointestinal nematode infection. IL-18 is a pleiotropic cytokine and has different roles depending on where and when it is induced. It plays a role in negatively regulating Th2 cytokine secretion in *Trichuris* infection. More specifically, IL-18 plays a role in downregulating IL-13, thereby promoting host susceptibility to *T. muris* infection, and this response is independent of IL-12 and IFN-γ (59).

Likewise, IL-10 is critical in the polarization of Th2 responses to *T. muris* infection and survival. Mice lacking IL-10 displayed significant morbidity and mortality

possibly due to the overproduction of systemic IFN-γ and TNF-α. It appears that IL-10 is an immunoregulatory cytokine, and plays a critical role in nematode resistance (140). However, further evaluations are needed to determine the mechanisms involved in possible IL-10 dependent down regulation of Th1 and Th2 responses associated with *T. trichiura* infection (150). Early results in studies on immune regulation in infectious diseases suggest that T-regulatory (T-reg) cells also produce significant amounts of IL-10 and lead to downregulation of detrimental proinflammatory responses.

It has been observed that the well-defined *T. muris*-mouse model system has provided critical information towards further understanding the mechanisms involved in the host immune response to *Trichuris* infection. On the other hand, there have been very few experiments done in humans pertaining to *T. trichuris* infection and host immune responses. However, it is importance to discuss the information that is known whether it be obtained through the results of laboratory experiments or epidemiological observation. It has been shown that children chronically and heavily infected with *T. trichiura* develop a dysentery syndrome. In the colons of these infected children, a local immediate hypersensitivity response is elicited resulting in severe growth retardation and bloody diarrhea with a high presence of mucus cells. IgE is secreted in response to this infection along with significant mast cell production, but these responses have not been shown to play a significant role in worm expulsion (34). Furthermore, a study was conducted to observe the effects of age and infection intensity on cytokine and antibody production in humans infected with *T. trichiura*. These studies indicated that the intensity of *Trichuris* infection was affected by age, and younger children (ages 4-7) were more heavily infected than adults. Also, adults that were less heavily infected showed a greater IgE

response than infected children, thereby implying an association of significant IgE production with the level of protection. Therefore, *Trichuris* host resistance is age-related. This phenomenon was also demonstrated and explained in *T. suis*-infected pigs, and controlled experimental evidence was presented (121). Here, there was positive correlation between IgG2 production and infection intensity, but no significant relationship demonstrated between cytokine production and age or infection intensity (45). Unfortunately, at this time no reagents were available for measuring IgE production in swine.

Overall, most *Trichuris* studies have been conducted using the well established *T. muris*-mouse model system. This has really promoted advances in understanding the mechanisms involved in clinical trichuriasis. Likewise, the *T. suis*-swine disease model system has also contributed to the growing knowledge of *Trichuris*-host interaction. With the use of these models and other resources, chronic gastrointestinal nematode infection will be better understood and more preventive measures can be taken to control the outcome of these infections.

III. Rationale

Because of the increasing health and economical impact that food-borne diseases are causing throughout the world, greater efforts are being executed towards elucidating the mechanisms of pathogenesis related to the source of food-borne illnesses (96). The human gastrointestinal tract is colonized by approximately 500 species of commensal bacteria which can cause disease when normal anatomic or immunologic defenses are abrogated (146). Bacteria that normally reside in cattle, swine, and poultry as commensals that sometimes cause invasive infection in animals and humans (6, 7, 33). There have been reports of overlaps between serotypes of *C. jejuni* found in humans, poultry, and cattle which suggest that foods of animal origin may play a significant role in transmitting *C. jejuni* to humans (7). *C. jejuni* is a food-borne pathogen that has been identified throughout the world as one of the leading causes of human gastroenteritis (6, 7). There have been significant advances in understanding the mechanisms by which *C. jejuni* causes disease, but few immunological studies have been pursued. This is especially true of those using the naturally occurring animal model systems and cultured primary intestinal epithelial cells. *C. jejuni* is commonly found in the colon of conventionally-reared pigs without signs of pathology. However, infection and pathology result in the presence of the swine whipworm, *T. suis* (103). We hypothesized that the severe pathology observed during this interaction mimics the clinical disease of *Campylobacteriosis* in swine and humans. Therefore, to address this hypothesis, I studied Th-1 cytokine responses of primary swine intestinal cells after single or dual challenge infections with *C. jejuni* and *T. suis* infection.

Preliminary studies suggested that *T. suis* infection may play a significant role in *C. jejuni* infection in weaning-aged swine (100, 101, 103). Additional *in vivo* studies using this existing swine model contributed to the understanding of Th2 cytokine responses following dual infection (Parathasarathy, dissertation, 2004). In addition, a primary swine intestinal epithelial cell line was also used to observe the expression of Th2 cytokines induced by *C. jejuni* and/or *T. suis* ESP (Parathasarathy, dissertation, 2004). Likewise, several other studies were done to observe *C. jejuni* colonization and localization in the distal colon and to measure localized cytokine expression following rectal or oral *C. jejuni* and *T.suis* challenges (Jones, dissertation, 2005).

Hence, this existing swine disease model has provided a better understanding of some host immunomodulatory responses triggered by *C. jejuni* and *T. suis* infections. However, there are still unanswered questions, and the model described herein will permit further study to characterize the immune responses that are possibly triggered by interactions between host factors of *C. jejuni* and/or *T.suis* which may have an effect on the development of enteric pathogenesis.

Hypothesis

The central hypothesis of this work is that *C. jejuni* interaction with the intestinal mucosal epithelium stimulates an acute proinflammatory cytokine response. A related hypothesis is that *T. suis* exposure alters this proinflammatory cytokine response thereby promoting *C. jejuni* infection.

Short-term goals

1. **To determine whether *C. jejuni* and/or *T. suis* infections induce a Th1 proinflammatory cytokine response *in vivo***
2. **To determine if cultured swine intestinal epithelial cells secrete proinflammatory cytokines (IL-8, TNF-α and IL-1-β) following *C. jejuni* exposure**
3. **To determine if recombinant swine IL-8, TNF-α or IL-1-β affects *C. jejuni* adherence, invasion, and/or the transepithelial electrical resistance (TER) of undifferentiated swine intestinal epithelial cells with persistent exposure, *in vitro*.**
4. **To determine if there is a significant, synergistic proinflammatory cytokine response triggered by *C. jejuni* and *T. suis* infection**

In summary, the goal of this dissertation work was to determine the proinflammatory cytokine response (IL-8, TNF-α and IL-1-β) to *C. jejuni* and *T.suis* infection, and determine whether IL-8, TNF-α and IL-1-β expression plays a significant role in promoting campylobacteriosis in swine that were dually infected with *C. jejuni* and *T.suis*.

Chapter 2 summarizes *in vivo* studies on the proinflammatory cytokine responses (IL-8, TNF-α and IL-1-β) elicited by *C. jejuni* and *T. suis* infection in swine. Four to five-week-old pigs were orally infected with *C. jejuni*, *T. suis* embryonated eggs, or both. There were 2 experimental challenges; they were conducted as a short-term challenge (Day 0-Day2) or as a long-term challenge (Day 0-Day 23). Experimental human studies have shown that pro- and anti-inflammatory cytokine levels can be detected in stools of

patients with *Shigella* infection or with inflammatory bowel disease (9, 132, 139). These findings support the hypothesis that the host response to invasion, injury, or infection is driven by the release of a series of cytokines. Therefore, we measured the secreted proinflammatory cytokine levels from the feces of pigs orally infected with *C. jejuni*, *T. suis* embryonated eggs, or both. The potential roles of these cytokines with significant levels of expression are discussed.

Chapter 3 summarizes *in vitro* studies on the proinflammatory cytokine responses (IL-8, TNF-α and IL-1-β) from swine intestinal epithelial cells infected with *C. jejuni*. This chapter also summarizes the specific roles of IL-8, TNF-α and IL-1-β in *C. jejuni* adherence and/or invasion of a primary swine intestinal epithelial cell line that mimics the basal epithelial crypt cells where *Campylobacter* resides. Studies have shown that IL-8, TNF-α and IL-1-β are proinflammatory cytokines secreted by cultured intestinal epithelial cells following bacterial stimulation. Also, it is known that cytokines are likely to function as a network and rarely as isolated mediators. It is suggested that during infection, cytokine networks are likely the main controlling elements in both inflammation and immune reactions. The cytokine networks are also believed to control the interactions of Th1 and Th2 lymphocytes . However, in order to elucidate the proinflammatory mechanisms of the cytokine networks, it is also relevant to understand the isolated cytokine mediator responses due to infection. Therefore, we measured secreted IL-8, TNF-α and IL-1-β protein from *C. jejuni*-infected, undifferentiated swine intestinal epithelial cells (IPEC-1). We also pretreated these cultured primary cells with recombinant swine IL-8, TNF-α and IL-1-β to determine whether these cytokines play a significant role in *C. jejuni* adherence, invasion, and/or the transepithelial electrical

resistance (TER) with persistent exposure. The nature of the responses from undifferentiated IPEC-1 cells and the potential roles of IL-8, TNF-α and IL-1-β in *C. jejuni* adherence and/or invasion are discussed.

Chapter 4 summarizes the previous chapters and elaborates on the proposed swine disease model and the possible mechanisms of pathogenesis with *C. jejuni* and *T. suis* in swine. It also covers important future directions for this research area.

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Chapter 2: "*Trichuris suis*-mediated cytokine dysregulation in concurrent *Campylobacter jejuni* and *Trichuris suis* infection"

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Abstract

Interactions between helminths and bacteria have been documented to induce gastrointestinal disease in swine. It has been shown that infections with *Trichuris suis* and *Campylobacter jejuni* in swine produce a mucohemorrhagic enteritis which appears clinically similar to human campylobacteriosis. The mechanism by which this synergistic relationship causes disease and pathology is unclear. Cytokine dysregulation is believed to play a significant role in this dual infection. We hypothesized that *C. jejuni* interactions with the intestinal mucosa stimulates an acute proinflammatory cytokine response, and that concurrent *T. suis* infection alters this pro-inflammatory cytokine response by inducing anti-inflammatory cytokines, thereby promoting *C. jejuni* infection. To test this hypothesis and determine if changes in mRNA and protein expression of pro- and anti-inflammatory cytokines are associated with the pathological lesions observed in this infection, we measured cytokine expression in the jejunum, proximal and distal colon tissues and feces of infected pigs using Real-Time polymerase chain reaction (PCR) analysis and enzyme-linked immunoabsorbent assays (ELISA). We used the following experimental designs: (1) a short term challenge experiment (day 0–day 2) and (2) a long term challenge experiment (day 0–day 23). For both experiments (short and long term challenges), four-week-old, weaned pigs were divided into 5 treatment groups: Group 1, *C. jejuni*; Group 2, *C. jejuni* and *T. suis*; Group 3, *T. suis*; Group 4, noninvasive *E. coli*

DH5-α; and Group 5, sterile milk (uninfected control). In the short term challenge experiments, on day 0, fecal samples were collected from all pigs, and the separate groups of pigs were then orally inoculated with either *C. jejuni* (2.0×10^8 cfu), *T. suis* (2500 embryonated eggs), *C. jejuni* and *T. suis*, noninvasive *E. coli* DH5-α (2.0×10^8 cfu) or sterile milk (uninfected control). Fecal samples were collected again on days 1 and 2 post-inoculation. In the long term challenge experiments, fecal samples were collected from all pigs on day 0, and the pigs in Groups 2 and 3 were given 2500 embryonated *T. suis* eggs orally while all other pigs received sterile milk. On day 21, fecal samples were first collected. Then, pigs assigned to Groups 1 and 2 were inoculated orally with 2.0×10^8 cfu of *C. jejuni*, and the pigs in Group 4 were given 2.0×10^8 cfu of *E. coli* DH5-α, while the remaining pigs were given sterile milk. On day 22, fecal samples were collected from all pigs. On day 23, fecal samples were taken for ELISA analysis of cytokine expression, all pigs were euthanized, and tissue samples were taken for Real-Time PCR analysis of cytokine expression.

Adult *T. suis* reside in the proximal colon and dominated immune responses in this site. The presence of *T. suis* in the colon also influenced responses in the jejunum. In the tissue samples taken on day 23 of the long term challenge experiments, *T. suis* stimulated increased expression of IL-13 mRNA in the proximal colon, while expression of IL-8, MCP-1, and IL-12 were decreased in pigs infected with *T. suis* alone or concurrently with *T. suis* and *C. jejuni*. Pigs given *C. jejuni* alone produced few responses here in the proximal colon. In the distal colon, increased expression of IL-1-β, IL-6, IFN-γ and IL-13 was measured in response to *T. suis* alone, while pigs dually infected with *T. suis* and *C. jejuni* had no significant response similar to that seen in pigs

given *C. jejuni* alone. In the jejunum, *C. jejuni* infection stimulated increased expression of MCP-1, TNF-α, IL-12p40, IFN-γ, IL-4, and IL-10, while pigs infected with *C. jejuni* and *T. suis* concurrently had no measurable proinflammatory cytokines and showed decreased expression of GM-CSF. These data suggest that *Campylobacters* or *Campylobacter*-like bacteria stimulate a proinflammatory response mainly in the jejunum and that *T. suis* down regulates proinflammatory responses in that site in concurrent infections with *C. jejuni*. *T. suis* also down regulates proinflammatory responses in the proximal colon, both in single infections and in concurrent infections with *C. jejuni*. At 48 hours after *C. jejuni* challenge, pigs showed few responses in the colon to this bacteria. Conversely, *T. suis* dominated responses in the colon in the long term experiments that lasted 23 days, but had little effect in the short term experiments when pigs were sacrificed 48 hours after challenge.

Also, we measured IL-8, TNF-α, IL-1-β, IL-4, IL-6, and IL-10 concentrations in the fecal supernatants of pigs in all groups in the short- and long-term experiments. IL-8, TNF-α, IL-1-β, IL-4, IL-6, and IL-10 were detected in the feces of all pigs in both experiments. However, IL-8, TNF-α, IL-1-β, IL-4, IL-6, and IL-10 were not significantly affected by the administered treatments in the short term challenge experiments. In the long term challenge experiments, neither IL-8, TNF-α, IL-4, IL-6 nor IL-10 expression was affected in response to the treatments. However, IL-1-β was significantly decreased in the pigs given *T. suis* alone by 23 days post-infection. In addition, the pigs dually infected with *C. jejuni* and *T. suis* had moderately decreased IL-1-β expression. In both experiments, we found that the pigs given *C. jejuni*, *T. suis*, or *T. suis* and *C. jejuni* had clinical diarrhea post-infection. The pigs that were given *T. suis*

alone or *T. suis* and *C. jejuni* had more frequent, more severe diarrhea than the pigs that received *C. jejuni*. Some of the *C. jejuni*-infected pigs had mild diarrhea. The pigs given *E. coli* DH5-α or milk only showed no signs of disease.

In addition, we found that the two distinct methodologies (Real-time PCR and ELISA for quantitating fecal cytokines) for assessing innate and adaptive responses did not show comparable results for cytokine expression in this study. The measurements of cytokines in fecal supernatants were less sensitive and more prone to problems with degradation of the measured product than the RT-PCR applied to tissues. However, despite the issues with the ELISA assay when applied to fecal supernatants, some changes in cytokine production could be observed. Using ELISA, RT-PCR, clinical assessment and gross and histopathology, we were able to conclude that *T. suis* infection likely contributes to the down regulation of pro-inflammatory cytokines in singly or dually-infected pigs and that *T. suis* is mainly responsible for the frequent, severe diarrhea observed in pigs infected concurrently with *C. jejuni* and *T. suis* or *T. suis* only. The overall findings suggest that *T. suis* interaction with the intestinal mucosa is likely mediating the cytokine dysregulation observed and may be playing a role in promoting a more conducive environment for secondary infection by *Campylobacter* species, other related *Campylobacter*-like bacteria, or other opportunistic enteric bacteria during a natural, concurrent infection.

Introduction

Throughout the world *Campylobacter jejuni* is known to be one of the leading bacterial causes of diarrheal disease in humans (6). The enterocolitis caused by this infection commonly occurs following the consumption of improperly cooked pork, poultry or beef; unpasteurized cow's milk; or contaminated water (6, 72). *C. jejuni*, *C. coli*, and *C. lari* can be found as commensals in the intestinal tracts of animals and thus can serve as sources of food contamination (6, 7, 26, 72). Recent studies have shown that there is a significant incidence of *C. jejuni* in the intestinal tract of pigs raised on integrated commercial hog farms. The mean incidence of *C. jejuni* was 76.8% in gilts, 87% in pregnant sows, and 31.7% in newborn piglets (78). This finding suggests that pigs are significant reservoirs of *C. jejuni.*

By 1991, approximately 45% of the US swine farms were infected with *T. suis*, a whipworm intestinal nematode primarily found in the cecum and proximal colon (Bliss, 1991). Manifestations associated with infection include weight loss, dysentery, anemia, and anorexia (18). Typically, pigs with light *T. suis* infections present mild clinical signs and can be treated with anthelminthics to induce nematode expulsion (Jones *et al.*, unpublished data). However, pigs with severe natural infections often die (24). These conditions contribute to economic losses in the swine industry. Reports from 1991 indicated that pork producers directly and indirectly lost approximately $1 billion dollars annually as a result of swine internal parasites (24). It was suspected that much of the pathology associated with this *T. suis* infection was due to syncytial tunnels created by larvae burrowing into the intestinal epithelium, which contributed to breakdown of the

epithelial barrier and secondary invasion by intestinal microflora, thereby amplifying the severity of the clinical signs (18). Later findings supported this hypothesis by demonstrating a synergism between *T. suis* and the microbial flora of the large intestine, causing dysentery in pigs (65). This phenomenon has greater significance because it is also known that the human whipworm, *T. trichiura*, affects an estimated one billion people globally, causing chronic disease (73).

Since pigs are similar to humans with respect to digestive anatomy and physiology, they can be used as a suitable animal model for studying bacterial and helminth infections that cause disease in humans. There have been several attempts to develop an animal model suitable for the study of immunological responses due to *Campylobacter* infection. Unfortunately, some of these models have proven less suitable for mimicking human campylobacteriosis. Some of these reported models using ferrets, rabbits, hamsters, dogs, chickens, and mice were not suitable because the investigators modified the gut flora in order to facilitate *C. jejuni* infection. Not only are some of these animal models altered so that they do not reflect natural infection, they are also expensive and lack anatomical and physiological similarities to the human colon (5, 12, 21, 43, 62, 65, 73). Unlike other studies, the pig *C. jejuni/T. suis* model system established by Mansfield and colleagues reliably reproduced the clinical symptoms associated with human *Campylobacter* infection (50). They have confirmed through several studies that *C. jejuni* is commonly found in the colon of conventionally reared pigs without signs of pathology. However, in the presence of *T. suis*, infection and pathology result (49-51). With the increasing awareness of concurrent bacterial/nematode interactions, the identification and characterization of the mechanisms by which these enteric pathogens

concurrently cause disease will be beneficial from both animal and human health perspectives. Studies will enable researchers to address such questions as why some animals or individuals develop disease due to such synergisms and others do not. For example, in one case, a recent study has shown that concurrent enteric helminth infection in mice can reduce helicobacter-induced gastric atrophy (34). Other investigators are also now suggesting that *T. suis* is a safe and effective treatment of inflammatory bowel disease, ulcerative colitis, and Crohn's disease (69, 70).

The mechanisms by which *C. jejuni* causes disease in humans are not clearly understood. However, it is believed that host cytokines play a pivotal role in eliciting immune responses during enteric infection. A localized acute inflammatory response is typically associated with *Campylobacter* enterocolitis and is believed to play an important role in the clinical symptoms elicited by enteric invasion, injury, or infection. Bacterial-host cell interactions have been studied using multiple well-established *in vitro* models, and various bacterial and host cell components have been identified as playing significant roles during infection (38, 39, 53). However, the *in vitro* model systems do not completely mimic the true physiologic conditions and the responses seen in animal model systems or, more importantly, mimic the clinical manifestations associated with human campylobacteriosis. Therefore, at this time it is necessary to use, both a cultured intestinal epithelial cell model and an animal model for more thorough studies of the overall effects of cytokine regulation in this disease.

Experimental human studies have shown that pro- and anti-inflammatory cytokine levels can be detected in stools of patients with inflammatory bowel disease (57, 66). It has also been shown that significant levels of proinflammatory cytokines (TNF-α, IL-1-

β, IL-1ra, IL-6, IL-8, and GM-CSF) have been detected in stool samples from patients with *Shigella* infection (57, 63). The findings of this *Shigella* study showed that the correlation of cytokine levels with severity of disease was significant; cytokine levels were higher in the stools of patients with severe disease as compared to the stools of patients with mild disease. These findings showed that an array of cytokines, particularly pro-inflammatory cytokines, play a role in initiating and amplifying acute mucosal inflammatory response following epithelial barrier invasion, injury, or infection. Hence, these reports showed that measurement of stool cytokines can be a reliable and noninvasive means of understanding the disease status in the intestine (66).

We hypothesized that *C. jejuni* infection elicits an innate immune response by up-regulating pro-inflammatory cytokines in weaned, conventionally-reared pigs, but during concurrent *T. suis* infection, anti-inflammatory cytokines are triggered that can down-regulate pro-inflammatory cytokines, thereby promoting *C. jejuni* infection. Therefore, the aim of this study was to detect and quantify pro- and anti-inflammatory cytokine mRNA levels in intestinal tissue samples and protein levels in the feces of pigs infected with *C. jejuni* and *T. suis* and to determine if the two cytokine analyses (ELISA on fecal supernatants and RT-PCR on tissues) were comparable and useful in correlating cytokine dysregulation with disease. We also documented clinical signs such as the occurrence of diarrhea during the infection as it related to the severity of disease. This study was designed based on previous evidence suggesting a synergistic effect involved in these infections and the differential development of pathology induced by *C. jejuni* and *T. suis* in the colon of naïve, germ-free pigs (49, 50). The mechanism by which this concurrent infection causes disease similar to human campylobacteriosis is unknown. However, we

do know that *T. suis* causes direct mechanical damage by invading the basal crypt epithelial cells of the proximal colon and that *C. jejuni* invades through the epithelium and proliferates in the lamina propria and submucosa surrounding the site of worm attachment (50). It is believed that these modes of action play a significant role in the infection and observed disease in these dual infections. Also, it is suspected that cytokine-mediated damage triggered when these pathogens breach the epithelium may also play a significant role in the pathology associated with these infections. Therefore, this investigation was important in determining the role of pro- and anti-inflammatory cytokine responses in concurrent infections with *C. jejuni* and *T. suis* in pigs.

Materials and Methods

Animals

In this study, the pigs were divided into 5 treatment groups (Table 2.1). In two replicate experiments each, there were 4 or 6 pigs per treatment group in the short term challenge experiments (2 days) and 5 or 6 pigs per treatment group in the long term challenge experiments (23 days). The four-week-old, weaned, conventionally-reared Duroc/Yorkshire/Landrace piglets were obtained from the Michigan State University Swine Farm. They were transported to the University Research Containment Facility in sterile dog cages and housed in 5 adjoining rooms in identical large isolation units to reduce cross contamination. Feed and water were provided to the pigs *ad libitum*, and they were handled under identical management conditions in accordance with university and National Institutes of Health guidelines for humane animal use; protocol number 06/01-096-00.

Bacterial strains, growth conditions, and experimental inoculum preparation for swine challenge

C. jejuni 33292 was grown at 37°C, 5% CO_2 on Bolton agar (Oxoid Inc., Basingstoke, Hampshire, England) plates containing 5% sheep's blood. *E. coli* DH5-α was grown on Bolton agar plates without sheep's blood at 37°C. The bacterial were grown for 18–20 hours, harvested with RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) and suspended in sterile milk at a final concentration of 1×10^8 cfu/ml.

Bacterial strains and growth conditions for enzyme-linked immunosorbant assay (ELISA) and 16S rRNA PCR analysis

For ELISA, *C. jejuni* NCTC 11168, *C. lari*, *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, and *Arcobacter butzleri* were grown on Tryptose soy agar (TSA)(Oxoid Inc., Ontario, Canada) plates with 5% sheep's blood at 37° C in Gas Pak jars with a Campy*Gen* pack for 48 hours. *Helicobacter hepaticus* was grown on Tryptose soy agar (TSA)(Oxoid Inc., Ontario, Cananda) plates with 5% sheep's blood for 5 days in a Gas Pak jar without catalyst at 37° C evacuated to -20 mmHg and then filled with a gas mixture, N_2, H_2, and CO_2 (80:10:10). The bacteria were harvested from agar plates by resuspending the organisms in sterile 10 mM phosphate buffered saline (PBS). As described below, protein was extracted from the bacterial suspensions using the Pierce (Rockford, IL) B-PER® midi-scale protein extraction protocol according to the manufacturer's instructions and later used in the ELISA.

For 16S rRNA PCR analysis, *Campylobacter* DNA extraction was performed as described by Ausubel *et al* (8). The *Helicobacter hepaticus* DNA was kindly provided by Dr. Vincent Young at the Food Safety and Toxicology Center at Michigan State University, East Lansing, MI.

Embryonated *T. suis* eggs inoculum

Adult whipworms were isolated from the colonic mucosa of experimentally infected pigs, and embryonated eggs were collected and prepared as described previously(40). *T. suis*-infected pigs were anesthetized via intramuscular injection of 4.4 mg/kg Telazole/2.2 mg/kg xylazine (MSU Veterinary Pharmacy, East Lansing, MI) and euthanized with an intravenous injection of 86 mg/kg sodium pentobarbital (Fatal Plus®,

Vortech Pharmaceuticals, Dearborn, MI). Adult worms were harvested from the proximal colon using forceps and cultured at 37°C, 5% CO_2 for approximately 10 days or until the worms showed no sign of movement. Throughout the culturing process, medium with eggs was collected and washed by centrifugation 2 or 3 times at 10,000 rpm for 5 minutes to remove excess debris. The *T. suis* eggs were suspended in sterile MilliQ water, placed in a vented tissue culture flask, and incubated at 34°C, 5% CO_2 for nineteen days to induce embryonation. On day 19, porcine bile extract (Sigma Chemical Co., St. Louis, MO) was added to the flask at 10 ug/ml and incubation was continued for an additional 3 to 5 days. Each day the eggs were examined microscopically for the maturation of the L1 stage nematode, which was detected as movement of the larva inside the egg. The development and movement of the worm indicated that embryonation had taken place. The embryonated eggs were collected and centrifuged at 10,000 rpm for 10 minutes, resuspended in sterile water in tissue culture flasks, and stored at 4°C in the dark until inoculation.

Animal inoculation

All pigs were inoculated with treatments as indicated in Table 2.1 by oral gavage using a sterile 12 gauge steel ball feeding needle placed over the back of the tongue. Prior to the experimental challenge, at each time point, and immediately prior to euthanasia, all pigs were rectally swabbed to test for the presence of *C. jejuni*. The rectal swabs were transported in Cary Blair medium (Difco Laboratories, Detroit, MI) on ice to the laboratory for *Campylobacter* isolation and identification. Also, at each time point, some of the collected fecal material was microscopically examined to detect the presence

of *T. suis* eggs. All inocula were prepared using nonfat dry milk reconstituted in sterile MilliQ water. *T. suis* embryonated eggs were examined microscopically and enumerated using a hemacytometer. The inoculum for the pigs that received *T. suis* was 2500 embryonated eggs suspended in sterile milk in a total volume of 2.0 ml. Eighteen to twenty hour cultures of *C. jejuni* 33292 or *E. coli DH5-α* were harvested from plates with RPMI 1640 and suspended in sterile milk at a final concentration of 1×10^8 cfu/ml. The pigs that received *C. jejuni* 33292 or *E. coli DH5-α* were inoculated with 2.0×10^8 cfu of bacteria in a total volume of 2.0 ml. Control pigs were inoculated with 2.0 ml of sterile milk.

Experimental design

These experiments were conducted as a short term challenge (2 days) or a long term challenge (23 days). For each of the experiments, the pigs were divided into 5 treatment groups for inoculation (Table 2.1): Group 1, *C. jejuni*; Group 2, *C. jejuni* and *T. suis*; Group 3, *T. suis*; Group 4, *E. coli* DH5-α; and Group 5, uninfected milk control. At the end of each experiment, all pigs were euthanized with an intravenous injection of 86 mg/kg sodium pentobarbital (Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI) on day 2 or 23 following fecal sampling. Following euthanasia on day 2 (from 2nd repetition of short term challenge only) or day 23, blood and plasma samples were obtained from all pigs and stored at -80°C until assayed. Also, following euthanasia on day 23 of the long term challenge only, tissue samples were taken from the jejunum, proximal colon, and distal colon of all the pigs and stored at -80°C until assayed.

Short term challenge (2 days)

Four pigs per group were used in the first experiment, and 6 pigs per group were used when the experiment was repeated for a total of 10 pigs per group. On day 0, prior to inoculation, feces were collected from all animals. Next, the pigs in each group were inoculated with 2.0 ml of the designated treatment. At 24 and 48 hours post challenge, fecal samples were collected from all animals. All pigs were euthanized at 48 hours post-challenge following fecal sampling. Blood samples were obtained after the pigs were euthanized to measure total anti-*Campylobacter* IgG in the plasma (samples taken only from the 2nd repetition of experiment). Table 2.2 illustrates the inoculation and fecal sampling protocol.

Long term challenge experiment (23 days)

Five pigs were used in the first experiment, and 6 pigs per group were used when the experiment was repeated for a total of 11 pigs per group. On day 0, prior to inoculation, feces were collected from all experimental animals. Next, each of the pigs in group 2 and 3 received 2500 *T. suis* embryonated eggs, and the pigs in groups 1, 4, and 5 were given sterile milk. On day 21 post-inoculation, fecal samples were collected from all animals. Then, each of the pigs in groups 1 and 2 was inoculated with 2×10^8 cfu *C. jejuni*, and the pigs in group 4 were given 2×10^8 cfu *E. coli* DH5-α. The pigs in group 5 received sterile milk. On days 22 and 23, feces were collected from all experimental animals. All pigs were euthanized on day 23 following fecal sampling, and tissue samples were collected as described below from the jejunum, proximal colon, and distal colon of all the pigs. Blood samples were also obtained after the pigs were euthanized to

measure total anti-*Campylobacter* IgG in the plasma. Table 2.3 illustrates the inoculation and fecal and tissue sampling protocol.

Sample collection

All fecal samples were collected in sterile 25 ml polystyrene tubes and placed on ice to be transported to the laboratory for processing using the fecal extraction procedure described below; fecal samples were stored at -80°C until analysis. Blood was collected by venipuncture into sterile red top tubes, and serum was removed and stored at 4°C and centrifuged; plasma was stored at -80°C until analysis.

"To eliminate cross contamination, a separate set of sterile dissection instruments was used for each group. For sample collection, the abdomen was opened by a midline incision and the small and large intestines were exteriorized. Full thickness samples of the jejunum, proximal colon, and distal colon were collected using sterile scissors and forceps. One set of samples was fixed in 3.7% formaldehyde for histology. A second set was placed in cryovials and snap frozen in liquid nitrogen for nucleic acid isolation." (Jones, dissertation, 2005, reprinted with permission)

Isolation and identification of *C. jejuni* from feces and tissue samples

Several assays were used to determine if pigs were free of *C. jejuni* prior to inoculation and to monitor the presence of the inoculated strain post-inoculation. The isolation and identification of *C. jejuni* from feces was conducted using previously described methods (31). Briefly, prior to inoculation and immediately prior to euthanasia, all pigs were tested for the presence of *C. jejuni* by culture of feces from

rectal swabs. The rectal swabs were streaked onto Preston selective agar (Oxoid Inc., Basingstoke, Hampshire, England) and incubated at 42°C, 5% CO_2 in humidified air for 48 hours. Isolated colonies were tested by PCR using primers specific for the quinolone resistance-determining region (QRDR) of the *gyrA* gene of *C. jejuni* (75).

"Tissue samples were also taken from each segment of the intestine of each pig to test for the presence of *Campylobacter* organisms. Total DNA was isolated from the tissue using a Qiagen DNEasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The DNA was quantified spectrophotometrically (Beckman model DU530, Fullerton, CA), and PCR was used to amplify the *C. jejuni* QRDR target from 500 ng of the total DNA using primers JL238 and JL239 (75). Electrophoresis, ethidium bromide staining, and UV illumination were used to visualize the results as previously described. A Restriction Fragment Length Polymorphism (RFLP) assay developed to detect and distinguish *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* by analyzing a portion of the 23S rRNA gene was used to identify thermophilic *Campylobacters* in the animals (31). The target DNA was amplified using primers THERM1 and THERM4. Electrophoresis, ethidium bromide staining, and UV illumination were used to visualize the results as previously described" (31)(Jones, dissertation, 2005, reprinted with permission).

ELISA for measuring total IgG in pig plasma

We developed an indirect ELISA protocol to measure total IgG in the plasma of the pigs to determine if there were cross-reacting antibodies against the challenge-dose *C. jejuni* or *C. jejuni*-related bacteria (ϵpsilon proteobacteria). The *C. jejuni* antigen was

prepared using the Pierce (Rockford, IL) B-PER® midi-scale protein extraction protocol as given by the manufacturer, and the Bradford assay was used to determine and optimize the protein concentration. The antigen concentration was adjusted by diluting the antigen 1:5000 to a final concentration of 1.9 ug/ml in 10 mM phosphate buffered saline (PBS), pH 7.4. 96-well Nunc MaxiSorp plates (Fisher Scientific, Hanover Park, IL) were coated with the antigen/coating solution at 100 ul/well and stored overnight at 4°C. Following overnight incubation, the antigen/coating solution was removed, and blocking buffer (3% BSA solution in PBS (10 mM, pH 7.4) containing 0.05% Tween 20) was added to the plates at 200 ul/well and incubated overnight at 4°C. Plates were washed 4 times with wash buffer containing 100 mM PBS, pH7.3, 0.05% Tween-20. The pig plasma samples served as the primary antibody. All plasma was diluted 1:10 in blocking buffer, added to the plates at 100 ul/well, and incubated at room temperature for 1 hour. Plates were washed 4 times with wash buffer containing 100 mM PBS, pH7.3, 0.05% Tween-20. The conjugate antibody (Biotin-SP-conjugated AffiniPure F $(ab')_2$ Fragment Goat Anti-Swine IgG (H+L); Jackson ImmunoResearch Labs, Inc., West Grove, PA) was diluted 1:20,000 in blocking buffer and used as a secondary antibody at 100 ul/well and incubated at room temperature for 1 hour. Plates were washed 4 times with wash buffer containing 100 mM PBS, pH7.3, 0.05% Tween-20. The optimal titers for the primary and secondary antibodies were determined from preliminary experiments. The color reaction was produced using extravidin peroxidase (Sigma, St. Louis, MO) diluted 1:2000 in blocking buffer and incubated for 1 hour at room temperature at 100 ul/well. Plates were washed 4 times with wash buffer containing 100 mM PBS, pH7.3, 0.05% Tween-20. TMB substrate (Sigma, St. Louis, MO) was warmed to room temperature,

added to plates (100 ul/ml) and incubated at room temperature for ~10 minutes or until color changed. Immediately, the color reaction was stopped by adding 100 ul/well of 2N H_2SO_4. A microplate reader (EL800 Universal Microplate Reader, Bio-Tek instruments, Winooski, Vermont) was used to measure the absorbance at 450 nm, and the total IgG in each sample was quantified using KCjunior® software (Bio-Tek instruments, Winooski, Vermont).

The ELISA for measuring total IgG in pig plasma was repeated as described above using protein extracted from *C. jejuni* and various related bacteria (*C. lari*, *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, *Arcobacter butzleri*, and *Helicobacter hepaticus*). The extracted protein from these bacteria was used as bacterial antigen in this assay to determine if the pigs were exposed to *C. jejuni*-related bacteria prior to the experiments. The plasma samples tested were taken from 8 experimentally *C. jejuni*-infected pigs (randomly chosen from the short and long term challenge experiments) to represent the known positive control. Plasma samples from two newborn piglets (< 6 hours old) from the MSU swine farm were used as negative controls. We also used plasma samples from 6 control pigs that were given milk only throughout the challenge experiments; these pig plasma samples were also randomly chosen from the short and long term challenge experiments.

Identification of *Campylobacters* and *Helicobacters* by 16S rRNA PCR amplification

Species-specific identification of *Campylobacters* and *Helicobacters* using PCR assays have been described previously (47, 64). The PCR assay for identifying *Campylobacter* species is described as follows: all reactions were performed in a total

volume of 25 ul containing 25 ng genomic DNA; 20 mM Tris-HCL (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 200 uM (each) dATP, dCTP, dGTP and dTTP; 0.4 uM of each primer; and 0.625 units of *Taq* DNA polymerase. DNA was amplified in a thermocycler for 25 cycles. The following parameters were used: denaturation, 94°C, 1 min; annealing, 55-65°C depending on the primer pair, 1 min; extension, 72°C, 1 min. The PCR products were separated by electrophoresis on agarose gels stained with ethidium bromide and visualized by UV light (47). The PCR assay for identifying *Helicobacter* species is described as follows: all reactions were performed in a total volume of 50 µl containing: 1.25 µg of template DNA (unless otherwise stated), 3.0 units of *Taq* polymerase, 10 mM Tris-HCL (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; and 1 µM of each oligonucleotide primer. The time and temperature parameters were as follows: samples were heated at 94°C for 5 min, and the DNA was amplified for 35 cycles under the following conditions: denaturation, 94°C, 2 sec; annealing, 60°C, 2 sec; primer extension, 72°C, 30 sec. The PCR products were separated by electrophoresis on agarose gels stained with ethidium bromide and visualized by UV light (64).

Histology

"Tissues were examined microscopically to detect *T. suis* larvae, to evaluate histological changes, and to localize specifically stained *Campylobacter* organisms. Fixed tissue samples were embedded in paraffin and 5 µm sections were cut and adhered to charged slides. Slides were routinely dehydrated in progressively higher concentrations of ethanol, deparaffinized and rehydrated. For histological evaluation, slides were stained

with Mayer's hematoxylin and eosin and cover slipped." (Jones, dissertation, 2005, reprinted with permission).

Real-time PCR

"*Trichuris suis* resides in the cecum and proximal colons of pig (19), while *C. jejuni* can colonize throughout the small and large intestine of pigs (10, 50). Therefore, to determine the effect of infection with *T. suis*, *C. jejuni*, or both on local immune responses in areas where these pathogens are present, we took samples from the jejunum, proximal colon, and distal colon. mRNA expression of 15 cytokines, chemokines, and iNOS was measured from each tissue using a real time PCR assay (29). Total RNA was isolated from frozen tissue samples from each pig using Trizol reagent (GibcoBRL Life Technologies). Briefly, $3mm^3$ tissue samples were homogenized in Trizol using a Polytron® tissue homogenizer (Kinematica, Cincinnati, OH). RNA was extracted with chloroform, precipitated with isopropanol, and washed with ethanol. Pellets were air dried, then resuspended in DEPC treated H_20 (GibcoBRL Life Technologies). DNA contamination was removed by treatment with approximately 27 Kunitz units of DNase I for 15 minutes. Samples were repurified on a Qiagen RNEasy column following the manufacturer's protocol (Qiagen, Valencia, CA). RNA concentration and integrity were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). For each sample, 10 μg of total RNA was reverse transcribed with random primers using a Stratagene First Strand RT-PCR kit (Stratagene, La Jolla, CA). First strand cDNA was used as the template in real time PCR reactions. Real time PCR primers and probes designed using the Primer Express (Applied Biosystems, Foster City, CA) software

package was used to analyze 14 cytokines (Chapter 2, Table 1). The 18S rRNA probe was obtained from Applied Biosystems and was used as a constitutively active gene for normalization. PCR was performed using a commercially available kit (Brilliant kit, Stratagene, La Jolla, CA) on an ABI PRISM 7900HT (Applied Biosystems). Amplification conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C 15 seconds and 60°C for 1 minute. Fluorescence signals measured during amplification were processed post-amplification. Threshold cycle (C_t) values were determined using values of 0.2 for TET, 0.1 for VIC, and 0.05 for FAM reporter dyes" (Jones, dissertation, 2005, reprinted with permission).

ELISA for fecal cytokine analysis

IL-8, IL-1-β, TNF-α, IL-4, and IL-10 were measured in the supernatants of the fecal samples using swine sandwich ELISA kits according to the manufacturer's instructions (BioSource International, Inc., Camarillo, CA). Indirect ELISA was performed to measure secreted IL-6. The swine IL-6 cytokine and primary anti-swine IL-6 antibody (rabbit polyclonal, IgG isotype) were purchased from BioSource International, Camarillo, CA. The secondary biotinylated antibody (anti-rabbit IgG (whole molecule)), Bovine Serum Albumin (BSA), extravidin peroxidase, and tetramethyl benzidine (TMB) were purchased from Sigma Chemical Co., St. Louis, MO. The fecal supernatants were used to coat the 96-well plates and stored overnight at 4°C. Each plate also contained IL-6 protein standard controls (in duplicate), at 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 ng/ml, in RPMI 1640 without phenol red and FBS. Following the overnight incubation, supernatants were discarded and the plates were blocked with 3% BSA solution (in

Phosphate Buffered Saline (PBS, 0.01 M) containing 0.1% Tween 20) and incubated for 90 minutes at 37°C. Primary rabbit anti-swine IL-6 antibody (1/100 titer) was used to capture the IL-6 protein (37°C, 90 minutes) and a goat anti-rabbit antibody conjugated with biotin (1/5000) was used as a secondary antibody at 37°C, 90 minutes). The optimal titers for the primary and secondary antibodies were determined from preliminary experiments (data not shown). The color reaction was produced using extravidin peroxidase (1 ug/ml, 45 minutes at room temperature) and a ready-to-use peroxide-containing TMB substrate (30 minutes, room temperature). The color reaction was stopped with 6N H_2SO_4. Plates were washed after each step with PBS containing 0.1% Tween-20. A microplate reader (EL800 Universal Microplate Reader, Bio-Tek instruments, Winooski, Vermont) was used to measure the absorbance at 450 nm, and the concentration of IL-6 protein in each sample was determined using KCjunior® software (Bio-Tek instruments, Winooski, Vermont).

Fecal extraction procedure for cytokine measurement

The fecal extraction procedure for cytokine measurement has been described previously (63). The feces were collected from the anus of pigs into sterile 25 ml polystryrene tubes. Five to ten grams of the fecal sample was diluted 1:3 in sterile phosphate buffered saline (PBS, pH 7.2) containing trypsin inhibitor from soybeans (1 mg/ml) and phenylmethylsulfonyl fluoride (PMSF, 1 mg/ml) and centrifuged at 16,500 rpm at 4°C for 15–30 minutes. Supernatants were collected and stored at -80 °C until further use in ELISA. The PBS was obtained from Gibco BRL, Rockville, MD. The trypsin inhibitor and PMSF were obtained from Sigma Chemical Co., St. Louis, MO.

Fecal cytokine recovery

In order to control for breakdown of cytokines in the fecal material, a cytokine recovery assay was performed. This was done by spiking the fecal samples from uninfected pigs with known quantities of commercial recombinant cytokine before mixing the samples in the fecal extraction procedure as discussed above. The cytokines were added to the fecal contents at the following concentrations: 50 pg/ml, 100 pg/ml, 250 pg/ml, 500 pg/ml, and 1000 pg/ml of IL-8, TNF-α, IL-1-β, IL-4, IL-6, or IL-10. The fecal supernatants were tested by ELISA to estimate the recovery rates of the cytokines added.

Statistical analysis

"The cytokine mRNA expression data for each tissue was analyzed separately using Statview 5.0 for Macintosh (Abacus Concepts, Berkeley, Calif.). The C_t value for the 18S ribosomal subunit was subtracted from the C_t value for each cytokine message to normalize for RNA content prior to statistical analysis. One-way analysis of variance (ANOVA) was performed to determine the statistically significant differences between treatments. A Fisher's least squares difference test was performed to determine differences between infected and control animals at each time point. P values of 0.05 or less were considered statistically significant" (Jones, dissertation, 2005, reprinted with permission).

The total IgG ELISA data were analyzed using descriptive statistic routines in Windows Excel.

The cytokine protein expression data were analyzed using Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Inc., Cary, NC). Statistical analysis was performed using the mixed model analysis with repeated measurements. The fixed effects in this model were the: experiment, treatment, day, and all of the possible interactions between these 3 fixed factors. The random effect in this model was the individual animal. The short (2 days) and long (23 days) term challenge experiments were each conducted twice. The mixed model analysis allowed the experimental data from the two short term challenge experiments to be combined for analysis and likewise for the two long term challenge experiments. The data could be combined because the main effect of the interaction term with the experiment was insignificant ($P > 0.05$). Cytokine levels are expressed as mean (in picograms per milliliter) ± standard error of the mean (SEM). Differences were considered statistically significant if $P \leq 0.05$.

Results

C. jejuni isolation and detection

We were unable to isolate *Campylobacter jejuni* from the fecal cultures of any pigs before or after infection (Table 2.4). To enhance sensitivity of detection, we used PCR assays to determine the presence of *C. jejuni* in the tissue samples taken separately from the jejunum, proximal colon, and distal colon. Polymerase chain reaction (PCR) using specific primers for the QRDR of the *gyrA* gene of *C. jejuni* did not detect *C. jejuni* in any tissue samples from any pig group, except in 1 out of 6 pigs in the *T. suis*-infected group (Table 2.4). However, *Campylobacter* 23S rRNA RFLP analysis suggested that *C. jejuni*, *C. coli*, and an unidentified *Campylobacter* species that gave a unique RFLP pattern were present in our animals (Figure 2.1). These findings in pigs handled rigourously as specific pathogen free by various methods lead us to further explore these results.

Immunologic recognition of *C. jejuni*

To confirm *C. jejuni* infection in experimentally inoculated pigs and to discriminate between *C. jejuni* and related epsilon proteobacter infection in preinoculated and control pigs, we pursued ELISA measurements of bacteria specific IgG. Figures 2.2 A and B show that IgG antibodies in the plasma of all the pigs in each treatment group from the 2nd repetition of the short term challenge experiments (days 0, 1 and 2) reacted with *C. jejuni* antigen (samples were not available from the first independent experiment). IgG antibodies in the plasma of most pigs in the treatment groups from the long term (days 0, 21, 22 and 23) challenge experiments (from 2 independent

experiments) also reacted with *C. jejuni* antigen. The ELISA was conducted using the plasma samples collected 2 days post *C. jejuni* inoculation on day 2 (short term challenge) and day 23 (long term challenge) of the experiments. Given that we detected high levels of anti-*Campylobacter* IgG antibodies in the plasma of our pigs and that it has been shown that the induction of an IgG response requires more than 2 days post-*Campylobacter* infection (about 8–14 days)(23), our data suggest that the pigs had established humoral immune recognition of *C. jejuni* before being experimentally infected. Therefore, we also wanted to distinguish whether these IgG antibodies measured in the pig plasma were *C. jejuni* specific or resulted from cross-reactions with other *Campylobacter* spp. or other environmental *Campylobacter*-like bacterial antigens. After randomly selecting plasma samples from some of the pigs in the short (days 0, 1 and 2) (2^{nd} repetition only) and long term (days 0, 21, 22 and 23; from 2 independent experiments) challenge experiments, we found that IgG antibodies in the plasma of 8 pigs randomly chosen from the *C. jejuni*-infected groups (positive control) and 6 pigs randomly chosen from the pig groups that received milk alone reacted with *C. jejuni*, *C. lari*, *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, *A. butzleri*, and *H. hepaticus* antigen by ELISA (Figure 2.3). Conversely, plasma samples taken from 2 newborn piglets (< 6 hours old) at the MSU swine farm (negative control) did not elicit any IgG response against *Campylobacter spp.*, *A. butzleri*, and *H. hepaticus* antigen. In these studies of our experimental pigs, the greatest IgG response was directed against *H. hepaticus* antigen (a related epsilon proteobacteria), suggesting that these bacteria or closely related *Helicobacters* were present in reasonably high numbers in the pigs prior to inoculation

with *C. jejuni*. Once again, these results stimulated further studies to distinguish whether the pigs carried a *Helicobacter spp.*

Recognition of *Campylobacter* spp. and *Helicobacter* spp. in feces

In order to confirm the previous ELISA findings, we performed subsequent species-specific PCR assays to identify *Campylobacter spp.* or *Helicobacter spp.*, which could have possibly had an effect on the cytokine immune response of the pigs. We conducted *Campylobacter* 16S rRNA PCR analysis and found that *Campylobacter* species were undetectable in the fecal samples from any pig group before or after bacterial infection (Tables 2.5 and 2.6). However, after *Helicobacter* 16S rRNA PCR analysis, we found that *Helicobacter* DNA could be detected in the feces of some of the pigs from the uninfected control group, suggesting that they were likely exposed to *Helicobacter* species (*Campylobacter*-like bacteria) prior to any experimental exposure to *C. jejuni* (Tables 2.5 and 2.6). The presence of these closely related bacteria likely contributed to the cross-reacting IgG antibodies detected in the plasma of the pigs by ELISA. The IgG cross-reacting antibodies may have contributed to the immune response against the experimentally-administered *C. jejuni*, thereby having an effect on the outcome of the cytokine responses elicited throughout the study. In the pig, *Helicobacter spp.* are found mainly in the stomach and small intestines of swine so larger effects are expected in these compartments.

Histopathology

"Haemotoxylin and Eosin (H&E) stained sections of jejunum, proximal colon, and distal colon of each pig were examined microscopically for the presence of *T. suis* larvae and histological changes due to infection. *T. suis* larvae were seen in proximal colon sections from 5 of 6 pigs in group 3, and 5 of 6 pigs in group 4. No larvae were seen in groups 1 or 2 (Figure 2.4).

Control animals (Group 1) had no proliferative, inflammatory, or degenerative lesions in any of the tissues examined and were therefore used as a normal comparison for all infection groups. All lesions were confined to the colon. Colonic lesions due to *C. jejuni* or *T. suis* infection were similar to those described in germ free piglets (50). Pigs in group 2 (*C. jejuni* only) developed focal mild crypt distension with sloughed epithelial cells, mucus and bacteria in the crypts in the distal colon (Figure 2.5A) and LGCs (Figure 2.6A). There was no significant inflammatory infiltrate into the lamina propria on day 23 after inoculation. Pigs infected with *T. suis* only developed mild to moderate colitis with goblet cell hyperplasia and crypt distension with mucus in superficial lamina propria. Lesions were found only in the areas where *T. suis* larvae were located. A mixed inflammatory infiltrate was present in the lamina propria and included lymphocytes, plasma cells, neutrophils, and eosinophils (Figure 2.5 B). Where present, crypts of LGCs were not distended (Figure 2.6 B). Pigs infected with both *C. jejuni* and *T. suis* developed mild colitis with goblet cell hyperplasia and crypt distension with mucus in superficial lamina propria in the areas where *T. suis* larvae were located. A mixed inflammatory infiltrate in the lamina propria included lymphocytes, plasma cells, neutrophils, and

eosinophils. Entrapped crypts of the LGCs were distended with mucus and few sloughed epithelial cells" (Figure 2.6 C). (Jones, dissertation, 2005, reprinted with permission)

Clinical observation

All piglets were observed prior to infection and prior to fecal sampling to assess clinical signs. Diarrhea was the main clinical sign and was assessed as any evidence of liquid feces ranging from soft stool to pipe stream diarrhea. The results are summarized in Tables 2.7 (short term challenge experiment) and 2.8 (long term challenge experiments). The occurrence of diarrhea was also indicated by subjective evaluation of collected loose feces and large amounts of loose fecal discharge in the cages of the pigs. Over a 2-day period (short term challenge), the occurrence of diarrhea was assessed on days 0, 1 and 2 during fecal sampling. Pigs given *C. jejuni* only had 2 episodes of diarrhea, and the dually infected pigs that received *C. jejuni* and *T. suis* had 5 episodes of diarrhea. There were no signs of clinical diarrhea in the uninfected pigs, the *T. suis* only infected pigs, or the noninvasive *E. coli* DH5-α infected pigs. The occurrence of diarrhea was also assessed on days 0, 21, 22 and 23 of fecal sampling for the long term challenge experiments and more diarrheic episodes were seen. Diarrhea was most prominent in the pigs infected with both *T. suis* and *C. jejuni* (19 episodes) and the pigs given *T. suis* only (20 episodes). Pigs given *C. jejuni* only had 6 episodes of diarrhea over the observation period. Pigs that were uninfected or received noninvasive *E. coli* DH5-α had no signs of diarrhea.

Cytokine expression following Real-time PCR analysis

"We measured expression of mRNA from 15 cytokines, chemokines, and iNOS in each segment of intestine sampled in an effort to determine if *T. suis* or *C. jejuni* had a significant effect on cytokine expression, and to determine if the affected cytokines were indicative of a specific response.

Cytokine expression patterns were distinct in each segment of the intestine tested (Figure 2.7). In the proximal colon of the *T. suis* only and dual infection groups, IL-13 expression was increased 3 to 4 fold while IL-12 and IL-8 expression was decreased 2 to 4 fold when compared to uninfected controls. In the dual infection group, expression of IL-5 and MCP-1 were each decreased 2 fold compared to controls. These data indicate that *T. suis* stimulates IL-13 in its local environment while decreasing expression of proinflammatory cytokines.

In the distal colon of pigs infected with *T. suis* only, expression of IL-1-β, IL-6, IFNγ, and IL-13 was increased 3 to 4 fold over controls. Infection with *C. jejuni* only or dual infection with *C. jejuni* and *T. suis* did not significantly affect expression of any cytokines, chemokines, or iNOS in the distal colon. These data indicate that a predominately proinflammatory response has been initiated in the distal colons of pigs infected with *T. suis* only.

In the jejunum, expression of MCP-1, TNFα, IL-12p40, IFNγ, IL-4, and IL-10 were increased 3 to 5 fold in the *C. jejuni* only group when compared to controls. Expression of GM-CSF was decreased 5 fold in the dual infection group when compared to controls. These data indicate that *Campylobacter* species present at low levels in the

tissues or related *Campylobacter*-like bacteria stimulate a predominately proinflammatory response in the jejunum, while *T. suis* may be down regulating proinflammatory cytokines." (Jones, dissertation, 2005 reprinted with permission)

Cytokine rate recovery from feces

Table 2.9 shows the recovery rate of exogenously added recombinant IL-8, IL-1-β, TNF-α, IL-4, IL-6, and IL-10 present in the feces. In general, these exogenously added recombinant cytokines were recovered from feces at low rates (10-20% recovery). These findings suggest that the low recovery rate may have an impact on accurately measuring cytokine expression in the feces of the pigs. Additionally, preliminary experiments showed that when water was experimentally evaporated from either solid or loose fecal samples, there were no significant changes in the weight of the samples. Thus, these results suggested that the consistency of the feces would not likely have an effect on adequately measuring changes in fecal cytokine levels (data not shown).

Cytokine Secretion–short term challenge (2 days)

IL-8, IL-1-β, TNF-α, IL-4, IL-6, and IL-10 concentrations were measured in the feces of all piglets throughout the experiment. However, there were no significant changes in IL-8, IL-1-β, TNF-α, IL-4, IL-6, and IL-10 expression due to any of the administered treatments (IL-8, $P = 0.56$; IL-1-β, $P = 0.27$; TNF-α, $P = 0.15$; IL-4, $P = 0.26$; IL-6, $P = 0.45$; IL-10, $P = 0.79$). The results are shown in Figures 2.8 and 2.9 and 2.10 and 2.11 and 2.12 and 2.13, respectively. These results represent the combined data of two independent experiments.

Cytokine secretion–long term challenge (23 days)

IL-8 secretion

IL-8 was detected in all fecal samples throughout the experiment (23 days). The results are illustrated in Figures 2.14. By day 23, pigs infected with *C. jejuni* alone showed a significant increase in IL-8 expression compared to levels on day 0 (pre-inoculation; $P = 0.02$); however, this increased expression was not statistically significant compared to the milk only control group on days 0, 21, 22 and 23 ($P > 0.05$). The *T. suis* only infected group showed a significant decrease in IL-8 expression by day 23 compared to levels on day 21 ($P = 0.01$) and day 22 ($P = 0.01$), but this decrease was not significantly different ($P > 0.05$) compared to day 0 and the milk only control group on day 23. Also, *E. coli* DH5-α did not induce any changes in IL-8 expression. Therefore, the overall changes of IL-8 expression due to the administered treatment over a period of time were insignificant with a P value of 0.08. These results represent the combined data of 2 independent experiments.

TNF-α secretion

TNF-α was detected in all fecal samples. The results for these experiments are shown in Figure 2.15. The effects of each treatment on TNF-α expression over a period of time showed moderate changes within the treatment groups. The pigs infected with *C. jejuni* showed a significant increase in TNF-α expression from day 0 to days 22 and 23, and the *E. coli* DH5-α group showed a significant increase in TNF-α expression from day 0 to day 22, but these increases in TNF-α expression were not significantly different in comparison to the milk only control group. From day 0 to day 23, the pigs that were

given milk only showed a significant increase in TNF-α. From day 21 to day 22, the *T. suis* group showed an increase in TNF-α and a decrease from day 22 to day 23, but there were no significant differences compared to day 0 or to the milk only control group. In addition, the pigs infected with *C. jejuni* and *T. suis* showed a significant increase in TNF-α expression from day 0 to day 22 and then showed a decrease by day 23; however, these changes were not significant compared to the milk control. Hence, the overall changes in TNF-α expression due to the administered treatment over 23 days were not significant in comparison to the milk only and day 0 control groups with P values > 0.05.

IL-1-β secretion

IL-1-β was detectable in all stool samples throughout the experiment (23 days). The results are shown in Figure 2.16. By day 23, the pigs infected with *T. suis* alone showed a significant decrease in IL-1-β expression compared to day 0 and the milk only control group on days 0, 21, 22 and 23. This result indicates that infection with this helminth was successful, and triggered down-regulation of a pro-inflammatory cytokine. The *T. suis* group also showed significantly decreased IL-1-β expression on day 23 compared to the pigs that received *C. jejuni*. The dual infected group and the *E. coli* DH5-α group showed a significant decrease in IL-1-β expression by day 23 compared to day 0, but the decrease was not significant when compared to the milk only group. Furthermore, the significant decrease in IL-1-β expression implies that *T. suis* suppressed the IL-1-β response. These results represent the combined data from 2 independent experiments.

IL-4 secretion

IL-4 was detectable in all stool samples throughout the experiment (23 days). The results are shown in Figure 2.17. The effects of each treatment on IL-4 expression showed moderate changes within the treatment groups. By day 23, *T. suis*-infected pigs showed a significant decrease in IL-4 expression compared to IL-4 levels on days 0, 21, and 22. Also, pigs given *C. jejuni*, *E. coli* DH5-α, or *C. jejuni* and *T. suis* showed a decrease in IL-4 expression by day 23 compared to levels on day 0 within each of the treatment groups. However, the overall changes of IL-4 expression were not statistically significant compared to the milk only control group ($P \geq 0.05$). These results represent the combined data of 2 independent experiments.

IL-6 secretion

IL-6 was detectable in all stool samples throughout the experiment (23 days). There were no significant changes in IL-6 expression due to the administered treatments ($P > 0.05$). The results are shown in Figure 2.18.

IL-10 secretion

IL-10 was detectable in all stool samples throughout the experiment (23 days). The results are shown in Figure 2.19. There were some moderate changes in IL-10 expression within the treatment groups throughout the experiment. Pigs given *C. jejuni*, *T. suis*, or *C. jejuni* and *T. suis* showed increased IL-10 levels by day 22 compared to day 0. Also, *E. coli* DH5-α-infected pigs showed an increase in IL-10 expression by day 22 that continued through day 23. These moderate changes in IL-10 expression within the

treatment groups were not significant when compared to the IL-10 levels detected in the pigs given milk only ($P > 0.05$).

Discussion

In our *Campylobacter* swine disease model, there have been repeated observations suggesting that there is enhanced disease and pathology in the gut mucosal epithelium and LGCs in response to a concurrent infection with *C. jejuni* and *T. suis* (49, 50)(Jones, dissertation, 2005). In this study, clinical signs and histopathology also support an interaction between these two pathogens. The most severe clinical signs and histopathological lesions were observed in dually infected pigs. Lesions were similar to that seen previously with germ free pigs with major changes observed in the proximal colon surrounding the worms and in the distal colon in the LGCs (50). It is thought that cytokine dysregulation plays a significant role in this response. In the present study, we hypothesized that weaned piglets infected with *C. jejuni* demonstrate an innate protective immune response via pro-inflammatory cytokine up-regulation and simultaneous *T. suis* infection mediates the down-regulation of the pro-inflammatory cytokine response, thereby promoting *C. jejuni* infection.

In this study, we used standard culture procedures, PCR assays, and immunological testing to determine whether the weaned piglets used in our experiments had been exposed to *C. jejuni*, other *Campylobacter* species, or *Campylobacter*-like organisms (epsilon proteobacteria) at the MSU swine farm facility prior to experimental infection. The piglets apparently had low level exposures to *Helicobacter spp.* We must consider the possibility that the piglets were also exposed to related *Campylobacter* species or other environmental *Campylobacter*-like bacteria from the sow. This was an important factor because it may have caused control pigs to have falsely elevated

cytokine responses elicited following exposure to *Helicobacter*. Also after experimental infections, we attempted to use standard culture methods to identify *C. jejuni* prior to and throughout the experiment, but in spite of our best efforts, culture alone was not sensitive enough to detect *C. jejuni*. Others have also found it difficult to recover *C. jejuni* from feces by culture alone and have used PCR assays and immunohistochemical examination to detect *C. jejuni* (20, 79). Because of problems detecting *C. jejuni* in pigs after inoculation, some researchers in recent work used culture of supernantants from 5-10 gram samples of feces to enhance the sensitivity (Moller-Nielson, personal communication). To increase the specificity and sensitivity of detection in our studies, we used *C. jejuni* specific *gyrA* Real-time PCR (TaqMan) for detection and RFLP analysis of the *Campylobacter* 23S rRNA gene for thermophilic *Campylobacter* species differentiation. TaqMan analysis of post infection tissue samples from necropsy gave negative results for most pigs, but a positive result for *C. jejuni* in distal colon samples from 16% (1 of 6) of the pigs that received *T. suis*-only. In follow-up analysis of post infection samples from necropsy, 23S rRNA RFLP analysis gave positive results for *C. jejuni* in jejunum samples from the dually infected pigs (16%, 1 of 6) and in proximal colon samples from 16% (1 of 6) of the pigs that received *T. suis*-only. Likewise, *C. jejuni* was detected in proximal colon samples from 66% (4 of 6) of the dually-infected pigs. RFLP analysis also showed positive results for *C. coli* and a possible *Campylobacter* spp. that gave a unique banding pattern that was not consistent with *C. jejuni*, *C. lari*, *C. coli*, or *C. upsaliensis* in jejunum, proximal and distal colon samples. These RFLP findings conflicted somewhat with the *gyrA* TagMan assay results and suggested that multiple *Campylobacter* species could have been present in the colon of

these pigs. RFLP results also implied that the pigs may have been exposed to *Campylobacter* spp. or *Campylobacter*-like organisms (epsilon proteobacteria) prior to the experiment. Subsequent immunological testing only partially clarified the issue. It demonstrated the presence of IgG antibodies against *C. jejuni* protein antigen in the plasma of all pigs in the short and long term challenge experiments. In addition, we detected significant levels of IgG antibodies that reacted with antigen from other *Campylobacter* species and *Campylobacter*-like bacteria (*Arcobacter butzleri* and *Helicobacter hepaticus*) in the plasma of some of the pigs given *C. jejuni* (a total of 8 pigs randomly selected from the short and long term challenge) or milk only (a total of 6 pigs randomly selected from the short and long term challenge). These findings further suggest that these pigs may have had a previous exposure to an epsilon proteobacterium possibly *C. jejuni* or other closely related member of the group with similar outer membrane proteins. It has been shown in humans that IgA and IgG are produced in response to *C. jejuni* infection, and serum and salivary IgG antibody responses are detectable up to 1 year post-infection while long-term IgA antibody responses are not detectable (14, 27). The greatest IgG reaction was obtained with *H. hepaticus* protein antigen, thereby suggesting that this was the main contaminating bacterium that was present in these pigs. It also suggests that the IgG antibodies detected in the plasma of the pigs cross-reacted with the protein antigen of other *Campylobacter* species and *Campylobacter*-like bacteria. Because of the short time period between initial bacterial inoculation (short term challenge-day 0; long term challenge-day 21) and sampling (short term challenge-day 2; long term challenge-day 23), the experimentally inoculated pigs would not have enough time to trigger an IgG response against the administered *C. jejuni*.

As in *C. jejuni*-infected humans, it has been shown that IgG antibody titers generally peak at approximately 8-14 days postexposure, but in some cases may not peak until 3 to 4 weeks postexposure, and remain elevated and persist at high levels for several weeks or months in patients (23, 45). Taken together, it appears that the pigs had been exposed to the putative contaminating bacterium (epsilon proteobacteria), possibly a *Helicobacter* spp., at birth or shortly after birth at the MSU swine farm prior to the experiment. Indeed, these ELISA results show increased levels of IgG antibodies reacting with multiple bacterial protein antigens, including *C. jejuni*, other *Campylobacter* species, *A. butzleri*, and *H. hepaticus*, and could not distinguish between the occurrence of cross reactivity and infection with multiple epsilon proteobacteria. Because no published work exists comparing homologies between outer membrane proteins of bacteria within this group, we cannot distinguish between these possibilities based on these assays. In a final attempt to distinguish the identity of the early bacterial exposure in these pigs, we tested fecal supernatants for the presence of other *Campylobacter* species and *Helicobacter* species using 16S rRNA PCR analysis. Here we found that almost all pigs in the short- and long-term challenge experiments tested negative for the presence of *Campylobacter* species pre- and post-infection. However, several pigs tested positive for the presence of *Helicobacter* species pre- and post-infection. This data provides the best evidence to suggest that the pigs were exposed to *Helicobacter* spp. prior to the experiment and that the cytokine responses measured were likely affected by this prior bacterial exposure. This also suggests the possibility that the effects seen actually represent host resistance to secondary bacterial infection. In this scenario, it is likely that these pigs acquired immunity after early exposure to an epsilon proteobacteria such as *Helicobacter*, and

elicited a humoral immune response which provided partial protective immunity to subsequent *C. jejuni* challenge. For example, it is possible that systemic IgG arising from early exposures may have bound to the experimentally administered *C. jejuni* or to shed *C. jejuni* surface material and limited *C. jejuni*-enterocyte interactions which is the main mechanism for increased inflammatory cytokine production in bacterial infections (38, 39, 42).

In the short-term challenge experiments, there were no significant changes in secreted IL-8, IL-1-β, TNF-α, IL-4, IL-6, or IL-10 expression due to any treatment when compared to the uninfected pigs that received milk only. We hypothesized that *C. jejuni* exposure would trigger an increase in the expression of pro-inflammatory cytokines, such as IL-8, IL-1-β, and TNF-α, and simultaneous *T. suis* infection would stimulate increased production of anti-inflammatory cytokines, such as IL-4, IL-6, and IL-10. Based on literature and previous findings in our laboratory, we believe that the anti-inflammatory cytokine response can down regulate the pro-inflammatory cytokine response and thereby facilitate *C. jejuni* infection. Consequently, the dysregulation of cytokine expression due to this concurrent infection could cause immunomodulatory effects that may contribute to the observed disease and pathology, which is similar to that seen in campylobacteriosis. However, because the data here provide some evidence suggesting that these pigs were exposed to *Helicobacter spp.* or a *Campylobacter*-like bacteria prior to the experiment, it is reasonable to believe that the administered *C. jejuni* was a secondary bacterial challenge. Therefore, in the groups of pigs that received bacteria alone (*C. jejuni* only or *E. coli* DH5-α only), the cytokine expressions measured were likely a result of host resistance to secondary bacterial infection. In the short- term challenge experiment, we

found that *C. jejuni* alone did not induce the expected increase in secreted pro-inflammatory cytokine expression in feces. There were also no significant changes in cytokine expression in the pigs that received *E. coli* DH5-α only. Likewise, in a previous study, Withanage et al. demonstrated that there were no significant changes in the expression of proinflammatory cytokines or chemokines, such as IL-1β, IL-8, or K60, in the gastrointestinal tract of chickens following secondary *Salmonella* infection. However, there was notable expression of MIP family chemokine and IL-6 in the ileum and cecal tonsils (76). Additionally, in our study, we found that there were increased levels of IgG in the plasma of almost all the pigs regardless of treatment that reacted in the *C. jejuni* ELISA. It has been shown in previous studies that significant levels of circulating IgG antibodies specific for *Salmonella* were high in infected chickens following primary infection and remained high with little change following secondary infection (15, 17, 76). In addition, significant levels of specific IgG antibodies have been detected in patients with *Campylobacter* enteritis, and IgG levels have been reported to be detectable in most patients up to a year post infection (23, 27). Therefore, our results appear to be similar to previous reports that suggest that humoral responses may play a significant role in protective immunity during homologous and heterologous re-challenge (16). The current finding also raises speculation about the possibility that there may be cross-reactive humoral immune responses to *Campylobacter* species or other epsilon proteobacteria that are associated with protective immunity. As for the two groups of pigs that received the *T. suis* in the short-term challenge (day 0-day 2), the results were not surprising. Studies have shown that clinical signs mostly begin 13-14 days following *T. suis* infection. The severe, bloody diarrhea associated with swine trichuriasis from the

20^{th} day onwards is due in part to the burrowing activities of the larval and adult stages in host colonic tissues and secretion of proteolytic enzymes and proteases (ESP). During this phase of *T. suis* development, the posterior end of the helminth freely protrudes into the lumen while the anterior body is embedded in the mucosa (18). At this phase, the third stage larvae have just moulted to the fourth larval stage. Following the direct mucosal damage by the helminth, localized lesions have been observed surrounding the site of worm attachment. It is at this time that resident bacteria have been demonstrated to amplify the severity of the infection (3, 4, 56, 77). The evidence provided by these reports suggests that *T. suis* acts synergistically with *C. jejuni* in producing the pathogenesis of swine mucohemorrhagic colitis. Thus, it is reasonable to believe that results from the long-term challenge experiments (23 days) would likely reflect the immune response patterns of natural infections more than that seen in the short-term challenge experiments (2 days).

In the long-term challenge experiments (day 0-day 23), we found that *T. suis* infection significantly decreased secreted IL-1-β by 23 days post-inoculation. Also, on day 23, IL-1-β was down-regulated in the pigs that received *T. suis* alone compared to the pigs that only received *C. jejuni.* Furthermore, the dually infected pigs showed moderately decreased IL-1-β expression by day 23, but the response was not significant. The significant decrease seen in IL-1-β expression in response to *T. suis* alone is characteristic of most helminth infections. In general, helminth infections elicit a Th2 response which induces the production of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 during infection (33, 74). This pattern of cytokine expression is characteristic of a Th2 response which predominantly limits Th1 cytokine-induced inflammation (Th1 cytokines- Il-12,

IFN-γ, IL-8, TNF-α, and IL-1-β) and protects the host by causing worm expulsion, reducing worm fecundity and enhancing host resistance mechanisms (33, 44). Tissue samples were also taken from the pigs in the long-term challenge experiments on day 23 following fecal sampling, and using these samples, Real Time PCR analysis showed increased IL-13 mRNA (Th2 cytokine) in the proximal colon of *T. suis*-infected pigs and decreased IL-8 and IL-12 expression (Th1 cytokines) (Jones, dissertation, 2005). This supports the hypothesis that *T. suis* stimulates Th2 anti-inflammatory cytokine production which can down-regulate the production of Th1 pro-inflammatory cytokines. We have also found in our laboratory that intestinal pig epithelial cells stimulated with *T. suis* ESP secrete IL-6 and IL-10 (60). Therefore, it is possible that the down regulation of IL-1-β in response to *T. suis* alone is likely due to IL-4, IL-10 and IL-13 production in response to the helminth infection. *In vitro* and *in vivo* studies have demonstrated IL-4, IL-10 and IL-13 inhibition of intestinal inflammatory responses such as Th1-type cytokine production (22, 46, 48, 68). Taken together, our fecal cytokine ELISA results suggest that *T. suis* likely plays a significant role in the down-regulation of IL-1-β production, and this response may play a contributing role in altering host conditions which promote secondary bacterial infection. However, these results need to be confirmed in further studies because of the low cytokine recovery rate (10-20%) determined for these fecal ELISAs. Several factors may have contributed to the low cytokine recovery rate: protein degradation associated with freezer storage, the loss of protein during the fecal preparation due to the cytokines binding to the solid fecal matter, or protein degradation due to protease activity. Because of the drawbacks associated with measuring cytokines in feces using ELISA, we are unable to confidently support the future use of ELISA for

this purpose and believe that our results from this analysis are notable, but must be confirmed with further experimentation. Nevertheless, in the long-term challenge experiments, cytokine mRNA expression measurements in the tissues of these weaned piglets were more informative. We observed significant changes in cytokine expression in different intestinal compartments based on treatment group, specifically the jejunum, proximal and distal colon. *T. suis* resides in the proximal colon while *C. jejuni* has been found in all of these sites. We found that *T. suis* infection predominantly influenced cytokine expression in the proximal colon with some effects in the distal colon. *C. jejuni* mainly affected the cytokine expression in the jejunum with minimal changes in the colon. In the proximal colon, *T. suis* stimulated increased IL-13 expression (3 to 4 fold) in the pigs that received *T. suis* alone or those that were dually infected, and IL-8 and IL-12 expression was decreased 2 to 4 fold in both groups when compared to the uninfected control pigs that received milk only. MCP-1 expression was also decreased in the dually-infected pigs. This is similar to other results that show that during intestinal nematode infection, IL-13 plays a significant role in affecting Th-2-cell-mediated responses which are associated with goblet cell hyperplasia and worm expulsion (11, 37, 52). Th-2 responses are associated with the production of anti-inflammatory cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and these anti-inflammatory cytokines can down regulate Th-1 responses (IFN-γ, IL-2, IL-12, lymphotoxin, and IgG2a production) which subsequently have a diminishing effect on pro-inflammatory cytokine responses (IL-8, TNF-α, MCP-1, IL-1, and IL-6) (25, 28, 59). These types of cytokine modulations can generally be seen during concurrent bacterial-helminth infections. Previously reported data from our laboratory showed that pigs rectally challenged with *C. jejuni* showed

increased IL-8 expression at 1 and 24 hours post-inoculation suggesting the importance of IL-8 production in early host resistance to *C. jejuni* in immunologically naïve swine (Jones, dissertation, 2005). It has also been shown that *C. jejuni* stimulates cultured intestinal epithelial cells (INT 407) to secrete IL-8 and MCP-1 (38, 42). Likewise, *Helicobacter pylori* stimulates cultured gastric epithelial cells to secrete IL-8 and MCP-1 (35, 58, 61). In another enteric bacteria with similar pathogenesis, it has been demonstrated in a rabbit ligated intestinal loop model of *Shigella flexneri* infection that IL-8 appears to play a critical role in controlling the spread of infection (67). Because bacteria have been shown to invade and proliferate in the lamina propria and submucosa surrounding the site of worm attachment in previous studies (50), we expected to see increases in proinflammatory cytokines like IL-8 in the proximal colon of pigs given *C. jejuni* alone. However, no changes in IL-8 were observed in either study design associated with *C. jejuni* infection alone in this tissue. This may have been affected by the suspected early exposure to *Helicobacter* spp. in these studies or perhaps the inoculated strain of *C. jejuni* invaded mainly the small intestines based on the proinflammatory responses measured there. Based on the tissue RT-PCR results it is also possible that *T. suis* contributed to decreased IL-8 expression and may have facilitated bacterial invasion and proliferation in the lamina propria and submucosa, similar to what is observed in the *Shigella* model. IL-13 production due to *T. suis* infection likely contributes to the decreased expression of IL-8, given that it has been demonstrated that the production of IL-8 from intestinal epithelial cells can be inhibited by IL-13 and IL-4 (48).

We also found that expression of both IL-12 subunits was significantly decreased in the proximal colon of pigs given *T. suis*. IL-12 is predominantly produced by monocytes, macrophages, and dendritic cells and plays a role in inflammation and acquired immune responses (36, 71). IL-12 drives the development of Th1-type immune responses by stimulating T cells to produce IFN-γ, and *in vitro* and *in vivo* studies have shown that IL-12 plays a significant role in developing specific immunity against intracellular pathogens (36, 71). However, IL-13 has been shown to down-regulate IL-12 production (54). Increased expression of IL-13 has been documented in resistant mice strains infected with *T. muris* suggesting its critical protective role in nematode infection (11). Taken together, decreased expression of both IL-12 subunits in the proximal colon of *T. suis*-infected pigs can be attributed to the immunomodulatory effects of IL-13.

Similar to the decreased expression of pro-inflammatory cytokines in the proximal colon, IL-5 expression was significantly decreased in the dually infected pigs. In general, IL-5 is up-regulated along with other classical Th2 cytokines during helminth infections, and IL-5 has been shown to characteristically induce eosinophil production and activation (32). However, experimental studies have shown that *C. jejuni* intestinal colonization is reduced in Balb/c ByJ female mice that are orally pretreated with IL-5, suggesting that IL-5 may play a protective role for the host (13). It is possible that *C. jejuni* elicits the down regulation of IL-5 as a mechanism to evade host immune responses and establish infection. In the proximal colon of the pigs that received *C. jejuni* only, IL-5 expression was decreased, but the decrease was not statistically significant. Even so, the decreased expression of IL-5 observed in the dually-infected pigs and the *C. jejuni*-infected pigs suggests that this cytokine may play a significant role

in the pathology associated with *C. jejuni* infection *in vivo* and that further studies into this hypothesis are warranted.

Unlike the decreased pro-inflammatory cytokine response observed in the proximal colon, IL-1-β, IL-6, IFN-γ and IL-13 were up-regulated in the distal colon of the pigs infected with *T. suis* only. We speculate that this immune response is likely due in part to the effects of *T. suis* ESP in the distal colon. The up-regulation of IL-1-β, IL-6, IFN-γ is indicative of an inflammatory response. It has been demonstrated *in vitro* that ESP treatment of differentiated IPEC-1 cells causes a dose dependent cytotoxic response and significantly decreased transepithelial electrical resistance (TER)(1, 2). Also, cultured differentiated and undifferentiated IPEC-1 cells secrete IL-6 following ESP exposure (Parthasarathy, 2005). Therefore, it is reasonable to consider that the cytotoxic effects of ESP on intestinal epithelial cells can stimulate an inflammatory cytokine response in the distal colon. Another possible reason for the inflammatory response observed in the distal colon is secondary bacterial infection that is augmented by *T. suis* infection. In the distal colon of *T. suis*-infected pigs in previous studies, several resident bacterial species, including *C. jejuni*, *C. coli*, an unidentified *Campylobacter* species, and *E. coli*, have been cultured from abscessed lymphoglandular complexes (51). It has been shown that *C. jejuni* and *C. coli* stimulate cultured human INT-407 intestinal epithelial cells to secrete IL-8 (39). Therefore, it is possible that bacterial invasion of the distal colon tissues in this study initiated an inflammatory response and was responsible for the histopathological changes in the LGCs. It has been shown that some components of the ESP consist of antibacterial agents, and a possible scenario is that the antibacterial effects of ESP could kill residential bacteria thereby making the host environment more

conducive to facilitating the proliferation of pathogenic bacteria. Once the ESP antibacterial activity has killed the residential bacteria, these pathogenic secondary bacteria could more effectively interact with the intestinal epithelium and stimulate these cells to produce proinflammatory cytokines. Also, the effects of ESP produced by 4th stage larvae that have traveled to the distal colon could contribute to increased expression of IL-13. It has been shown that *T. suis*-infected pigs have serum antibodies as early as 21 days post inoculation that recognize a 20kDa glycoprotein isolated from ESP collected from adult worms cultured *in vitro* (41). This indicates that an immune response is likely triggered by the larval stages present in pigs 21 days post-*T. suis* infection. Altogether, these data suggest that ESP may have direct effects on intestinal epithelial cells and could account in part for the increased cytokine production *in vivo* at sites distal to worm attachment.

In the jejunum, the pigs infected with *C. jejuni* only showed increased expression of MCP-1, TNF-α, IL-12p40, IFN-γ, IL-4, and IL-10. Although we did not confirm the presence of *C. jejuni* or other *Campylobacters* in this tissue by culture, results from the histopathology, immunological testing (IgG ELISA), and the 23S rRNA PCR analysis suggest that it is still likely that the inoculated strain of *Campylobacter* triggered this response. *C. jejuni* is notoriously difficult to recover from colonized pigs without disease because of low bacterial numbers and the preferred predilection site that is strictly at the base of mucosal crypts. *In vitro*, *C. jejuni* induces INT 407 cells to produce intracellular MCP-1, TNF-α, IFN-γ, IL-4, and IL-10 (42), and *Helicobacter pylori* induces gastric epithelial cells to produce IL-8 and MCP-1 (35, 55, 58, 61). Thus, the cytokine changes observed could be due to *Campylobacter* interaction with the intestinal epithelial cells in

the jejunum. *Helicobacter* responses, if present, would be expected to be similar based on previous studies (34). In the jejunum of the dually-infected pigs, GM-CSF expression was decreased 5 fold compared to the uninfected milk controls, suggesting that the *T. suis* down-regulated pro-inflammatory cytokine expression similar to the response seen in the proximal colon.

Furthermore, clinical diarrhea was a prominent sign of disease throughout the experiments. During fecal sampling in both experiments, diarrhea was observed in pigs that received *C. jejuni* only, *T. suis* only or both. However, the dually infected pigs and the *T. suis* only-infected pigs had more frequent, more severe diarrhea than the other treatment groups, thereby implying that *T. suis* is mainly responsible for causing the observed diarrhea. Additionally, diarrhea was mostly observed in the long term challenge experiments where *Trichuris* infection had progressed for 21 days. As has been reported frequently in literature reports on *Trichuris*-induced disease, diarrhea usually begins around 21 days post-infection and is commonly called the "21 day scours" (24). In both experiments, some of the *C. jejuni*-infected pigs had mild diarrhea, which is commonly seen in campylobacterosis (10). In a germ free *Campylobacter* swine disease model experiment of similar design, Mansfield et al. previously reported that some of the *C. jejuni*-infected pigs showed clinical signs of mild diarrhea less severe than that in dually-infected or *T. suis* only-infected pigs (50). Uninfected and *E. coli* DH5-α infected pigs showed no signs of diarrhea. The observation of severe diarrhea in pigs infected dually or with *T. suis* alone suggests that *T. suis* contributed significantly to diarrheal disease.

Additionally, the histopathologic lesions observed in the tissues of pigs infected with *T. suis* alone or dually infected with *T. suis* and *C. jejuni* were consistent with what has been reported in the literature(10, 50). The lesions in the tissues from the *C. jejuni*-infected pigs were mild, and there was no crypt distention or goblet cell hypertrophy. The lesions observed in the tissues of *T. suis*-infected pigs showed signs of mild colitis with crypt distention and increased thickness of all the layers of the colon. However, the lesions in the tissues of dually-infected pigs showed the greatest signs of disease with the greatest crypt distention and increased thickness of all layers of the colon, and greater infiltration of immune cells. Also, in the groups of pigs that were dually-infected or received *T. suis* alone, histological evaluation (H&E staining) confirmed the presence of *T. suis* in the tissues (Figure 2.4). Altogether, these results confirmed that all the pigs inoculated with *T. suis* became infected, and we were able to correlate the presence of the worm with cytokine changes seen in the colon.

In summary, we found it necessary to use multiple diagnostic tests to detect and identify *Campylobacter* species or other epsilon proteobacteria in the feces and intestinal tissues of pigs, and despite this, definitive diagnosis was not possible. However, the data presented here suggest that throughout the intestine, *T. suis* appears to mediate most changes in pro- and anti- inflammatory cytokine expression during concurrent *T. suis* and *C. jejuni* infection, and it is possible that the immunomodulatory effects associated with these changes play a role in promoting a favorable host environment for secondary bacterial infection. One possible scenario is that *T. suis* interacts with the resident bacteria and down regulates the pro-inflammatory cytokine response which is generally elicited by bacterial infection, and the immunomodulatory effects of these pro-

inflammatory cytokines are diminished which could thereby facilitate bacterial invasion of intestinal cells and tissues causing cellular destruction and disease, as seen in human *campylobacteriosis*. Although our hypotheses cannot be unequivocally accepted, the data presented in this study provide some evidence to support this scenario. The occurrence of frequent, severe diarrhea in pigs infected with *C. jejuni* and *T. suis* or *T. suis* alone is a clear sign of disease and shows that *T. suis* contributes significantly to this syndrome. We were also able to detect pro- and anti- inflammatory cytokines in the feces of all the pigs regardless of treatment using ELISA, in agreement with data reported in the literature (9, 30, 57, 63, 66). However, measuring cytokine expression in feces by ELISA was found to be insufficient to directly address our hypothesis; unfortunately, this noninvasive method for investigating fecal cytokine expression during infection does not appear to be useful. In this study, the use of RT-PCR to measure cytokine expression in the tissues served as a more accurate and reliable method to address the overall hypothesis. Future studies will be conducted to describe the roles of these pro- and anti-inflammatory cytokines in this concurrent infection.

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Table 2.1. Experimental treatment groups for the short (2 days) and long (23 days) term challenge experiments.

Treatment Groups	Treatment	
Group 1	*C. jejuni*	(2×10^8 cfu)
Group 2	*C. jejuni* and *T. suis*	(2×10^8 cfu)and (2500 embryonated eggs)
Group 3	*T. suis*	(2500 embryonated eggs)
Group 4	*E. coli* DH5-α	(2×10^8 cfu)
Group 5	Uninfected milk control	(2 ml)

Table 2.2. Inoculation timeline for short term challenge experiments (2 days): 4-week old weaned, conventionally reared pigs were divided into 5 treatment groups with a total of 10 pigs per group. The inoculations were administered via oral gavage in 2.0 ml of sterile milk. The pigs were given 2×10^8 cfu *C. jejuni*, 2500 *T. suis* embryonated eggs, both, 2×10^8 cfu *E. coli* DH5-α, or milk.

Day 0	**Day 1**	**Day 2**
1. Rectal swabbing and fecal sampling	Rectal swabbing and fecal sampling of all animals	1. Rectal swabbing and fecal sampling of all animals
2. Inoculation protocol: Group 1: *C. jejuni* Group 2: *C. jejuni* and *T. suis* Group 3: *T. suis* Group 4: *E. coli* DH5-α Group 5: Milk		2. All pigs were euthanized and necropsied.

Table 2.3. Inoculation timeline for long term challenge experiments (23 days): 4-week old weaned, conventionally reared pigs were divided into 5 treatment groups with a total of 11 pigs per group. The inoculations were administered via oral gavage in 2.0 ml of sterile milk. The pigs were given 2×10^8 cfu *C. jejuni*, 2500 *T. suis* embryonated eggs, both, 2×10^8 cfu *E. coli* DH5-α, or milk.

Day 0	**Day 21**	**Day 22**	**Day 23**
1. Rectal swabbing and fecal sampling	1. Rectal swabbing and fecal sampling	Rectal swabbing and fecal sampling of all animals	1. Rectal swabbing and fecal sampling of all animals
2. Inoculation protocol: Group 1: Milk Group 2: *T. suis* Group 3: *T. suis* Group 4: Milk Group 5: Milk	2. Inoculation protocol: Group 1: *C. jejuni* Group 2: *C. jejuni* Group 3: Milk Group 4: *E. coli* DH5-α Group 5: Milk		2. All pigs were euthanized and necropsied. Tissue samples were taken from all pigs.

Table 2.4. Summary of *Campylobacter* detection and identification. Campylobacters were detected and identified from pigs by fecal culture, PCR for the *C. jejuni* QRDR on intestinal lumen contents obtained from each intestinal region and tissue DNA, and RFLP analysis of the thermophilic *Campylobacter* 23S rRNA gene using tissue DNA. Samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, reprinted with permission).

			QRDR		*Campylobacter* 23S rRNA RFLP (tissue DNA)		
	Infection	Culture	Fecal DNA	Tissue DNA	*C. jejuni*	*C. coli*	Unidentified *Campylobacter sp.*
Jejunum	Control	0/6	0/6	0/6	0/6	5/6	2/6
	C. jejuni	0/6	0/6	0/6	0/6	2/6	5/6
	T. suis	0/6	0/6	0/6	0/6	3/6	2/6
	Dual	0/6	0/6	0/6	1/6	4/6	1/6
Proximal colon	Control	0/6	0/6	0/6	1/6	4/6	5/6
	C. jejuni	0/6	0/6	0/6	1/6	2/6	4/6
	T. suis	0/6	0/6	0/6	1/6	4/6	1/6
	Dual	0/6	0/6	0/6	4/6	4/6	2/6
Distal colon	Control	0/6	0/6	0/6	0/6	0/6	6/6
	C. jejuni	0/6	0/6	0/6	0/6	0/6	6/6
	T. suis	0/6	0/6	1/6	0/6	2/6	3/6
	Dual	0/6	0/6	0/6	0/6	2/6	6/6

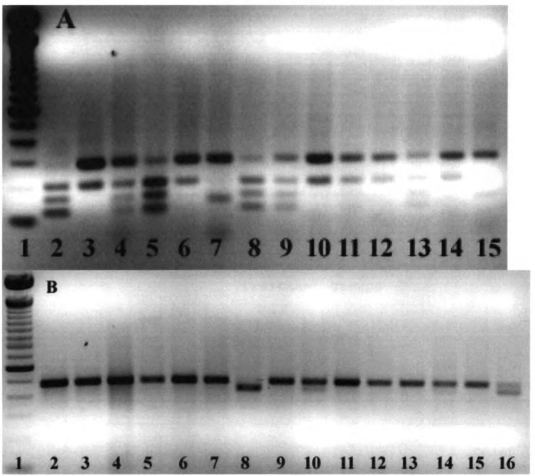
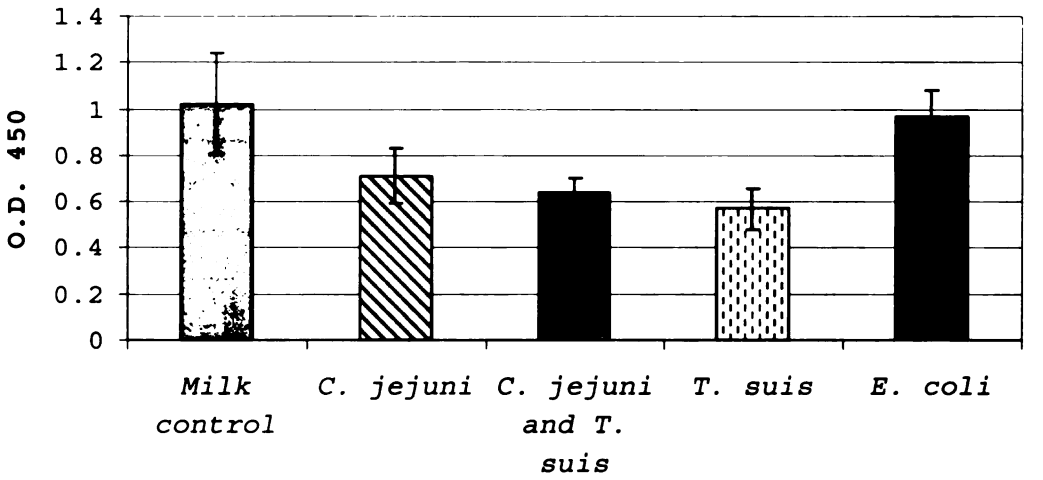
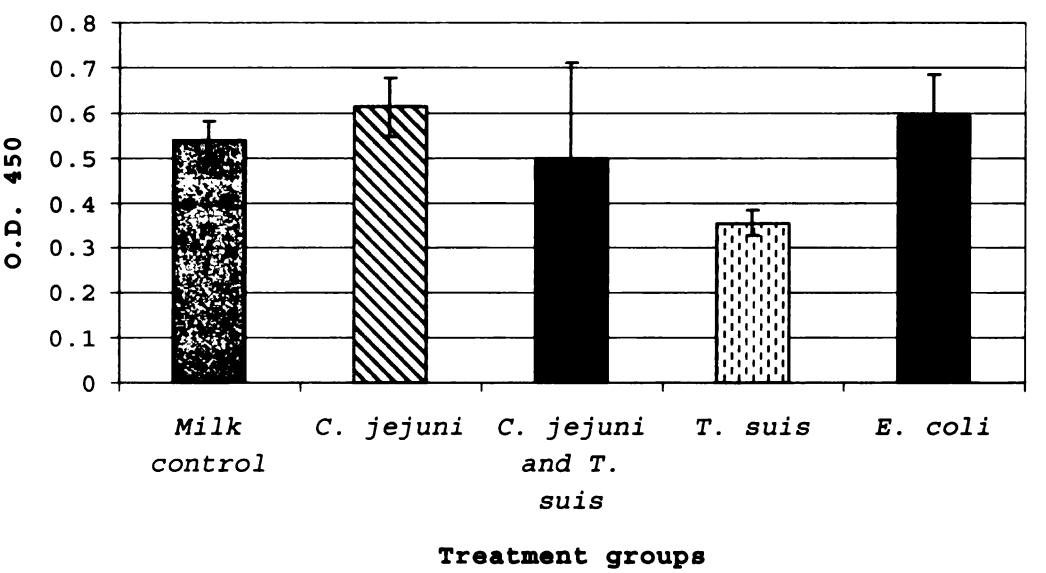


Figure 2.1. RFLP analysis of the *Campylobacter* 23S rRNA gene. Panel A: *AluI* digest of proximal colon samples from infected pigs . Lane 1: 100 bp DNA ladder; Lane 2: *C. jejuni*; Lane 3: *C. coli*; Lanes 4 through 15: DNA from infected pigs. Panel B: *Tsp5091* digest of proximal colon samples from infected pigs. Lane 1: 100 bp DNA ladder; Lane 2: *C. jejuni*; Lane 3: *C. coli*; Lane 4: *C. hyoilei*; Lanes 5 through 16: DNA from infected pigs. Samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, reprinted with permission).

A



B



Figures 2. 2 A and B. (A) Short term challenge experiment and (B) long term challenge experiment ELISA results indicating levels of plasma IgG antibodies detectable against *C. jejuni* protein antigen. IgG levels were measured in the plasma of all pigs from each treatment group. The data represent the mean ± SEM.

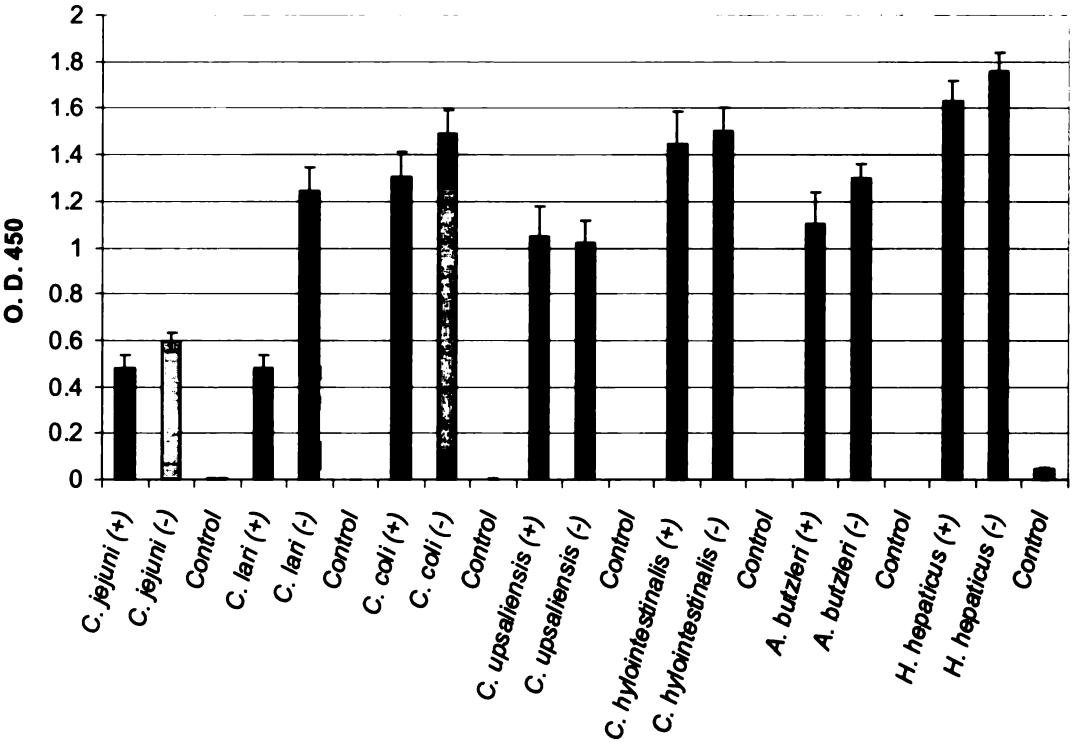


Figure 2.3. ELISA results indicating levels of plasma IgG antibodies detectable against protein antigen of various *Campylobacter* species, *Arcobacter butzleri*, and *Helicobacter hepaticus*, suggesting IgG cross-reactivity. IgG levels were measured in the plasma of 8 *C. jejuni*-infected pigs randomly selected from the short- and long-term challenge experiments (+), 6 uninfected pigs randomly selected from both challenge experiments that received milk only(-), and 2 newborn piglets < 6 hours old from the MSU swine farm (control). The data represent the mean ± SEM. **"Images in this dissertation are presented in color."**

Table 2.5. Short term challenge experiment 16S RNA PCR analysis results for rapid identification of *Campylobacter* and *Helicobacter* DNA in pig feces.

	Campylobacter		*Helicobacter*	
Pig #	Pre-infection	Post-infection	Pre-infection	Post-infection
1	-	-	-	-
2	-	-	-	-
3	-	-	-	+
4	-	-	-	-
5	×	-	×	-
6	-	-	-	+
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	+	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	-	-	-
24	-	-	-	-
25	-	-	-	-
26	-	-	-	-
27	×	-	×	-
28	-	-	-	-
29	×	-	×	-
30	-	-	-	-

a. + tested positive
b. - tested negative
c. × represents missing sample

Table 2.6. Long term challenge experiment 16S RNA PCR analysis results for rapid identification of *Campylobacter* and *Helicobacter* DNA in pig feces.

	Campylobacter		*Helicobacter*	
Pig #	Pre-infection	Post-infection	Pre-infection	Post-infection
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	+	-
19	-	-	-	-
20	-	-	-	-
21	-	-	+	-
22	-	-	-	-
23	-	-	-	-
24	-	-	-	-
25	-	-	-	-
26	-	-	-	+
27	-	-	-	-
28	×	-	×	-
29	-	-	-	-
30	×	-	×	-
31	-	-	-	weak +
32	-	-	-	-
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	-	-
38	-	-	-	-
39	-	-	-	-

a. + tested positive
b. - tested negative
c. × represents missing sample

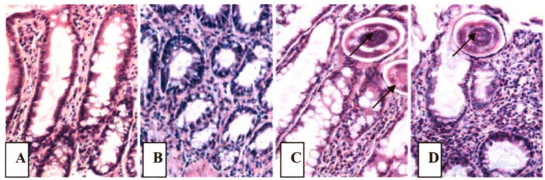


Figure 2.4. H&E stained sections from the colons of pigs were examined for *T. suis* larvae. Panel **A**: Control; **B**: *C. jejuni* only; **C**: *T. suis* only; **D**: Dual infection, 10X magnification. Larvae (arrows) were detected in 5 of 6 pigs from each of the *T. suis* only and dual infection groups. Samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, reprinted with permission). **"Images in this dissertation are presented in color."**

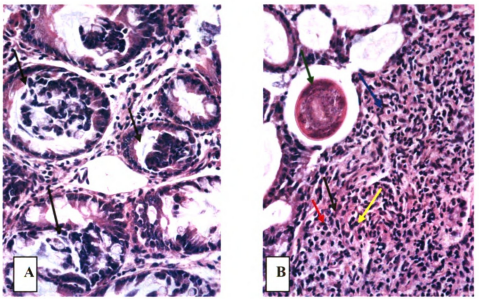


Figure 2.5. H&E section from a *C. jejuni* infected pig (panel A) showing sloughed epithelial cells in the crypts (black arrow), and a *T. suis* infected pig (panel B) showing the mixed inflammatory infiltrate in the lamina propria associated with the larvae (green arrow). Inflammatory cells included eosinophils (black arrow), plasma cells (blue arrow), lymphocytes (red arrow), and neutrophils (yellow arrow) (40X magnification). Samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, reprinted with permission). **"Images in this dissertation are presented in color."**

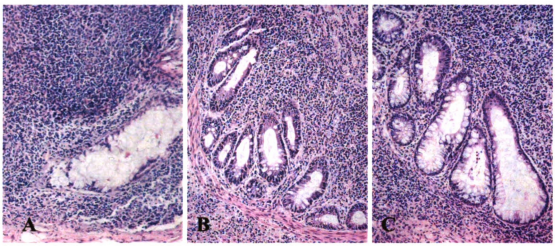


Figure 2.6. H&E sections of LGCs from infected pigs. Crypt distension was present in the pigs that received *C. jejuni* only (panel A) and in the dually-infected pigs (panel C), but crypts were not distended in the pigs that received *T. suis* only (Panel B). (10X magnification). Samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, modified and reprinted with permission). **"Images in this dissertation are presented in color."**

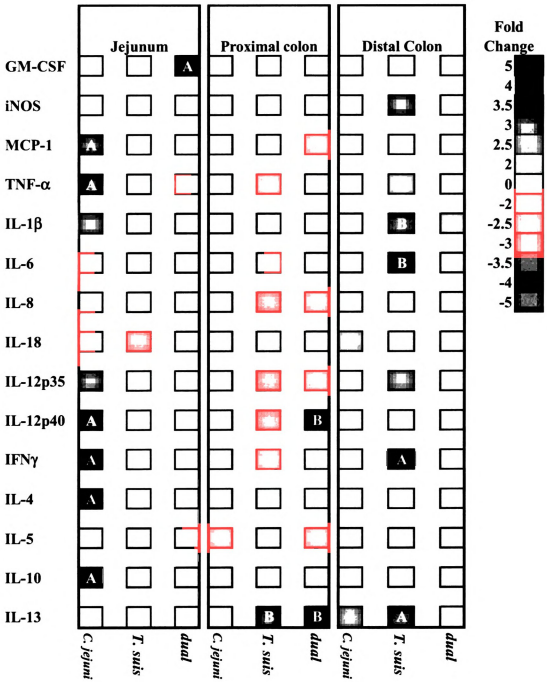


Figure 2.7. Expression of mRNA from a panel of cytokines was measured by Real Time PCR. The data represent the average fold change in expression from infected animals when compared to the milk control animals. Letters indicate statistically significant changes: A: $p \leq 0.05$; B: $p \leq 0.01$. Tissue samples were taken from the pigs in the 2nd repetition of the long term challenge experiments only (Jones, dissertation, 2005, reprinted with permission). **"Images in this dissertation are presented in color."**

Tables 2.7. Short term challenge: clinical assessment of diarrhea in weaned, conventionally-reared swine infected with *T. suis* and/or *C. jejuni*.

Experiment	Treatment group	# of pigs with diarrhea		
		Day 0	Day 1	Day 2
Short (2 days)[a] 2nd repetition of experiment only	Group 1 *C. jejuni*	0/6	2/6	0/6
	Group 2 *C. jejuni* and *T. suis*	0/6	1/6	4/6
	Group 3 *T. suis*	06	0/6	0/6
	Group 4 *E. coli* DH5-α	06	0/6	0/6
	Group 5 Uninfected	06	0/6	0/6

All animals were assessed for diarrhea during fecal sampling on days 0, 1, and 2 of the short term challenge experiment.

[a] The four pigs from each treatment group in the first repetition of the short term challenge experiment were not assessed for diarrhea.

Tables 2.8. Long term challenge: clinical assessment of diarrhea in weaned, conventionally-reared swine infected with *T. suis* and/or *C. jejuni*.

Experiment	Treatment group	# of pigs with diarrhea			
		Day 0	Day 21	Day 22	Day 23
Long (23 days) Both repetitions combined	Group 1 *C. jejuni*	0/11	1/11	2/11	3/11
	Group 2 *C. jejuni* and *T. suis*	0/11	6/11	6/11	7/11
	Group 3 *T. suis*	0/11	5/11	5/11	10/11
	Group 4 *E. coli* DH5-α	0/11	0/11	0/11	0/11
	Group 5 Uninfected	0/11	0/11	0/11	0/11

All animals were assessed for diarrhea during fecal sampling on days 0, 21, 22, and 23 of the long term challenge experiments.

Table 2.9. Fecal cytokine recovery rate.

Cytokine	Fecal cytokine recovery rate (%)
IL-8	10
TNF-α	20
IL-1-β	10
IL-4	10
IL-6	20
IL-10	10

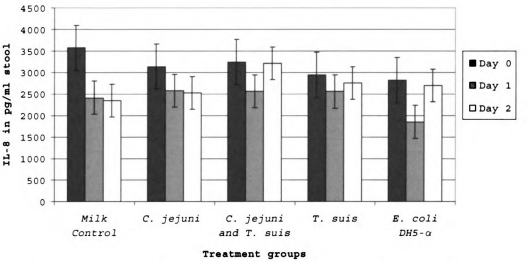


Figure 2.8. Changes in IL-8 levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± standard error of the mean (SEM); P=0.56.

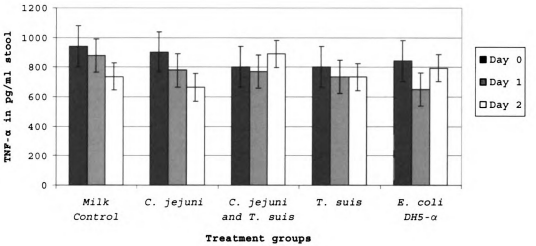


Figure 2.9. Changes in TNF-α levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± SEM; P=0.15.

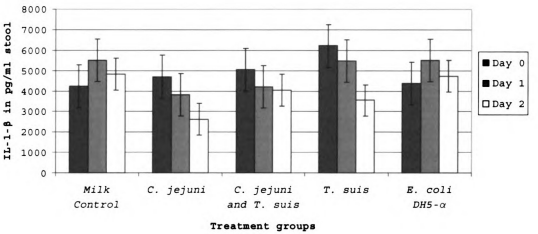


Figure 2.10. Changes in IL-1-β levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± SEM; P=0.27.

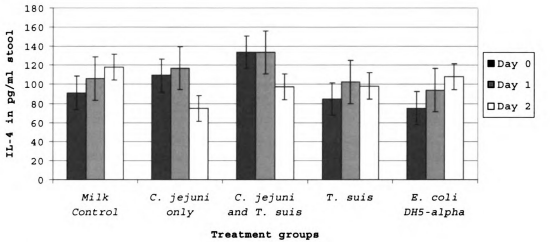


Figure 2.11. Changes in IL-4 levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± SEM; P=0.26.

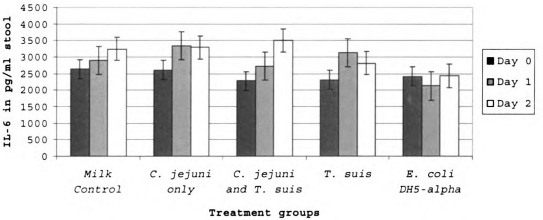


Figure 2.12. Changes in IL-6 levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± SEM; P=0.45.

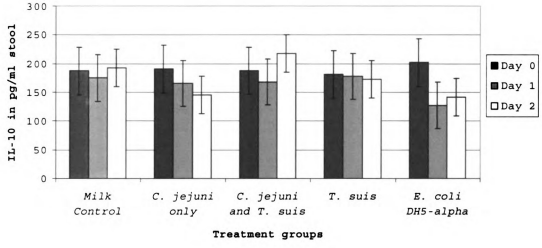


Figure 2.13. Changes in IL-10 levels were quantified in the feces of weaned piglets from the short term challenge experiments. The pigs were inoculated via oral gavage with various treatments. Fecal samples were collected on days 0, 1, and 2 for further processing to be used in ELISA. Values shown are means of two identical short term (2 days) challenge experiments with a total of 10 pigs in each group. The data represent the mean ± SEM; P=0.79.

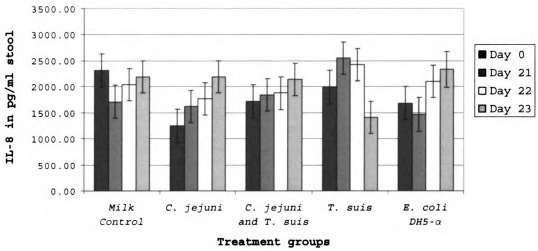


Figure 2.14. Changes in fecal IL-8 levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the *E. coli* DH5-α group). The data represent the mean ± SEM; $P \geq 0.05$.

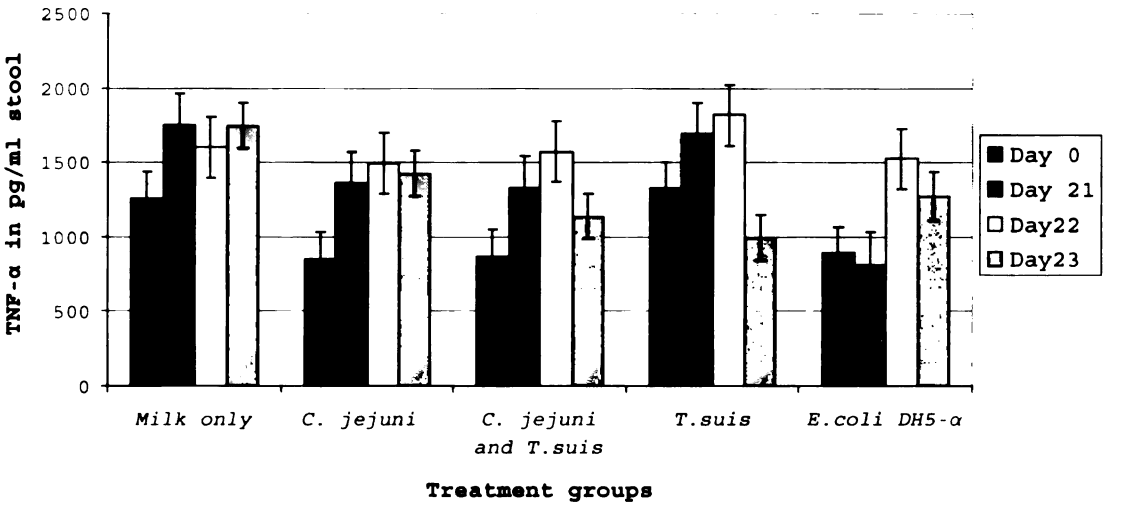


Figure 2.15. Changes in fecal TNF-α levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the *E. coli* DH5-α group). The data represent the mean ± SEM; $P \geq 0.05$.

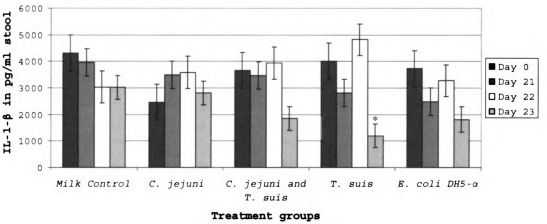


Figure 2.16. Changes in fecal IL-1-β levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the *E. coli DH5-α group).* The data represent the mean ± SEM. * Represents the significant difference of the values for the *T. suis*-infected pigs on day 23 in comparison to the values for the milk only control pigs; $P \leq 0.05$.

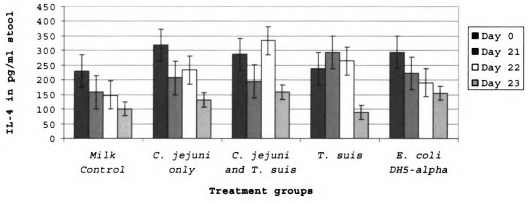


Figure 2.17. Changes in fecal IL-4 levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the *E. coli* DH5-α group). The data represent the mean ± SEM; $P \geq 0.05$.

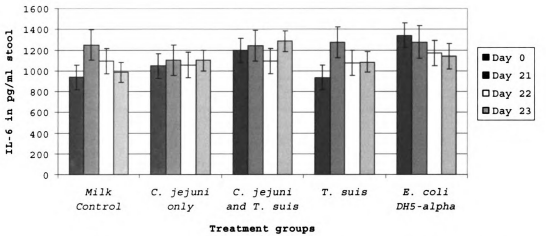


Figure 2.18. Changes in fecal IL-6 levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the *E. coli* DH5-α group). The data represent the mean ± SEM; $P \geq 0.05$.

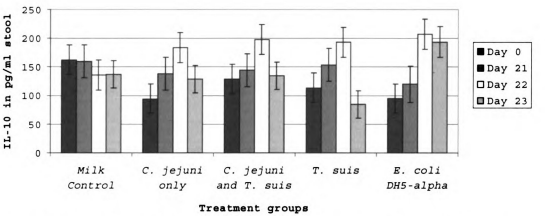


Figure 2.19. Changes in fecal IL-10 levels were measured using ELISA at various time points over a 23-day period. Fecal samples were collected from infected, weaned piglets. The values shown are means of two identical long term (23 days) challenge experiments with a total of 11 pigs in each group (n=8 in the E. coli DH5-α group). The data represent the mean ± SEM; $P \geq 0.05$.

Chapter 3: "Evaluation of proinflammatory cytokine production in intestinal pig epithelial cells infected with *Campylobacter jejuni*"

Abstract

C. jejuni is one of the leading causes of gastroenteritis throughout the world. Enterocytes serve as the first line of defense during bacterial infection, and *C. jejuni*-enterocyte interaction may play a significant role in the production of an acute inflammatory response. It has been shown *in vitro* that *C. jejuni* interacts with intestinal epithelial cells and stimulates the production of proinflammatory cytokines and chemokines. Therefore, we hypothesized that cultured undifferentiated, crypt-like intestinal pig epithelial cells (IPEC-1 cells) secrete TNF-α, IL-1-β, and IL-8 following *C. jejuni* infection. To test this hypothesis, we infected cultured IPEC-1 cells with *C. jejuni* and measured IL-8, TNF-α, and IL-1-β production. Supernatants were collected from IPEC-1 cells at 0, 8, 24, and 48 hours post-*C. jejuni* infection, and cytokine expression was measured using an enzyme-linked immunoabsorbant assay (ELISA). *C. jejuni* infection of IPEC-1 cells significantly induced TNF-α and IL-1-β production. In addition, we found that IPEC-1 cells constitutively secrete IL-8, and IL-8 production was not induced by *C. jejuni* infection. Our data suggest that induced TNF-α and IL-1-β production and constitutively secreted IL-8 may play a role in an innate inflammatory response that generates or modulates immune responses during *C. jejuni* infection *in vivo*. We also pretreated undifferentiated IPEC-1 monolayers with recombinant swine IL-8, TNF-α, or IL-1-β to determine the direct effects of these cytokines on *C. jejuni* adherence, invasion, and the transepithelial electrical resistance of IPEC-1 cells. Our

findings suggested that neither IL-8 nor IL-1-β pretreatments had a significant effect on *C. jejuni* invasion, adherence, or the transepithelial electrical resistance of IPEC-1 cells. In addition, we found that TNF-α did not significantly affect *C. jejuni* adherence to IPEC-1 cells or the transepithelial electrical resistance of IPEC-1 cells. Taken together, the data presented here suggest that the induced production of TNF-α and IL-1-β and the constitutive production of IL-8 from intestinal epithelial cells are indicative of an acute innate host immune response that may be responsible in part for eliminating these bacteria from the host *in vivo*.

Introduction

Campylobacter jejuni is a Gram negative, microaerophilic, rod-shaped bacterium which is known throughout the world as one of the leading causes of acute gastroenteritis (34). Documented clinical symptoms related to *C. jejuni* infection are abdominal cramps, diarrhea, fever, general malaise, backache, headache, and arthralgias (5, 6, 11, 12, 34). In human clinical infection studies, fresh blood, mucus, and polymorphonuclear leukocytes are found in diarrheic stool samples (34). In most cases, illness is self-limiting, and the duration of the illness is usually 1 week or less (34). In addition to the immediate effects of acute *Campylobacter* infection, there are long-term sequelae associated with campylobacteriosis such as reactive arthritis (ReA), Reiter's syndrome, Fisher syndrome, and Guillain-Barré Syndrome (41). These post-infectious sequelae result in serious health problems and have a significant economic impact (19, 28, 35, 41, 49, 54). The causative mechanisms of disease by Campylobacter infections are not well understood. However, it is believed that host immune responses, such as cytokine production, may play an important role in the pathogenesis of the disease.

Presently, there is limited knowledge about immune responses elicited during *C. jejuni* infection. *In vitro*, human intestinal epithelial cells (INT 407), monocytic cells, avian primary chick kidney cells, and an avian macrophage cell line secrete pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1-β and IL-6) and chemokines (IL-8, MCP-1, and MIP-1) following infection with viable *C. jejuni* (4, 25, 27, 31, 43). In these systems, *C. jejuni* invade and are found within vacuoles. *C. jejuni* also invades intestinal pig

epithelial cells (IPEC-1) with a differentiated phenotype and stimulates IL-6, IL-10, and IL-18 production (Parthasarathy, dissertation, 2004 and Jones, dissertation, 2005).

In a *Campylobacter* swine disease model, weaned piglets infected orally with *C. jejuni* show MCP-1, TNF-α, IL-12p40, IFNγ, IL-4, and IL-10 mRNA expression in the jejunum at 48 hours post-infection, and *C. jejuni* infection causes lesions in the jejunum and mild crypt distension with sloughing epithelial cells and mucus production. (Jones, dissertation, 2005). These reported findings suggest that cytokines may play a significant role in *C. jejuni*-induced intestinal inflammation and disease pathology. Further evaluation of *C. jejuni*-host cell interaction and cytokine expression is necessary to elucidate the immune response elicited during infection.

There has been limited data reported which suggest that *C. jejuni* interaction with or invasion of epithelial cells play a role in stimulating cytokine production (4, 9, 25-27, 43). *C. jejuni* invasion of intestinal epithelial cells has been observed *in vitro* and *in vivo* and has been suggested as playing an important role in the pathogenesis of campylobacteriosis (56, 59). However, to date, a direct correlation between *C. jejuni* invasion of intestinal epithelial cells and subsequent cytokine production resulting in disease and pathology has not been established. These types of studies cannot be done in human patients. However, in *C. jejuni*-infected pigs, the bacterial cells have been found, via immunohistochemical staining, to be localized in the crypts of the distal colon, the lymphoglandular complexes (LGCs, and within the follicle-associated epithelium of the LGCs (10, 39)(Jones, dissertation, 2005). Also, haemotoxylin and eosin (H&E) staining of intestinal tissue samples from these *C. jejuni*-infected pigs showed mild crypt distension with sloughed epithelial cells and mucus. Likewise, in these experiments,

proinflammatory cytokine production was associated with *C. jejuni* infection of tissues (Jones, dissertation, 2005). Because it has been shown that *C. jejuni*-host-cell interaction can trigger an immune response (such as stimulating intestinal epithelial cells to produce pro-inflammatory cytokines and chemokines) and these events are associated with disease and pathology, we hypothesize that pro-inflammatory cytokine production of IECs may also affect epithelial cell integrity and play a role in affecting *C. jejuni* adherence, invasion, and/or the transepithelial resistance of intestinal epithelial cells. Others have shown that specific cytokines (e. g. TNF-α, IFN-γ, and IL-1-β) can alter intestinal epithelial cell function or integrity of the epithelial barrier, thereby facilitating bacterial-enterocyte interactions and causing serious pathological consequences (24, 48, 51).

We designed this study to better understand the role played by undifferentiated, crypt-like intestinal epithelial cells in a natural host in immunity and immunomodulation following *C. jejuni* infection. We used undifferentiated, crypt-like IPEC-1 cells derived from an unsuckled newborn piglet to evaluate pro-inflammatory cytokine production following *C. jejuni* infection and some of the possible roles these cytokines play in *C. jejuni* infection. Because *C. jejuni*-enterocyte interaction has been shown to trigger an acute immune response *in vitro* and *in vivo*, we hypothesized that undifferentiated, crypt-like IPEC-1 cells secrete TNF-α, IL-1-β, and IL-8 in response to *C. jejuni* infection. Our findings show that *C. jejuni* induces these swine intestinal epithelial cells to produce TNF-α and IL-1-β and that IL-8 is not induced by *C. jejuni* exposure but is constitutively secreted by these cells. These findings suggest that this early, innate response may participate in stimulating adaptive Th1-type adaptive immune responses which play an

important role in bacterial elimination in other hosts. In addition, to elucidate the roles of pro-inflammatory cytokines in *C. jejuni*-intestinal epithelial cell interaction, we also evaluated how the treatment of IPEC-1 cells with recombinant swine TNF-α, IL-1-β, or IL-8 directly affects *C. jejuni* adherence, invasion and/or the transepithelial electrical resistance. We found that these pro-inflammatory cytokines do not influence *C. jejuni* adherence and/or invasion of intestinal epithelial cells, nor do they stimulate changes in the monolayer integrity to facilitate *C. jejuni* infection.

Materials and Methods

Bacteria and growth conditions

C. jejuni strain ATCC 33292 was grown at 37°C, 5% CO_2 on Bolton agar plates. This strain was originally isolated from a human with enteritis and was rederived by a single passage in a gnotobiotic pig. *Escherichia coli* DH5-α was a noninvasive negative control for the invasion assay, and *E. coli* O157:H43 was a positive control for the adherence assay. *E. coli* O157:H43 (DEC7A) was kindly provided by Dr. Thomas Whittman of Michigan State University. The *E. coli* strains were grown on Bolton agar at 37°C. All bacterial culture media were obtained from Oxoid (Oxoid Inc, Basingstoke, Hampshire, England) unless otherwise stated. All bacteria were taken from frozen stocks, streaked for isolation onto agar and incubated for 24-48 hours. For inoculation, the *C. jejuni* and *E. coli* isolated colonies were subcultured for 20 hours for confluent growth, harvested with sterile swabs, and suspended in RPMI 1640 medium (Invitrogen, Invitrogen, Frederick, MD) without phenol red, fetal bovine serum (Invitrogen, Frederick, MD) or insulin-transferrin selenium (Invitrogen, Frederick, MD). The optical density (OD_{560}) was adjusted to 0.1 to achieve 5×10^8 colony forming units per milliliter. Dilutions of the bacteria were made in RPMI 1640 medium (Invitrogen, Frederick, MD) without phenol red, fetal bovine serum (FBS; Invitrogen, Frederick, MD) and insulin-transferrin selenium (ITS; Invitrogen, Frederick, MD) or Bolton broth (*C. jejuni* and *E. coli* strains) as required to achieve a particular bacterial dose.

Cultured epithelial cells

IPEC-1 (intestinal pig epithelial cells) is a non-immortalized cell line originally derived from the small intestine of an unsuckled neonatal piglet. In this study, these cells were used in an undifferentiated state to represent the rapidly dividing basilar intestinal epithelial crypt cells. Two-to-three days after seeding, they form confluent monolayers but do not have tight junctions or distinct apical or baso-lateral surfaces, which is characteristic of the morphology of intestinal epithelial crypt cells.

All culture media and growth supplements were obtained from Invitrogen (Frederick, MD) unless otherwise stated. Growth flasks, flat bottom polystyrene plates, and transwells were obtained from Corning (Costar, Corning, NY). IPEC-1 cells were routinely maintained in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and supplemented with 1% insulin-transferrin selenium (ITS) and grown to a confluent monolayer in T75 flasks at 37°C in a humidified atmosphere with 5% CO_2. For infection studies, the cells were gently washed with versene and released from the flask with 0.05% Trypsin-EDTA. The cells were resuspended in RPMI 1640 supplemented with 5% FBS and 1% ITS. A subsample (50 ul) of the cells was mixed 1:1 with volume of trypan blue (Sigma, St. Louis, MO), then loaded onto a hemocytometer (Hausser Scientific, Horsham, PA), and viable cells were counted on a Nikon inverted microscope (Mager Scientific, Dexter, MI). Cells were seeded onto fibronectin coated (20 ug/ml) (Sigma, Inc., St. Louis, MO) 12-well Costar tissue culture plates (Costar, Corning, NY) at the appropriate density for each experimental design. The cells were incubated at 37°C in humidified atmosphere with 5% CO_2 and allowed to grow 2-3 days to form confluent, undifferentiated monolayers.

Experimental Design

C. jejuni infection of IPEC-1 cells for IL-8, IL-1-β, and TNF-α analysis

To determine the effect of *C. jejuni* on IL-8, IL-1-β, and TNF-α secretion from undifferentiated crypt-like cells, confluent IPEC-1 monolayers were seeded at a density of 4×10^6 per well and were infected with *C. jejuni* at multiplicity of infection (MOI) of 40:1 and 200:1 and 400:1. The *C. jejuni* inocula were prepared by diluting the stock (5×10^8 CFU/ml, OD_{560} 0.1) to the desired multiplicity of infection. The negative control treatment was RPMI 1640 medium without phenol red, FBS or ITS. The positive control treatment for IL-8 and TNF-α induction was phorbol myristate acetate (PMA) and calcium ionophore (CI), each at a final concentration of 100 ng/ml. The positive control treatments for IL-1-β induction were recombinant swine TNF-α (500 pg/ml per well) or phytohemagglutinin (PHA; 50 ug/ml per well) (Table 3.1). All treatments were prepared and diluted in RPMI 1640 medium without phenol red, FBS or ITS to eliminate any false positive results. Each treatment was applied in a final volume of one milliliter, and three replicates of each treatment were analyzed for cytokine production. The IPEC-1 cells were exposed to these treatments for 0, 8, 24 and 48 hours. The supernatants were collected from the wells at the time intervals described. In order to measure cytokine expression at 0 hours, the growth medium containing RPMI 1640 supplemented with 5% FBS and 1% ITS was removed, and the treatments were immediately applied to the confluent cell monolayer, and the supernatants were immediately collected. All supernatants from each treatment group were centrifuged at 4°C, 10,000 rpm for 5 minutes to remove cell debris, transferred to fresh 1.5 ml tubes, and stored at -80°C.

Levels of secreted IL-8, IL-1-β, and TNF-α were measured from the supernatants by enzyme-linked immunoabsorbant assay (ELISA).

Assay for IL-8, IL-1-β, and TNF-α secretion

IL-8, IL-1-β, and TNF-α were measured using swine sandwich ELISA kits obtained from Biosource International (Camarillo, CA); assays were performed according to the manufacturer's instructions. Each assay contained a IL-8, IL-1-β, or TNF-α standard protein control. A microplate reader (EL800 Universal Microplate Reader, Bio-Tek Instruments, Winooski, Vermont) was used to measure the absorbance at 450 nm, and the concentration of IL-8, IL-1-β, or TNF-α in each sample was quantified using KCjunior® software (Bio-Tek Instruments, Winooski, VT).

Invasion assay

To determine the direct effect of exogenous IL-8, IL-1-β, or TNF-α on *C. jejuni* invasion of IPEC-1 cells, confluent monolayers were pretreated with recombinant swine IL-8 (0, 100, 250, 500, 750, or 1000 pg/ml); IL-1-β (0, 3000, 30,000, or 300,0000 pg/ml); or TNF-α (0, 50, 500, or 5000 pg/ml) (Biosource International, Camarillo, CA) in a dose-response design (Table 3.2). The cytokine concentrations used in this experiment were determined by reports from the literature and preliminary data from previously conducted dose-response experiments in our laboratory. Each cytokine was diluted to the appropriate concentration in RPMI 1640 medium without phenol red, FBS or ITS and applied to the cells in a final volume of 1 ml. The monolayers were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 hours. The cytokine pretreatment medium

was removed; the monolayers were not washed in order to avoid cell damage or detachment. *C. jejuni* ATCC 33292 or *E. coli* DH5-α (noninvasive positive control) inocula were added to the confluent IPEC-1 cells at a MOI of 100:1 (approximately 2×10^8 CFU/ml) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 hours to allow bacterial invasion of the cells. The medium was removed and gentamicin (Invitrogen, Frederick, MD), diluted in RPMI 1640 without phenol red, at a final concentration of 100 ug/ml, was added to the monolayers. The monolayers were incubated for 1 hour to kill the extracellular bacteria, and then the gentamicin was removed. The monolayers were lysed with 0.1% sodium deoxycholate (Sigma, Inc., St. Louis, MO) in 1X Phosphate Buffered Saline (0.01 M). The internalized bacteria were enumerated by serial dilution and spreading on Bolton agar. After 48 hours incubation, the colonies were counted to determine the number of internalized bacteria. All treatments were performed in triplicate.

Adherence Assay

To determine the direct effect of exogenous IL-8, IL-1-β, or TNF-α on *C. jejuni* adherence to IPEC-1 cells, confluent monolayers were pretreated with each recombinant swine cytokine as previously described for the invasion assay (Table 3.2). After cytokine pretreatment, *C. jejuni* ATCC 33292 or *E. coli* O157:H43 (DEC7A) (adherence positive control strain) inocula were added to the confluent IPEC-1 cells at a MOI of 100:1 (approximately 2×10^8 CFU/ml) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 1 hour to allow bacterial adherence to the cells. The medium was then removed; the monolayers were not washed in order to avoid cell damage or detachment.

The monolayers were lysed with 0.1% sodium deoxycholate (Sigma, Inc., St. Louis, MO) in 1X Phosphate Buffered Saline (0.01 M). The cell-associated (adherent) bacteria were enumerated by serial dilution plating on Bolton agar and counting CFUs at 48 hours after incubation. All treatments were performed in triplicate.

Transepithelial resistance assay

To determine the effect of exogenous recombinant cytokines on IPEC-1 monolayer integrity, we measured the transepithelial resistance (TER) across the monolayers before and after treatment with recombinant swine IL-8, IL-1-β, or TNF-α (Biosource International, Camarillo, CA). IPEC-1 cells were seeded at a density of 2×10^6 per well onto 24-Transwell inserts (6.5 mm diameter, 3 μm pore size, Costar, Corning, NY) coated with 20 ug/ml fibronectin in order to measure the TER. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and allowed to grow 2-3 days to form confluent monolayers. All treatments were prepared and diluted in RPMI 1640 medium without phenol red, FBS or ITS and applied to the cells in a final volume of 1 mL.

The TER across the IPEC-1 monolayers was measured before cytokine treatment using an electrode (EVOMX, World Precision Instruments, Sarasota, FL) to assess monolayer integrity. TER values between 150-600 Ωcm^2 were considered evidence of confluent monolayers. The monolayers were exposed to recombinant swine IL-8 (250 pg/ml), IL-1-β (3000 pg/ml), TNF-α (500 pg/ml), or 0 pg/ml. They were next incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 hours, and then the TER was measured again. All treatments were performed in quadruplicate.

Statistical Analysis

ELISA data were analyzed using the mixed model procedure in the Statistical Analysis System (SAS)(SAS Institute Inc., Cary, NC) program. The fixed effects in this model were time and treatment. The random effect in this model was the plate. P values ≤ 0.05 were considered significant.

Invasion and adherence data were analyzed using the mixed model procedure in the Statistical Analysis System (SAS, SAS Institute Inc., Cary, NC) program. The fixed effects in this model were experiment and treatment. The random effect in this model was the plate. P values ≤ 0.05 were considered significant.

Transelectrical resistance (TER) data were analyzed in the Statistical Analysis System (SAS)(SAS Institute Inc., Cary, NC) program. A generalized linear model with one factor (treatment) was used to determine significance. P values ≤ 0.05 were considered significant.

Results

Sensitivity of TNF-α, IL-1-β, and IL-8 ELISAs

Figures 3.1 to 3.3 show the standard curves for the TNF-α, IL-1-β and IL-8 ELISAs, respectively. The regression curves for these assays were obtained using KCjunior® software (Bio-Tek instruments, Winooski, Vermont). The TNF-α, IL-1-β and IL-8 regression curves showed significant dose response curves with the concentration range and antibody titer used, respectively, R = 1.000; R = 0.9999; R = 0.9999. The lower limit of detection of the TNF-α and IL-1-β assays is ≥ 6 pg/ml, and the lower limit of detection of the IL-8 assay is ≥ 10 pg/ml.

Cytokine secretion from *C. jejuni*-infected IPEC-1 cells

Different ratios of bacteria to IPEC-1 cells were used in these experiments to determine the time course of induction and optimize the assay (Table 3.1).

We exposed undifferentiated IPEC-1 cells to *C. jejuni* 33292 for 0, 8, 24, and 48 hours to determine levels of TNF-α, IL-1-β, and IL-8 expression following bacterial infection. Figure 3.4 shows that IPEC-1 cells significantly secreted TNF-α post-*C. jejuni* infection at an optimal MOI of 400:1 at 8 hours (176.97 ± 15.25 pg/ml), 24 hours (354.98 ± 15.25 pg/ml), and 48 hours (181.81 ± 15.25 pg/ml) as compared to the RPMI 1640 medium control at 8 hours (16. 56 ± 18.67 pg/ml), 24 hours (22.83 ± 15.25 pg/ml), and 48 hours (28. 07 ± 15.25 pg/ml); all $p < 0.0001$. We found that *C. jejuni*-infected IPEC-1 cells showed maximum induction of TNF-α production at a bacterial MOI of 400:1 at 24 hours post-infection. We also note that at 0 hours an elevated level of TNF-α was detected in our RPMI 1640 medium control, and we speculate that this response was

likely due to physical stress on the IPEC-1 cells induced by the rapid manipulation during the experimental technique (described in the materials and methods) used to challenge the cells.

We also found that exposure of IPEC-1 cells to *C. jejuni* 33292 significantly induced IL-1-β (Figure 3.5). By 24 hours post-infection, IL-1-β was induced at a *C. jejuni* MOI of 400:1(134.27 ± 16.12 pg/ml) as compared to the RPMI 1640 medium control (40.47 ± 16.12 pg/ml; $p = 0.025$). In preliminary trials, IPEC-1 cells were tested for positive IL-1-β induction using phytohemagglutinin (PHA 50 ug/ml) or recombinant swine TNF-α (500 pg/ml); over a wide range of time points. Levels of IL-1-β were found to be beneath the level of detection of the assay (TNF-α stimulation) or were not significantly different from the RPMI 1640 control (PHA stimulation). Therefore, induction in this assay was defined as a positive response that was more than 3 fold greater than the control. Because the possible positive control treatments were weak or unavailable, this could be an experimental drawback. Follow-up experiments were conducted to determine if the recombinant TNF- α was active, and results showed that administered recombinant TNF- α actively stimulated IPEC-1 cells to induce IL-8 (data not shown). However, in this case, recombinant swine TNF- α did not effectively stimulate IPEC-1 cells to significantly produce IL-1-β.

IPEC-1 cells were not stimulated by *C. jejuni* 33292 exposure to secrete significant levels of IL-8; however, IL-8 protein was constitutively expressed by IPEC-1 cells. The positive control (PMA/CI) significantly induced IL-8 by 8 hours, and IL-8 levels remained significantly elevated through 48 hours as compared to the RPMI 1640 medium control (Figure 3.6A, all $p < 0.0001$). Figure 3.6B shows that IL-8 is

constitutively expressed by IPEC-1 cells. From the supernatants of the RPMI 1640 (control), *C. jejuni* MOI 40:1, and *C. jejuni* MOI 200:1 treatment groups, we detected IL-8 production at 24 hours post-treatment and continued detecting increased IL-8 levels through 48 hours. From the supernatants of the *C. jejuni* 400:1 treatment group, we detected IL-8 production as early as 8 hours post-treatment and continued detecting a rise in IL-8 levels through 48 hours. Although increased IL-8 levels were detected in the supernatants of each treatment group, there were no statistically significant changes in IL-8 secretion in response to *C. jejuni* infection of IPEC-1 cells (Figure 3.6B, $p > 0.05$). In these experiments, we determined that IL-8 secretion and accumulation was apparently time related and not dependent on the *C. jejuni* challenge dose given.

Effects of cytokine pretreatment on *C. jejuni* invasion and adherence of IPEC-1 cells

IPEC-1 cells were pretreated with IL-8, TNF-α, or IL-1-β to determine if these cytokines have a direct effect on the interaction between *C. jejuni* and crypt-like basal epithelial cells (IPEC-1 cells), specifically, effects on the integrity of the epithelial cell barrier. Treatment of IPEC-1 cells with recombinant swine IL-1-β or IL-8 did not have a significant effect on *C. jejuni* adherence and/or invasion (IL-1β, Figures 3.10 and 3.11; IL-8, Figures 3.13 and 3.14; all $p > 0.05$). Therefore, *C. jejuni* 33292 did adhere to and invade IPEC-1 cells regardless of recombinant swine IL-1-β or IL-8 pretreatment of IPEC-1 cells. Also, treatment of IPEC-1 cells with recombinant TNF-α did not have an effect on *C. jejuni* adherence to IPEC-1 cells (Figure 3.8, $p > 0.05$), and we could not draw a conclusion about its effects on *C. jejuni* invasion of IPEC-1 cells because of the variability observed in the number of internalized bacteria (Figure 3.7, $p > 0.05$). *E. coli*

DH5-α (a noninvasive strain) was the control for the invasion assays and neither TNF-α, IL-1-β, nor IL-8 affected *E. coli* DH5-α interaction with IPEC-1 cells; *E. coli* DH5-α did not invade IPEC-1 cells regardless of cytokine pretreatment of the IECs. *E. coli* O157:H43 DEC7A was the control for the adherence assays, and neither TNF-α, IL-1-β, nor IL-8 affected *E. coli* O157:H43 DEC7A adherence to IPEC-1 cells (TNF-α, Figure 3.9; IL-1β, Figure 3.12; IL-8, Figure 3.15)(all $p > 0.05$).

Effects of cytokine pretreatment on IPEC-1 transepithelial resistance (TER)

IPEC-1 cells were pretreated with IL-8, TNF-α, or IL-1-β to determine whether these cytokines have a direct effect on the transepithelial electrical resistance of IPEC-1 cells. Treatment of IPEC-1 cells with recombinant swine IL-8, TNF-α, or IL-1-β did not have a significant effect on the transepithelial electrical resistance of IPEC-1 cells (Figure 3.16; $p > 0.05$). IPEC-1 cells pretreated with RPMI 1640 medium alone had a TER of ~300 Ωcm^2. Experimental TER values between 150-600 Ωcm^2 were considered as evidence of confluent monolayers with no evidence of damage to the integrity of the cells post-treatment with recombinant swine cytokines.

Discussion

C. jejuni invasion or stimulation of intestinal epithelial cells (IECs) has been shown to significantly induce the expression and up-regulation of pro-inflammatory cytokines and chemokines (4, 25-27, 32). *In vivo*, a robust pro-inflammatory cytokine response is detected in crypt-like epithelial cells (Jones, dissertation, 2005), and *Campylobacter* organisms are associated with these surface epithelial cells within the crypts and were found within the crypts of lymphoglandular complexes in the distal colon (Jones, dissertation, 2005)(40). Pro-inflammatory cytokines such as IL-8, TNF-α and IL-1-β can act as mediators of inflammation and regulators of immune responses which are associated with an innate immune response that facilitates bacterial elimination during infection (37). In this study, we hypothesized that intestinal pig epithelial cells (IPEC-1 cells), which serve as a model of undifferentiated, crypt-like cells, secrete pro-inflammatory cytokines (specifically IL-8, TNF-α, and IL-1-β) in response to *C. jejuni* infection when challenged *in vitro*. We also examined the treatment of confluent IPEC-1 monolayers with recombinant swine IL-8, TNF-α, or IL-1-β to determine the direct effects of these cytokines on *C. jejuni* adherence, invasion, and/or the transepithelial electrical resistance of the IPEC-1 cells.

IPEC-1 cells were exposed to 5×10^8 CFU/ml of *C. jejuni* 33292 for 0, 8, 24 and 48 hours. At 8, 24, and 48 hours post-infection, we measured significantly increased levels of TNF-α compared to the uninfected controls. Maximum TNF-α induction was detected 24 hours following bacterial infection at an MOI of 400:1. Likewise, we measured significant levels of IL-1-β at 24 hours post-infection. Based on the data

presented here, *C. jejuni* challenge induces IPEC-1 cells to secrete TNF-α and IL-1-β at significant levels. This finding is comparable to other *in vitro* reports that demonstrate the production of pro-inflammatory cytokines by intestinal epithelial cells following *C. jejuni* infection (4, 9, 25, 27). Human intestinal epithelial cells, INT 407 cells, secrete an array of pro-inflammatory chemokines (IL-8, MCP-1, GROalpha, GROgamma, and gammaIP-10) when stimulated with *C. jejuni* (4, 27). *C. jejuni* has also been shown to stimulate INT-407 cells to produce intracellular IFN-γ, IL-10, TNF-α, and IL-4 (4, 47). Induction of pro-inflammatory cytokines to *C. jejuni* challenge is also observed *in vivo*. Pigs rectally challenged with *C. jejuni* for 1 hour show significant up-regulation of intracellular TNF-α, IL-18, IL-8, GM-CSF, IL-1-β, iNOS, and MCP-1 in the distal colon and lymphoglandular complexes (LGCs)(Jones, dissertation, 2005). It has also been demonstrated in Balb/c mice that *C. jejuni* induces a peritoneal inflammatory cytokine response and increased peritoneal phagocyte oxidative activity (31, 47). Given that increased pro-inflammatory cytokine expression elicited by *C. jejuni* infection of intestinal cells has been demonstrated *in vitro* and *in vivo*, it was not surprising that we detected significant elevated TNF-α and IL-1-β levels secreted by IPEC-1 cells following *C. jejuni* exposure; the production of these cytokines likely plays a significant role in host innate immunity to this bacteria.

Furthermore, in this *in vitro*, *C. jejuni*-challenge study, the cytokine response of the undifferentiated IPEC-1 cells is consistent with other *in vivo* and *in vitro* findings (4, 9, 25, 27, 53) (Parthasarathy, dissertation, 2004 and Jones, dissertation, 2005). Because IPEC-1 is a non-immortalzied cell line, the cytokine secretion pattern is likely more reflective of the physiology in the host than that represented by an immortalized cell line.

This is strengthened by the fact that concurrent studies conducted in our laboratory have shown that *C. jejuni* also invade differentiated IPEC-1 cells and stimulate these cells to produce cytokines such as IL-18, IL-6, or IL-10 (Parthasarathy, dissertation, 2004 and Jones, dissertation, 2005). Likewise, previous *in vivo* data from our laboratory show that *C. jejuni* stimulates a proinflammatory cytokine response after invading intestinal tissue and has been identified in the intestinal crypt cells of infected pigs (Kathryn, dissertation, 2005). Our findings support the hypothesis that primary, undifferentiated intestinal pig epithelial cells (IPEC-1) can serve as a more suitable model to mimic the enteric immune response of human undifferentiated intestinal crypt cells as compared to other *in vitro* systems that involve the use of immortalized cell lines, such as INT 407, T84, and HEp-2 cells. IPEC-1 cells were derived from a newborn unsuckled piglet, and pigs are monogastric and have dietary habits and anatomical and physiological characteristics similar to humans (7). Therefore, the overall similarities between cultured primary intestinal pig epithelial cells (IPEC-1) and human intestinal cells allow for a better evaluation of bacterial virulence, host immune mechanisms, and the possible role played by intestinal epithelial cells as an important source and initiator of enteric immune responses following assault.

The functions of IL-1-β and TNF-α are similar and play a significant role in enteric immune responses. TNF-α activates neutrophils, mononuclear phagocytes and eosinophils which can amplify the mucosal inflammatory response to efficiently eradicate infectious microorganisms (1). *In vivo*, mice exposed to a primary *L. monocytogenes* or *C. jejuni* infection produced significant plasma TNF-α which is believed to enhance innate immune functions(37). Together or independently, TNF-α or IL-1-β can also

stimulate inflammatory cells to produce IL-8 which contributes to neutrophil influx, and the combined action of TNF-α, IL-1-β and IL-6 contribute to systemic acute-phase effects which can induce fever (increased body temperature is not conducive for the growth of some pathogenic bacteria)(57). In addition, it is suggested that IL-1-β plays a pivotal role in early events leading to inflammation (50, 57). *In vitro*, nontransformed epithelial cells rapidly express IL-1-β mRNA, and secreted IL-1-β initiates and enhances the acute inflammatory response following bacterial infection (16, 29, 57). IL-1-β has also been shown to be predominantly expressed in undifferentiated cells located in the basal mucosal crypts following acute experimental colitis . Therefore, in agreement with these findings, the present data suggest that the significant production of TNF-α and IL-1-β in response to *C.jejuni* infection of IPEC-1 cells may play a significant role in initiating and modulating immune responses that facilitate the elimination of *C. jejuni* infection *in vivo*.

IL-8 is a proinflammatory cytokine that is a chemoattractant which induces adherence to vascular endothelium and extravasation into tissues (17). We found that IPEC-1 cells constitutively secrete IL-8 protein, and IL-8 production was not induced by *C. jejuni* infection. The constitutive expression of IL-8 by the undifferentiated, crypt-like IPEC-1 cells is consistent with other reports showing that various intestinal epithelial cell lines (T84, Caco-2, SW620, and HT29) constitutively secrete IL-8. Also, some of these cell lines can significantly express IL-8 depending on the stimulant (17, 18, 25, 43). Even though IPEC-1 cells were not induced to secrete IL-8 following *C. jejuni* exposure, the constitutive expression of IL-8 protein constitutes a significant inflammatory response when one considers the number of epithelial cells lining the colon. *In vivo*, the gut epithelium is continuously exposed to a variety of harmless microflora, antigenic dietary

constituents, and bacterial pathogens that can damage it by directly invading the mucosa or by toxin production. It is possible that there is a continuous, basal level of inflammation within the intestinal mucosa which can be up-regulated when necessary in response to pathogens and noxious antigens thereby providing protective immunity (16, 60). The constitutive production of IL-8 at low levels along the gut mucosal epithelium could be partially responsible for a consistent low-level inflammatory cellular infiltration of neutrophils and monocytes which would be favorable to the host (16, 25, 43). Therefore, we suggest that intestinal pig epithelial cells (IPEC-1 cells) constitutively secrete IL-8 protein as a molecular inflammatory mediator of innate host defense which may play a role in limiting microbial exposure at the mucosal surface and maintaining tissue integrity for low level insults. On the other hand, mature differentiated epithelial cells lining the villus tips of the colon or dendritic cells and macrophages populating the lamina propria immediately underlying the epithelium may be responsible for the IL-8 expression hypothesized to draw neutrophils to the this site.

Because others have reported that IL-8 is significantly induced via NFκB activation through IκBα degradation following *Salmonella Dublin*, enteroinvasive *Escherichia coli*, *Yersinia enterocolitica*, *Helicobacter pylori*, or *C. jejuni* invasion or stimulation of IECs (15, 20), we believe it is necessary to discuss a few possible reasons why IL-8 was not significantly induced by *C. jejuni* infection of IPEC-1 cells in our study. High concentrations of diverse bacteria and bacterial products continually interact with the intestinal mucosal epithelium. Because of this intimate contact, it is critical that the intestinal epithelia facilitate the control and maintenance of a normal healthy state to avoid persistent, dysregulated inflammation. Gut epithelial cells express toll-like

receptors (TLRs) which are a family of transmembrane receptors also termed pattern-recognition receptors (PRRs) (33, 42). TLRs play an important role in host innate immunity by recognizing pathogen-associated molecular patterns (PAMPs) present on diverse microbes including Gram-positive and Gram-negative bacteria, fungi, and mycobacteria (33). In general, infection of IECs can lead to activation of the TLRs which then initiates intracellular signaling cascades which can lead to the activation of the NFκB/Rel family transcription factors and cytokine production (2, 3, 38).

NFκB has been described as the central regulator of the intestinal epithelial cell innate immune response induced by enteroinvasive bacterial infection (20, 38). NFκB is activated by many extracellular stimuli via TLR interaction, and there are many signaling pathways that lead to NFκB activation which leads to the expression of many genes, including those encoding inflammatory cytokines (14, 38). More specifically, IL-8 production has been associated with TLR4 receptor signaling via LPS stimulation and NFκB activation (2, 3, 13, 21). It has been shown that immune-mediated signals regulate TLR-4 and MD-2 (a TLR4 coreceptor accessory molecule) expression, and changes in TLR-4 and MD-2 expression impact host cell responses to bacterial lipopolysaccharide (LPS) (20, 46, 60). In addition, it has been reported that TLR4 is barely detectable in isolated primary intestinal epithelial cells (38). Therefore, it is possible that decreased expression of Toll-like receptor-4 (TLR-4) and MD-2 or inhibition of intracellular signaling via the transcription factor can affect assessment of proinflammatory gene expression in some *in vitro* systems (3, 33). Hence, if TLR4 and its accessory molecules are only present at low levels or absent from the cell surface of IPEC-1 cells (isolated primary intestinal epithelial cells), then *C. jejuni* LPS either weakly interacts with TLR4

and its accessory molecules or does not bind the receptor complex to induce significant production of IL-8 thereby causing IPEC-1 cells to be unresponsive to this particularly bacterial component (*C. jejuni* LPS). It is also reasonable to think that *C. jejuni*-infected IPEC-1 cells can significantly produce IL-1-β and TNF-α and do not significantly induce IL-8 expression because induction of these cytokines is likely regulated via different Toll-like receptor signaling or different intracellular signaling pathways. It has been demonstrated that the flagellin of other bacteria, such as *Listeria monocytogenes* and *Salmonella typhimurium* , can bind to TLR5 receptors (TLRs that specifically recognize bacterial flagellin) to stimulate TNF-α, IL-8, or IL-1-β production (22, 33, 55), whereas, *C. jejuni* flagellin has been reported to be a poor stimulator of TLR5 and does not play a significant role in stimulating IL-8 production nor has it been tested to determine if it stimulates TNF-α or IL-1-β production (58). Even though not tested in this study, we suggest that *C. jejuni*-infected IPEC-1 cells express IL-1-β, TNF-α, or IL-8 via different TLR ligand signaling recognition or other intracellular signaling pathways. Additionally, recent multilocus sequence typing studies of *C. jejuni* strains in our lab suggest that some strains have LOS variants that may be more stimulating than others. Further studies are needed to elucidate the regulatory mechanisms involved in the interaction between *C. jejuni* bacterial products or components and intestinal epithelial cell TLR activation, intracellular signaling pathways, and cytokine gene expression and secretion.

Secreted cytokines can exert autocrine (self-stimulation), paracrine (adjacent), intracrine (within the cell) and some endocrine (distant) actions (23, 33, 42). Cytokines have been shown to modulate bacterial-enterocyte interactions *in vitro*. For instance, cytokines can disrupt intestinal epithelial cell function or barrier integrity (24, 48, 51).

Increased bacterial adherence has been associated with IFN-γ pretreatment of IECs suggesting that IFN-γ augments bacterial adherence to the intestinal epithelium (24). In addition, IFN-γ and TNF-α act synergistically on the epithelial barrier by causing reduced TER and altering cell morphology (21). Likewise, concurrent studies in our laboratory have shown that recombinant swine IL-4 (rIL-4) pretreatment of differentiated IPEC-1 cells significantly reduced the TER of IPEC-1 cells, increased *C. jejuni* internalization, and caused differential effects on adherence of two *C. jejuni* strains (Parthasarathy, dissertation, 2004). Because of the impact cytokines have on enterocytes and the possible roles they play in bacterial-enterocyte interaction, in this study, we pretreated the IPEC-1 cells with recombinant swine IL-8, TNF-α, or IL-1-β to determine if these cytokines would affect *C. jejuni* invasion, adherence and/or the transepithelial electrical resistance (TER) of IPEC-1 cells. Our results showed that *C. jejuni* 33292 does adhere to and invade IPEC-1 cells, and these data support previous findings from our laboratory (Parthasarathy, dissertation, 2004). However, neither IL-8 nor IL-1-β has a significant direct effect on *C. jejuni* invasion, adherence, and/or changes in transepithelial electrical resistance of undifferentiated IPEC-1 cells. Also, treatment of IPEC-1 cells with TNF-α did not significantly affect *C. jejuni* adherence or changes in the TER of the IECs. However, we were unable to draw conclusions about the effects of TNF-α on *C. jejuni* invasion of IPEC-1 cells because of the variability observed in these experimental results. Additional experiments could be conducted to address this concern; however, the lack of a significant effect under the rigorous experimental conditions employed, suggest efforts might be better placed elsewhere. Even though there was not a direct effect on *C. jejuni* adherence to or invasion of IPEC-1 cells (in response to IL-8 or IL-1-β pretreatment) or a

direct effect could not be determined following TNF-α pretreatment *in vitro*, it is still possible that IL-8, TNF-α, or IL-1-β may affect epithelial function in a paracrine-like or another autocrine-like manner during *C. jejuni* infection *in vivo*. For instance, it has been demonstrated that cytokines can facilitate enterocyte proliferation, maturation, MHC class II expression, cytokine receptor expression or apoptosis in healthy and diseased states (21, 48, 51). In addition, TNF-α and TGF-α have been shown to stimulate immature crypt cell proliferation, and TGF-β and INF-γ can inhibit mitotic activity (52). IFN-γ can alter immature crypt epithelial cell turnover and upregulate MHC class II expression (51). Therefore, alterations of cytokine concentrations as a consequence of bacterial exposure and intestinal inflammation may contribute to serious pathologic consequences. Further elucidation of the effects of distinct cytokines on epithelial cell growth, phenotype, function and bacterial-enterocyte interactions would be of importance in the understanding of cytokine mediated regulation of mucosal immune responses associated with *C. jejuni* infection.

In summary, we showed that undifferentiated, crypt-like intestinal pig cells (IPEC-1) can serve as a suitable *in vitro* model for studying *C. jejuni*-enterocyte interaction and host enteric immune responses elicited by *C. jejuni* during infection. We also found that *C. jejuni* infection of these cells significantly induces TNF-α and IL-1-β production. In addition, we observed that IPEC-1 cells constitutively secrete IL-8. The data presented here suggest that TNF-α and IL-1-β production and constitutively secreted IL-8 are significant inflammatory responses which are associated with an acute innate immune response and may lead to a healing Th1-type adaptive immune response. It is likely that this inflammatory response can generate and/or modulate the immune response

to benefit the host by contributing to the elimination of the bacteria. In addition, we found that neither IL-8 nor IL-1-β had a direct effect on *C. jejuni* adherence, invasion, or the TER of IPEC-1 cells. Likewise, we found that TNF-α did not have a direct effect on *C. jejuni* adherence to IPEC-1 cells or the TER of IPEC-1 cells. Even though these findings showed no direct effect of these cytokines on *C. jejuni* interactions (specifically adherence, invasion, or changes in IPEC-1 TER) with the swine intestinal epithelial cells, they could possibly have other autocrine- or paracrine-like effects which could play a role in host inflammatory immune responses associated with *C. jejuni* infection. For example, TNF-α and IL-1-β can stimulate intestinal epithelial cells to produce C3, a complement component which is associated with host acute-phase inflammatory responses (21, 44, 45, 48, 51). So, given that our data support the hypothesis that TNF-α, IL-1-β, and IL-8 are cytokines that are important components of the inflammatory immune response to enteric pathogens, we can utilize this information to more specifically determine, characterize, and elucidate the possible roles played by IL-8, TNF-α, and/or IL-1-β during *C. jejuni* infection.

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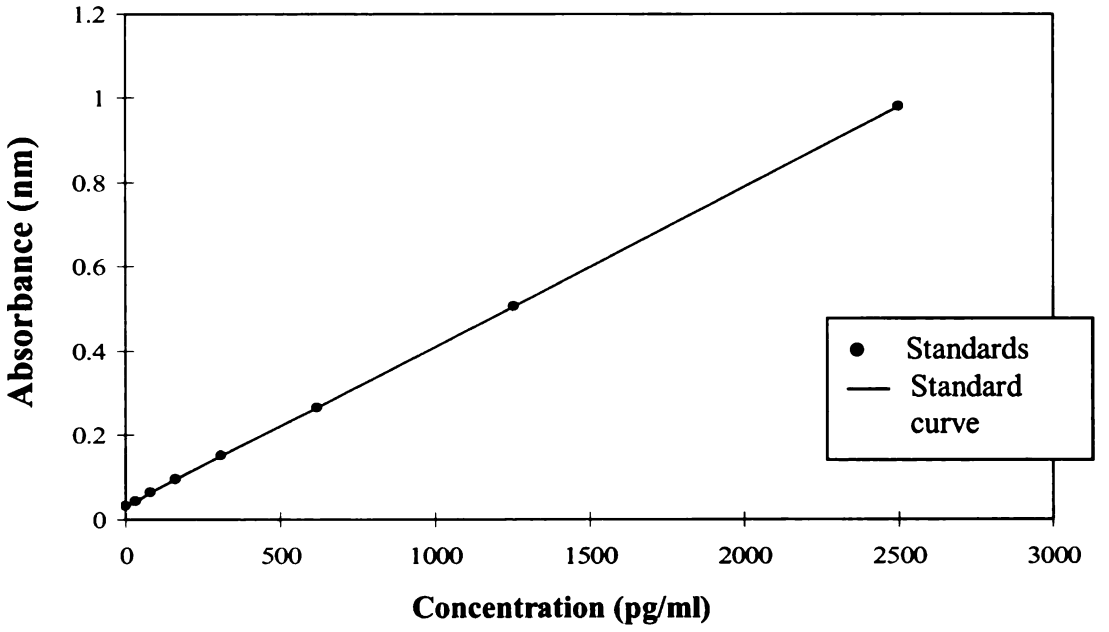
Table 3.1. IPEC-1 cells treatments for cytokine measurement (ELISA)

Treatment Group	Treatment
1	Positive controls: IL-8 and TNF-α phorbol myristate acetate (PMA) (100 ng/ml) calcium ionophore (CI) (100 ng/ml) IL-1-β phytohemagglutinin (PHA, 50 ug/ml) or recombinant swine TNF-α (rTNF-α, 500pg/ml)
2	*Campylobacter jejuni* 33292 (MOI 40:1)
3	*Campylobacter jejuni* 33292 (MOI 200:1)
4	*Campylobacter jejuni* 33292 (MOI 400:1)
5	Negative control: RPMI 1640 without phenol red, fetal bovine serum (FBS), or insulin-transferrin-selelium (ITS)

Table 3.2 Recombinant swine cytokine pretreatment concentrations for invasion and adherence assays

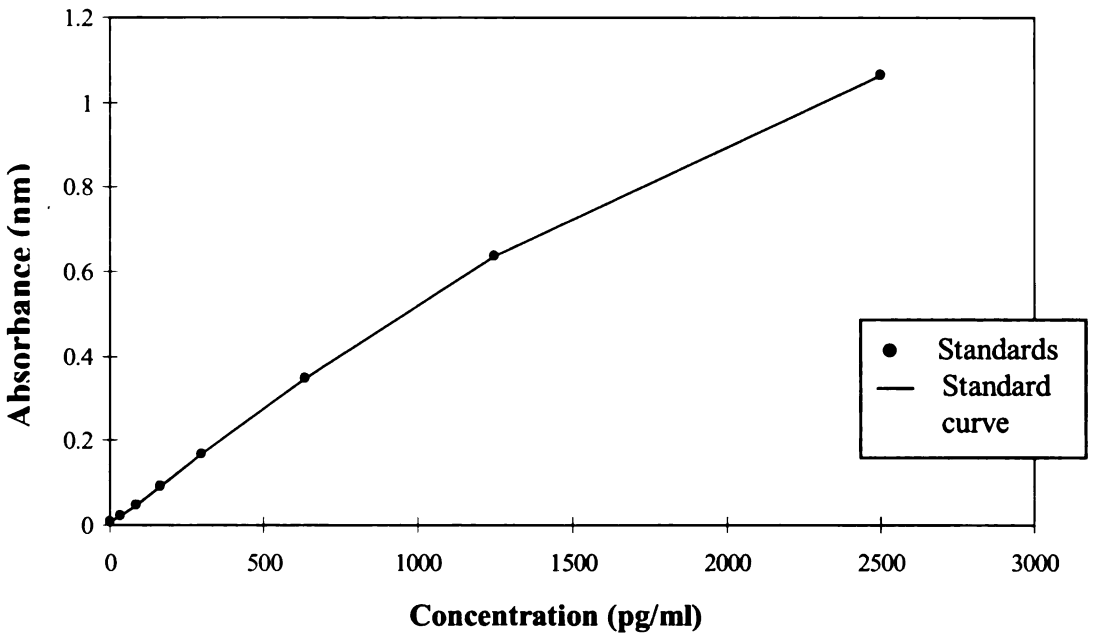
All treatments were suspended in RPMI 1640 without phenol red, fetal bovine serum (FBS) or insulin-transferrin-selenium (ITS). The negative control was identified as medium only.

Treatment Group	**Recombinant IL-8 (rIL-8)**	**Recombinant IL-1-β (rIL-1-β)**	**Recombinant TNF-α (rTNF-α)**
1	Negative control: Medium only 0 pg/ml	Negative control: Medium only 0 pg/ml	Negative control: Medium only 0 pg/ml
2	100 pg/ml	3000 pg/ml	50 pg/ml
3	250 pg/ml	30,000 pg/ml	500 pg/ml
4	500 pg/ml	300,000 pg/ml	5,000 pg/ml
5	750 pg/ml	Not applicable	Not applicable
6	1000 pg/ml	Not applicable	Not applicable



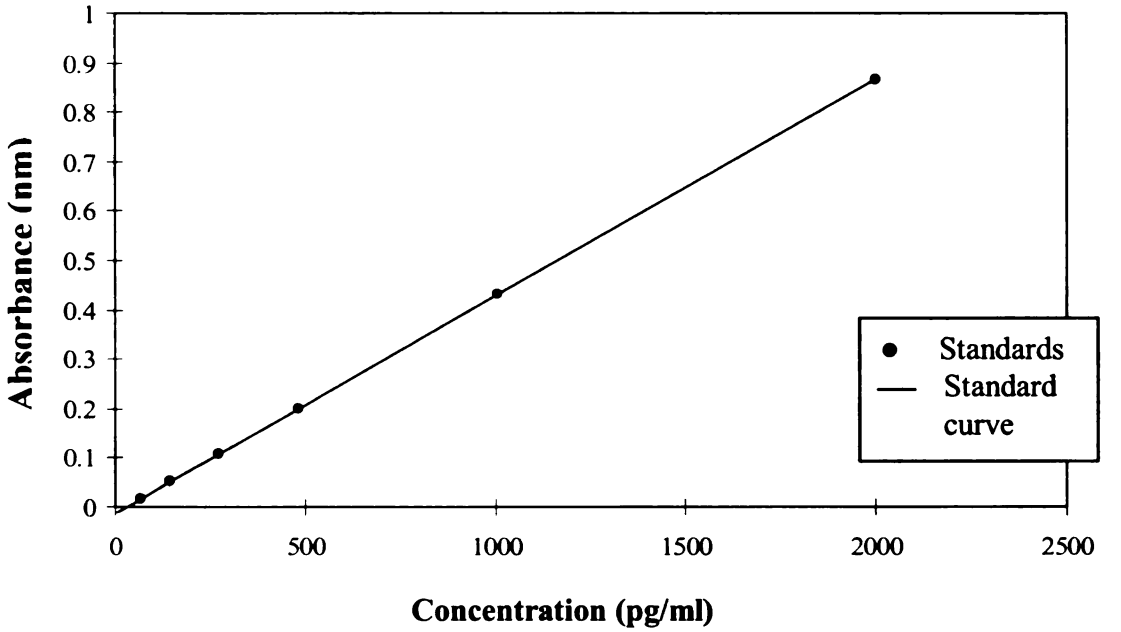
4 Parameter (y = (A - D) / (1 + (x/C)^B) + D)
A=19.1384 B=-1.0057 C=47262.147 D=0.0298, R-Square = 1.000

Figure 3.1. Standard curve for swine TNF-α used in the ELISA. The curve is representative of three identical experiments.



4 Parameter (y = (A - D) / (1 + (x/C)^B) + D)
A=2.5560 B=-1.1116 C=3407.3799 D=0.0077, R-Square = 0.9999

Figure 3.2. Standard curve for swine IL-1-β used in the ELISA. The curve is representative of three identical experiments.



4 Parameter (y = (A – D) / (1 + (x/C)^B) + D)
A=3.3492 B=-1.3873 C=3570.7411 D=0.0087, R-Square = 0.9999

Figure 3.3. Standard curve for swine IL-8 used in the ELISA. The curve is representative of three identical experiments.

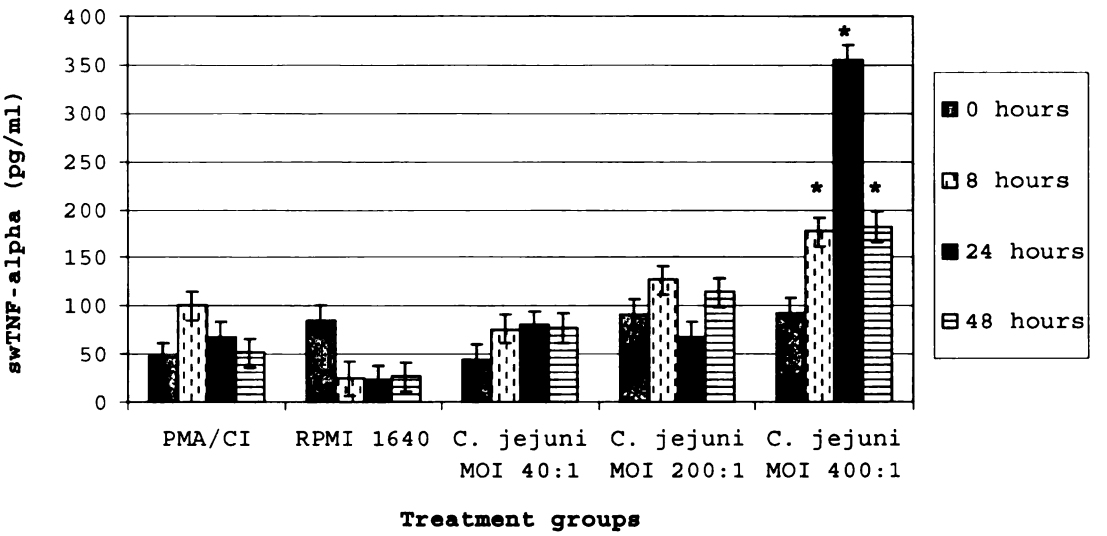


Figure 3.4. TNF-α secretion by IPEC-1 monolayers after exposure to *C. jejuni* 33292. Phorbol myristate acetate (PMA 100 ng/ml) and calcium ionophore (CI 100 ng/ml) were used as the positive control. RPMI 1640 medium was used as the negative control. All treatments were performed in triplicate and are representative of three identical experiments. Values shown represent the mean ± SD. An asterisk (*) indicates the significant difference between *C. jejuni* MOI of 400:1 and the RPMI 1640 (uninfected) control at 8, 24, and 48 hours post-infection ($P < 0.0001$).

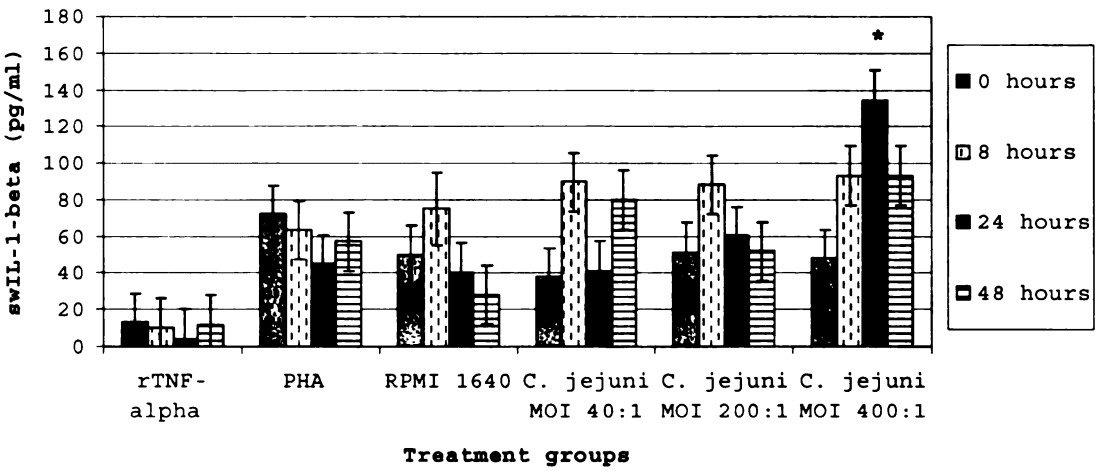


Figure 3.5. IL-1-β secretion by IPEC-1 monolayers after exposure to *C. jejuni* 33292. Recombinant swine TNF-alpha (500 pg/ml) or phytohemagglutinin (PHA 50 ug/ml) was used as the positive control. RPMI 1640 medium was used as the negative control. All treatments were performed in triplicate and are representative of three identical experiments. Values shown represent the mean ± SD. An asterisk (*)indicates a significant difference between *C. jejuni* MOI of 400:1 and the RPMI 1640 (uninfected) control at 24 hours post-infection ($P < 0.001$).

Figure 3.6 (A and B). The two graphs represent experiments conducted at the same time. **(A)** Positive control: IPEC-1 monolayer positively induced to secrete IL-8 following phorbol myristate acetate (PMA 100 ng/ml) and calcium ionophore (CI 100 ng/ml) exposure. RPMI 1640 medium is the negative control. Values shown represent the mean ± SD. An asterisk (*)indicates a significant difference between the positive control values at 8, 24, and 48 hours as compared to the RPMI 1640 control, P value was < 0.0001. **(B)** IL-8 secretion by IPEC-1 monolayers after exposure to *C. jejuni* 33292. RPMI 1640 medium was used as the negative control. All treatments were performed in triplicate, and the values shown represent three identical experiments. Values shown represent the mean ± SD. For all values, *P* was > 0.05 compared to the RPMI 1640 (uninfected) control.

Figure 3.6A.

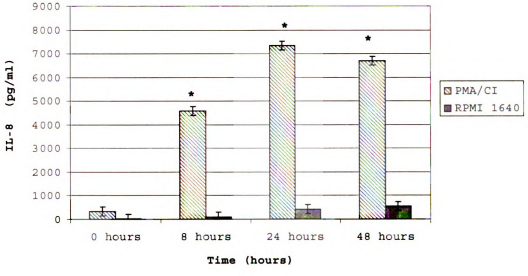
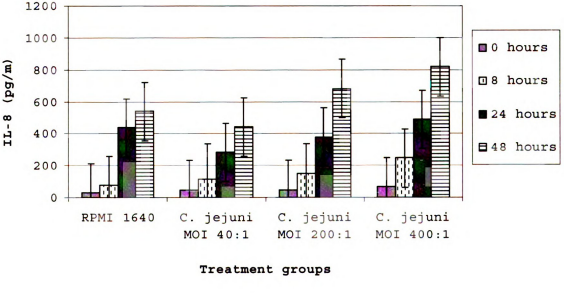


Figure 3.6B.



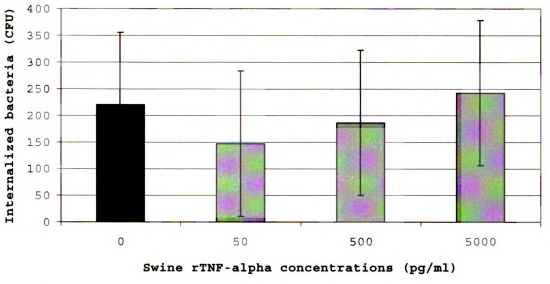


Figure 3.7. IPEC-1 monolayers were pretreated with increasing amounts of swine rTNF-α for 5 hours to determine the effect of swine rTNF-α on *C. jejuni* 33292 invasion of intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were performed in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, *P* was > 0.05 compared to the RPMI 1640 control.

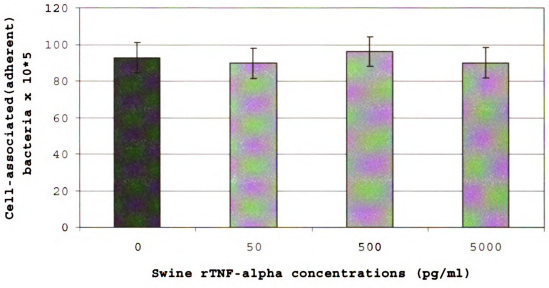


Figure 3.8. IPEC-1 monolayers were pretreated with increasing amounts of swine rTNF-α for 5 hours to determine the effect of swine rTNF-α on *C. jejuni* 33292 adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

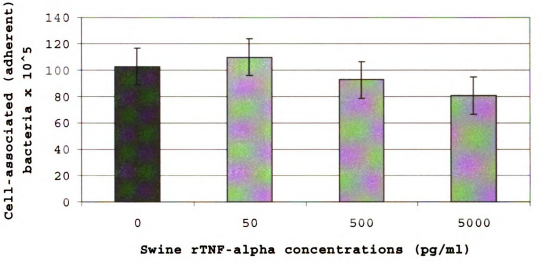


Figure 3.9. IPEC-1 monolayers were pretreated with increasing amounts of swine rTNF-α for 5 hours to determine the effect of swine rTNF-α on *E. coli* O157:H43 DEC7A adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

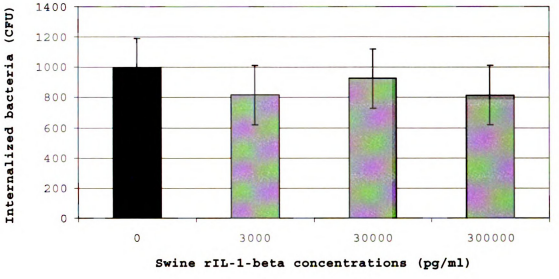


Figure 3.10. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-1-β for 5 hours to determine the effect of swine rIL-1-β on *C. jejuni* 33292 invasion of intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

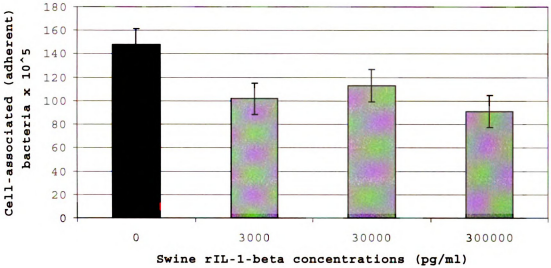


Figure 3.11. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-1-β for 5 hours to determine the effect of swine rIL-1-β on *C. jejuni* 33292 adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. For all values, P was > 0.05 compared to the RPMI 1640 control.

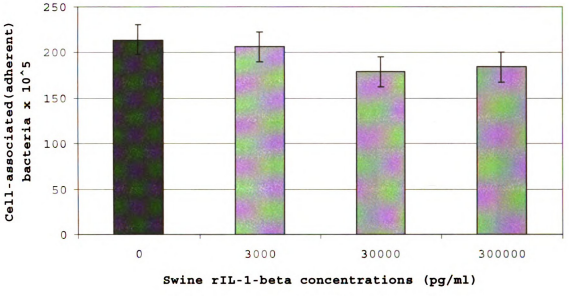


Figure 3.12. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-1-β for 5 hours to determine the effect of swine rIL-1-β on *E. coli* O157:H43 DEC7A adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

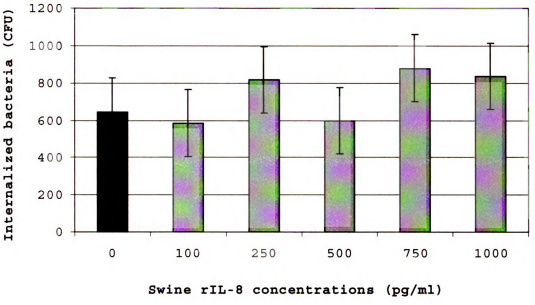


Figure 3.13. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-8 for 5 hours to determine the effect of swine rIL-8 on *C. jejuni* 33292 invasion of intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were done in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

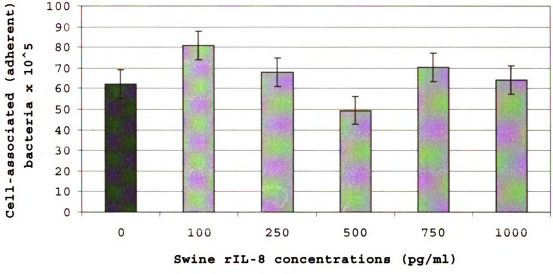


Figure 3.14. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-8 for 5 hours to determine the effect of swine rIL-8 on *C. jejuni* 33292 adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were done in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the RPMI 1640 control.

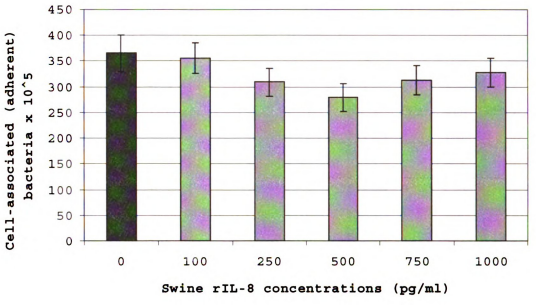


Figure 3.15. IPEC-1 monolayers were pretreated with increasing amounts of swine rIL-8 for 5 hours to determine the effect of swine rIL-8 on *E. coli* O157:H43 DEC7A adherence to intestinal epithelial cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were in triplicate. Results shown are representative of three experiments. The values shown represent the mean ± SD. For all values, *P* was > 0.05 compared to the RPMI 1640 control.

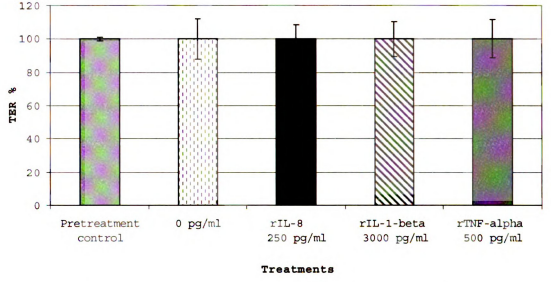


Figure 3.16. Effect of swine rIL-8, rIL-1-β, and TNF-α on transepithelial electrical resistance (TER) of IPEC-1 cells. The TER was measured before and after treatment. The pretreatment control represents the TER before any treatments were applied to the IPEC-1 cells. RPMI 1640 medium (0 pg/ml) was used as the negative control. All treatments were done in quadruplicate. The values shown represent the mean ± SD. For all values, P was > 0.05 compared to the pretreatment control and RPMI 1640.

Chapter 4: Summary and Conclusions

The long term goals of this laboratory project were to elucidate the mechanisms by which concurrent *C. jejuni* and *T. suis* infection cause severe disease and pathology in young swine. The identification and characterization of these mechanisms will be beneficial from both animal and human health perspectives. *C. jejuni* is a food-borne pathogen that has been identified throughout the world as one of the leading causes of human gastroenteritis (5, 6, 11). There have been significant advances in understanding the mechanisms by which *C. jejuni* causes disease, but few immunological studies have been pursued. This is especially true of those using a naturally occurring animal model and associated *in vitro* systems such as swine intestinal epithelial cells. *T. suis* is a nematode parasite found in the cecum and colon of swine. Young infected pigs can acquire catarrhal enteritis with serious pathology, such as anorexia, diarrhea, anemia, dehydration, retardation of growth, and death (32, 33). *C. jejuni* is commonly found in the colon of conventionally-reared pigs without signs of pathology; however, simultaneous *T. suis* infection results in severe disease and pathology (22-24). The severe pathology observed mimics the clinical disease of *Campylobacteriosis* in humans.

For this dissertation project, we addressed a two-fold hypothesis. The central hypothesis is that *C. jejuni* induces natural host resistance via pro-inflammatory cytokine responses, which arise as a result of infection. A related hypothesis is that pro-inflammatory cytokine responses elicited by *C. jejuni* can be down-regulated in the presence of *T. suis,* thereby promoting *C. jejuni* infection. These hypotheses were tested by evaluating pro- and anti-inflammatory cytokine expression in the feces and intestinal

tissues of pigs infected with *C. jejuni* and *T. suis* and more specifically by assessing the secretion of IL-8, TNF-α, and IL-1-β from cultured *C. jejuni*-infected intestinal pig epithelial cells (IPEC-1 cells), which served as a model system for undifferentiated, crypt-like cells. Additionally, we evaluated the effects of these secreted cytokines on bacterial-host cell interaction and intestinal mucosal integrity.

This dissertation project addressed three specific aims. The first specific aim was to evaluate secreted IL-8, IL-1-β, TNF-α, IL-4, IL-6, and IL-10 expression in the feces of pigs after single or dual challenge infections with *C. jejuni* and *T. suis*. Two experimental designs were used to test this specific aim: a short-term challenge experiment (2 days) and a long-term challenge experiment (23 days). In the short-term challenge experiments we assessed the acute pro-inflammatory response to *C. jejuni* and *T. suis* infection. In these experiments, conventionally, weaned piglets were assigned to separate treatment groups and were simultaneously infected with 2×10^8 cfu *C. jejuni* and/or 2500 *T. suis* embryonated eggs or 2×10^8 cfu *E. coli* DH5-α. Fecal samples were taken prior to inoculation (day 0), and on days 1 and 2 post-inoculation. The fecal samples were prepared for analysis, and the supernatants were used in an ELISA to measure pro- and anti-inflammatory cytokine responses following concurrent infection. In the long-term challenge we assessed the pro-inflammatory cytokine response to *C. jejuni* in the presence of adult stage *T. suis* larvae. The pigs were assigned to separate treatment groups. One group of pigs was orally inoculated with 2500 *T. suis* embryonated eggs, and 21 days post-*T. suis* inoculation, these pigs were orally inoculated with 2×10^8 cfu *C. jejuni*. The other assigned groups were singly inoculated with 2500 *T. suis* embryonated eggs, 2×10^8 cfu *C. jejuni*, or 2×10^8 cfu *E. coli* DH5-α. Fecal

samples were taken prior to inoculation (day 0), and on days 21, 22, and 23 post-inoculation. The fecal samples were prepared for analysis, and the supernatants were used in an ELISA to measure pro- and anti-inflammatory cytokine expression during concurrent infection. Also, on day 23 of the long-term challenge experiments, tissue samples were taken from the intestine, and cytokine mRNA expression was measured using real time PCR. The second specific aim was to measure secreted IL-8, TNF-α, and IL-1-β from cultured swine intestinal epithelial cells (IPEC-1 cells) following *C. jejuni* infection. In these *in vitro* experiments, IPEC-1 cells were cultured in an undifferentiated state to mimic intestinal crypt cells and infected with *C. jejuni* 33292. Culture supernatants were removed at 0, 8, 24, and 48 hours after infection and analyzed for IL-8, TNF-α, and IL-1-β protein expression by ELISA. The third specific aim was to determine whether pro-inflammatory cytokines (specifically, IL-8, TNF-α, and IL-1-β) play a significant role in *C. jejuni* adherence, invasion, and/or the transepithelial electrical resistance (TER) with persistent exposure. In the adherence and invasion assays, IPEC-1 cells were cultured in an undifferentiated state to mimic intestinal crypt cells and treated with various concentrations of recombinant swine IL-8, TNF-α, and IL-1-β for 5 hours. Recombinant swine IL-8, TNF-α, and IL-1-β supernatants were removed, and the pretreated IPEC-1 cells were infected with *C. jejuni* for 1 hour to allow bacteria to adhere to the cells or 3 hours to allow bacteria to invade the cells. After 1 hour, the adhered (cell-associated) bacteria were enumerated by serial dilution plating. After 3 hours, the gentamycin assay was performed, and intracellular *C. jejuni* were enumerated by serial dilution plating. Also, IPEC-1 cells were pretreated with recombinant swine IL-8, TNF-α, or IL-1-β to determine if cytokine exposure affects the transepithelial electrical

resistance (TER) of these cells. Undifferentiated IPEC-1 cells were grown to confluency, and the TER was measured prior to treating these cells with recombinant swine IL-8, TNF-α, or IL-1-β. Subsequently the IPEC-1 cells were treated with recombinant swine IL-8, TNF-α, and IL-1-β for 5 hours, and then the TER was measured.

To assess the effects of concurrent *T. suis* and *C. jejuni* infection on cytokine expression, we used our established swine infection model system set up as two experimental designs (short term; day 0–day 2 and long term; day 0–day 23) challenge experiments and measured pro- and anti-inflammatory cytokines in the feces and intestinal tissues of infected pigs. I*n vivo* results from the long-term challenge experiments (day 0-day 23) showed that pigs that received *T. suis* alone showed a significant decreased IL-1-β in the feces by 23 days post-inoculation, and the dually-infected pigs showed moderately decreased IL-1-β expression. Also, on day 23, IL-1-β was significantly decreased in the *T. suis*-infected pigs compared to IL-1-β expression in the *C. jejuni*-infected pigs. These and other findings suggest that *T. suis* likely predominates in driving the cytokine response by down regulating the expression of this pro-inflammatory cytokine during infection. This is not surprising because in most helminth infections a Th2 response is elicited with induced production of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 during infection (13, 38) This pattern of cytokine expression is characteristic of a Th2 response which has been shown to predominantly limit Th1 cytokine-induced inflammation (Th1 cytokines- Il-12, IFN-γ, IL-8, TNF-α, and IL-1-β)(13, 39) and result in worm expulsion.

In the short-term challenge experiments, there were no significant changes in secreted IL-8, IL-1-β, TNF-α, IL-4, IL-6, or IL-10 expression. Generally, in pigs that are

naturally infected with *T. suis*, signs of pathology are not seen until 21 days post-*T. suis* infection, it is reasonable to believe that results from the long term challenge (23 days) would likely reflect the immune response of the natural infections more than the short-term challenge (2 days). This also suggests that immunity to *T. suis* ova or first stage larvae may be limited.

In the swine model experiments, we determined the cytokine recovery rate for these fecal ELISAs to be 10–20%. This low recovery rate may have been due to protein degradation associated with freezer storage, the loss of protein during the fecal preparation due to the cytokines binding to the solid fecal matter, or protein degradation due to protease activity. Because of these possible drawbacks, the fecal ELISA data was only moderately useful and we relied instead on additional data obtained directly by measuring cytokine expression in gastrointestinal tract tissues in areas where the pathogens reside.

In the long-term challenge experiments, after fecal sampling on day 23, the pigs were euthanized and tissue samples were taken from the jejunum, proximal colon, and distal colon of our pigs. In the proximal colon of the two groups of pigs that received *T. suis*, Real Time PCR analysis showed increased expression of IL-13 mRNA (Th2 cytokine) and decreased IL-8 and IL-12 expression (Th1 cytokines). Compared to the uninfected control pigs, IL-13 expression was upregulated 3 to 4 fold, and IL-8 and IL-12 was decreased 2 to 3 fold and 2 to 4 fold, respectively. Additionally, expression of MCP-1, a chemokine, and IL-5, a Th2 cytokine, were significantly decreased 2 fold in the dual-infected pigs compared to the uninfected control pigs. Also, there were no significant

changes in cytokine mRNA expression in the pigs that received *C. jejuni* alone as compared to the uninfected controls.

These data provide evidence that supports the hypothesis that *T. suis* infection can stimulate the production of a Th2 cytokine (IL-13) which can down-regulate pro-inflammatory cytokines (IL-8 and IL-12) in the proximal colon of pigs. *In vitro*, it has been shown that IL-13 can decrease IL-8 secretion from human intestinal epithelial cells(21). It has also been shown that IL-13 protects BALB/c mice from LPS-induced lethal endotoxemia, and decreased IL-12 production is correlated with this protective effect (27). In addition, it has been shown in our laboratory that differentiated intestinal pig epithelial cells stimulated with *T. suis* excretory-secretory products secrete IL-10 (31). In general, IL-10 production has been associated with helminth infections, and more specifically, it has been shown to be a critical component in the development of a polarized Th2-type immune response which has been correlated with host resistance and survival during *Trichuris* infection (37). Although IL-10 mRNA was not significantly upregulated in any of the tissue samples from the pigs that received *T. suis*, it is possible that peak expression of IL-10 occurred before tissue sampling on day 23 of the experiment, and by day 23, IL-10 levels had possibly gone back down to baseline levels. Alternately, it is possible that low constant levels of IL-10 could play a role in downregulating pro-inflammtory cytokine expression in order to maintain intestinal mucosal integrity. More to the point, previous data showed that significant production of IL-10 from cultured IPEC-1 cells following *T. suis* ESP exposure occurred at 24 hours post-infection (31). *In vitro* and *in vivo* studies have demonstrated IL-10 and IL-13 inhibition of intestinal inflammatory responses such as Th1-type cytokine production,

and this inhibition is believed to diminish the severity of the inflammatory responses thereby reducing clinical disease activity (8, 20, 21, 37). Taken together, the data presented here are a supportive extension of previous findings in our laboratory, and collectively these findings suggest that it is possible that the down regulation of IL-8, IL-12 and possibly MCP-1 mRNA expression in response to *T. suis* infection may be due to IL-13 production and possibly IL-10 in response to helminth infection. Therefore, it is reasonable to suggest that *T. suis* plays a role in the down-regulation of pro-inflammatory cytokine expression, and that this response can alter host conditions which promote secondary bacterial infection.

In the distal colon, significant changes in cytokine expression were observed only in the pigs that received *T. suis* alone. IL-1-β, IL-6, and IFN-γ expression were increased 3 to 4 fold, and IL-13 expression was increased 3 fold compared to uninfected controls. IL-1-β, IL-6, and IFN-γ production is characteristic of an inflammatory response. The pigs showing these responses had diarrhea and lesions in these sites. This observed inflammatory response distal to the sites of worm attachment (in the cecum and proximal colon) may be the result of secondary bacterial infection. Previous data have shown that *T. suis* infection plays a role in facilitating secondary bacterial invasion of many species of resident bacteria, including *C. jejuni*, *C. coli*, *C. lari*, *Campylobacter spp.* (unidentified species), and *E. coli* (24).

In this study, in spite of our best efforts to support the organism in samples during transport, culture alone was not sensitive enough to detect or identify *C. jejuni* after infection. We found it necessary to use multiple diagnostic tests (TaqMan Real time PCR, RFLP analysis of Campylobacter 23S rRNA gene, 16S rRNA PCR analysis and

ELISA) to detect the inoculated strain of *C. jejuni* after infection. When *C. jejuni* was detected by TaqMan analysis in the distal colon of 1 of 6 pigs that received *T. suis*-only in screens of pigs post inoculation, we realized the possibility that prior infection with this organism or with other *Campylobacter spp.*, or other epsilon proteobacteria may have occurred in these pigs. Suspicions of a contaminating bacterium were further aroused when RFLP analysis also showed positive results for *C. coli* and a *Campylobacter* spp. that gave a unique banding pattern that was not consistent with *C. jejuni*, *C. lari*, *C. coli*, or *C. upsaliensis* in jejunum, proximal and distal colon samples from most of the pigs in the treatment groups. ELISA results for a wide range of these bacteria showed that the pigs were most reactive to *Helicobacter* spp. that may have caused problems in both serological and molecular assays. *Helicobacter* is a closely related member of the epsilon proteobacteria group with similar outer membrane proteins. Finally, we tested fecal supernatants for the presence of other *Campylobacter* species and *Helicobacter* species using 16S rRNA PCR analysis. Here we found that almost all pigs in the short- and long-term challenge experiments tested negative for the presence of *Campylobacter* species pre- and post-infection. However, several pigs tested positive for the presence of *Helicobacter* species pre- and post-infection. Taken together these data provide the best evidence to suggest that the pigs were exposed to *Helicobacter* spp. prior to the experiment and that the cytokine responses measured may have been affected by this prior bacterial exposure. We must consider that these results are consistent with the possibility that the increased inflammatory cytokine response seen in the distal colon of the pigs infected with *T. suis* alone may actually represent host resistance to secondary bacterial infection.

It is also possible that the effects of ESP produced by 4th stage larvae that have traveled to the distal colon could contribute to increased expression of IL-13 and IL-6. It has been shown that *T. suis*-infected pigs have serum antibodies as early as 21 days post inoculation that recognize a 20kDa glycoprotein isolated from ESP collected from adult worms cultured *in vitro* (17). It has been demonstrated *in vitro* that *T. suis* ESP treatment of differentiated IPEC-1 cells causes a dose dependent cytotoxic response and significantly decreased transepithelial electrical resistance (TER)(1, 2). Also, cultured differentiated and undifferentiated IPEC-1 cells secrete IL-6 and IL-10 following ESP exposure (31). Therefore, it is reasonable to consider that the cytotoxic effects of ESP on intestinal epithelial cells can stimulate an inflammatory cytokine response in the distal colon. We should consider that ESP may have direct effects on intestinal epithelial cells and could account in part for the increased cytokine production *in vivo* at sites distal to worm attachment.

Interestingly, we also found that there were no significant changes in cytokine expression in the distal colon of pigs that received *C. jejuni* alone or those that were dually-infected or in the proximal colon of pigs that received *C. jejuni* alone. In deed, we recognized through the use of multiple diagnostic tests that the pigs from our *in vivo* experiments were likely exposed to *Helicobacter* spp., a wildtype *C. jejuni*, other *Campylobacter* spp., or epsilon proteobacteria prior to the experiments. The timing and source of acquisition is not known, but pigs were farrowed and reared with their mothers in a solid floor shower-in shower-out confinement facility and then were moved directly to the containment colony for these studies. Because our data provides evidence suggesting that these pigs were likely exposed to *Helicobacter spp.* (epsilon

proteobacteria-campylobacter-like organisms) at birth or shortly after birth on the MSU swine farm, it is reasonable to believe that the *C. jejuni* administered during our experiments served as a secondary bacterial challenge. A possible scenario is that these pigs acquired immunity after early exposure to epsilon proteobacteria such as *Helicobacter*, and the host cytokine and chemokine responses to the experimental *C. jejuni* inoculum are more representative of host resistance to secondary bacterial infection associated with protective immunity. Therefore, this scenario affected the interpretation of the results, but it did not negate our findings.

Nevertheless, in the jejunum (a division of the small intestine), we found that pigs receiving *C. jejuni* alone showed significant changes in cytokine expression. The expression of MCP-1, TNF-α, IL-12p40, IFN-γ, IL-4, and IL-10 was increased 3 to 5 fold compared to uninfected controls. *In vitro*, *C. jejuni* induces INT 407 cells to produce intracellular MCP-1, TNF-α, IFN-γ, IL-4, and IL-10 (18), and *Helicobacter pylori* induces gastric epithelial cells to produce IL-8 and MCP-1 (15, 28, 29, 34). Thus, the cytokine changes observed could be due to *Campylobacter* or *Helicobacter* interaction with the intestinal epithelial cells in the jejunum. Additionally, it is likely that the jejunal samples taken from our pigs contained some Peyers patches along with surrounding epithelium. Peyers patches are lymphoid follicles in the small intestinal mucosa that serve as important components in local and systemic immune responses. Peyers patches are functionally similar to LGCs of the colon. It has been demonstrated in oral-challenged germfree piglets that *C. jejuni* targets LGCs and induces robust cytokine responses (22, 23) (Jones, dissertation, 2005). It is possible that *C. jejuni* or *Helicobacter* stimulated the jejunal Peyers patch to produce a similar cytokine response, which likely

influenced our results. Clearly, the net effect of *C. jejuni* exposure on the jejunum was elevation of proinflammatory cytokines. To distinguish the exact cell types from which this response arises, further work should be done to fractionate tissues in the jejunum prior to measuring cytokine responses. The data here suggest that in the absence of *T. suis*, *C. jejuni* or epsilon proteobacteria stimulate pro-inflammatory cytokine responses in the jejunum. Furthermore, in the gastrointestinal tract of chickens rechallenged with *Salmonella enterica* Serovar Typhimurium, it has been shown that significant changes in proinflammatory cytokine and chemokine (IL-6 and MIP family chemokine) expression occur in the ileum and cecal tonsils, which are also segments of the small intestine (40). Additionally, in the jejunum of dually-infected pigs, GM-CSF was down-regulated 5 fold compared to uninfected controls. The pattern of reactivity in the jejunum over the five groups of pigs suggests that *T. suis* is likely contributing to the down-regulation of pro-inflammatory cytokine expression in the jejunum which is similar to the immunomodulatory effects that occurred in the proximal colon.

In addition, we observed that clinical diarrhea was a prominent sign of disease throughout the experiments. Diarrhea was observed in pigs that received *C. jejuni* only, *T. suis* only or *both*. However, more frequent and more severe diarrhea was observed in the dually infected pigs and the *T.* suis only-infected pigs compared to the other treatment groups, thereby implying that *T. suis* is mainly responsible for causing the diarrhea. Additionally, diarrhea was mostly observed in the long-term challenge experiment where *Trichuris* infection had progressed for 21 days. As has been reported frequently in literature reports on *Trichuris*-induced disease, diarrhea usually begins around 21 days post-infection and is commonly called the "21 day scours" (9). In both experiments,

some of the *C. jejuni*-infected pigs had mild diarrhea which is commonly seen in campylobacterosis (7). It has also been reported by Mansfield et al. in a previous *Campylobacter* swine disease model experiment that some of the *C. jejuni*-infected pigs showed clinical signs of mild diarrhea at a less degree of severity than the dually-infected or *T. suis* only-infected pigs (23). The uninfected pigs and *E. coli* DH5-α infected pigs showed no signs of diarrhea. The observation of severe diarrhea in pigs infected dually or with *T. suis* alone has provided some evidence to suggest that *T. suis* plays a significant role in diarrheal disease.

Furthermore, *C. jejuni* invasion or stimulation of intestinal epithelial cells has been shown to induce pro-inflammatory cytokine production (16, 18). *In vivo, C. jejuni* invade the lymphoglandular complex (LGCs) entrapped crypts and the follicle-associated epithelium of the LGCs following a primary challenge infection (23)(Jones, dissertation, 2005). A robust pro-inflammatory cytokine response is expressed in the LGCs upon *C. jejuni* infection (Jones, dissertation, 2005). *In vivo* and *in vitro* studies have shown that *C. jejuni* interacts with enterocytes which serve as the first line of defense during bacterial infection (16, 19). My second specific aim was to assess IL-8, IL-1-β and TNF-α production from undifferentiated, crypt-like intestinal pig epithelial cells (IPEC-1 cells) following *C. jejuni* infection. This primary neonatal cell line was chosen because it complemented our swine model system. The cells were grown to form confluent monolayers and infected with *C. jejuni* for 0, 8, 24, and 48 hours. The supernatants were collected at each time point and analyzed using ELISA. TNF-α was secreted from the IPEC-1 cells by 8 hours post-inoculation, and continued production was measured through 48 hours post-inoculation. Likewise, IL-1-β was secreted by the IPEC-1 cells by

24 hours post-inoculation. We found that IL-8 was constitutively secreted by the IPEC-1 cells, and was not induced by *C. jejuni* infection. The data presented here suggest that the induced production of TNF-α and IL-1-β and the constitutive production of IL-8 from intestinal epithelial cells are indicative of an acute innate host immune response. It is likely that this inflammatory response can generate and/or modulate the immune response to benefit the host by contributing to the elimination of the bacteria.

In the third specific aim, we assessed whether IL-8, TNF-α, or IL-1-β treatment of IPEC-1 cells had an effect on *C. jejuni* invasion, adherence, and/or the transepithelial resistance (TER). IPEC-1 cells were grown on transwell inserts for 2 days to form confluent monolayers. The TER of the confluent monolayer was measured prior to treatment. Then, either IL-8, TNF-α, or IL-1-β were applied to the confluent monolayers and incubated for 5 hours, and the TER was measured again. The data presented from this work, showed that neither IL-8 nor IL-1-β have a direct effect on *C. jejuni* invasion, adherence, and/or the TER of IPEC-1 cells. Also, treatment of IPEC-1 cells with TNF-α did not significantly affect *C. jejuni* adherence or changes in the TER of the IECs. This finding suggests that these cytokines do not act in an autocrine-like manner to specifically affect *C. jejuni* adherence, invasion, and/or the TER of IPEC-1 cells. Even though there was not a direct effect on *C. jejuni* adherence to or invasion of IPEC-1 cells (in response to IL-8 or IL-1-β pretreatment) or a direct effect could not be determined following TNF-α pretreatment *in vitro*, it is still possible that IL-8, TNF-α, or IL-1-β may affect epithelial function in a paracrine-like or another autocrine-like manner during *C. jejuni* infection *in vivo*. For instance, it has been demonstrated that cytokines can facilitate enterocyte proliferation, maturation, MHC class II expression, cytokine receptor

expression or apoptosis in healthy and diseased states (14, 30, 35). In addition, TNF-α and TGF-α have been shown to stimulate immature crypt cell proliferation, and TGF-β and INF-γ can inhibit mitotic activity (36). IFN-γ can alter immature crypt epithelial cell turnover and upregulate MHC class II expression (35). Therefore, alterations of cytokine concentrations as a consequence of bacterial exposure and intestinal inflammation may contribute to serious pathologic consequences. Further elucidation of the effects of distinct cytokines on epithelial cell growth, phenotype, function and bacterial-enterocyte interactions would be of importance in the understanding of cytokine mediated regulation of mucosal immune responses associated with *C. jejuni* infection.

Future Directions

The results from this dissertation project opened other avenues for further research that would enhance understanding of several aspects of this dual infection phenomenon. In chapter 2 we orally infected immunocompetent, conventionally reared pigs that had a complete intestinal microbial population with *C. jejuni*, *T. suis*, or both and analyzed fecal cytokine expression and cytokine expression in the jejunum, proximal colon, and distal colon. In this study, we found that our pigs were probably exposed to *C. jejuni*, other *Campylobacter spp.* or epsilon proteobacteria prior to the experiments, and this finding significantly impacted the interpretation of our current results. Therefore, it would be imperative to use gnotobiotic pigs for future studies in order to definitively characterize and gain a better understanding of *in vivo* cytokine profiles and kinetics during concurrent *C. jejuni* and *T. suis* infection. Further analysis of cytokine expression using *in situ* hybridization and cytokine antibody arrays would allow us to determine if there is a correlation between cytokine mRNA expressed in intestinal tissues and secreted cytokine (protein) expression, respectively. During the concurrent infection, analysis of cytokine expression at different times throughout the course of a long term challenge experiment (days 0-45) would allow us to examine acute and long term inflammatory responses (beginning at 24 hours postinfection and continuing throughout the length of the experiment) to *C. jejuni* infection and would also allow us to examine changes in cytokine expression while *T. suis* developmental changes were occurring. Extending the evaluation time out to 41 or 45 days would allow a more thorough assessment of cytokine responses throughout the entire prepatent period of *T. suis* luminal development. In

addition, we would examine pathologic changes to correlate lesion progression with cytokine changes. This would entail having a large number of infected pigs so that a subset could be sacrificed at intervals after infection.

In general, *T. suis* infection triggers a Th2 type response involving the cytokine production of IL-4, IL-10, and IL-13 which plays a role in worm expulsion from the host(13, 38). This Th2-type response can down-regulate Th1-type innate host immune responses (e.g. IL-1-β, TNF-α, INF-γ, IL-8, and IL-12) which generally cause bacteria elimination from the host (13, 38, 39). In chapter 2 we discussed our results that suggest that *T. suis* likely mediates IL-8 and IL-12 down-regulation by producing IL-13 in the proximal colon of pigs, *in vivo*. Even though we did not measure IL-4 or IL-10 mRNA expression in any tissues from the pigs in the *in vivo* experiments, it would be useful to determine the possible roles of these cytokines *in vitro*. In previously reported data from our laboratory, recombinant swine IL-4 has been shown to weaken tight junctions between IPEC-1 cells, and this could allow bacteria to breach the epithelial barrier and gain access to the basolateral surface of cells. Undifferentiated and differentiated IPEC-1 cells could be treated with IL-4, IL-10 and/or IL-13 in a dose response experiment followed by infecting these cytokine pretreated IPEC-1 cells with *C. jejuni*. The supernatants of these *C. jejuni*-infected cells could be used in ELISA to measure IL-8, TNF-α, IL-1-β, IFN-γ, and IL-12 production. This experiment could determine if Th2-type cytokines (IL-4, IL-10, and IL-13) effect Th1-type cytokine production (IL-1-β, TNF-α, and IL-8) from *C. jejuni*-infected swine intestinal epithelial cells, *in vitro*. It would also be interesting to challenge these cells with multiple strains of *C. jejuni* from

patients to see if particular gene content or gene expression correlates with enhanced host response.

In chapter 3 we measured secreted IL-8, TNF-α, and IL-1-β from undifferentiated crypt-like swine intestinal epithelial cells (IPEC-1 cells) following *C. jejuni* infection. Previous reports from our laboratory have shown that *C. jejuni* specifically interact with colonic crypts and are associated with mucus near goblet cells (23). It has also been demonstrated that intestinal epithelial cells can be stimulated by *C. jejuni* to produce proinflammatory cytokines and chemokines (16, 18). We measured secreted IL-1-α and TNF-α from *C. jejuni*-infected IPEC-1 cells, but only showed that IPEC-1 cells constitutively secrete IL-8. This raises the question as to why IL-8 is not induced by *C. jejuni* infection of IPEC-1 cells. One way to address this question could be to determine if primary intestinal pig epithelial cells (IPEC-1 cells) lack or have decreased expression of Toll-like receptor 4 and MD-2 (accessory molecule) on the cell surface using PCR analysis or flow cytometry. It has been demonstrated that IL-8 production is associated with TLR4 receptor signaling via LPS stimulation and NFκB activation (3, 4, 10, 12, 14). Once it has been determined whether IPEC-1 cells lack or have decreased TLR4 and MD-2 on the cell surface, we could use western blot analysis of intracellular proteins to study the related intracellular signaling pathways of genes encoding proteins involved in inflammation. More specifically, we could evaluate the interaction of TLR4 and MD-2 binding to *C. jejuni* LPS, and determine if this interaction stimulates the NFκB signaling pathway to induce IL-8 gene expression (3, 4).

Also, in chapter 3, our data showed that IL-8, TNF-α, and IL-1-β had no direct effect on *C. jejuni* interactions (specifically adherence, invasion, or changes in IPEC-1

TER) with the swine intestinal epithelial cells, but they could possibly have other autocrine- or paracrine-like effects which could play a role in host inflammatory immune responses associated with *C. jejuni* infection. For example, it has been shown that TNF-α and IL-1-β can stimulate intestinal epithelial cells to produce C3, a complement component which is associated with host acute-phase inflammatory responses (14, 25, 26, 30, 35). Therefore, it would be interesting know if intestinal pig epithelial cells (IPEC-1 cells) could produce C3. It is known that IL-1-β is an initiator of inflammation and a regulator of immune responses, and it induces acute phase protein responses, such as the production of the acute phase protein complement C3. Undifferentiated and differentiated IPEC-1 cells could be pretreated with IL-1-β in a dose response experiment. Confluent monolayers could be exposed to the different concentrations of IL-1-β. C3 levels could be measured in the supernatants by ELISA, and C3 mRNA levels would be determined by Northern blot analysis. This experiment would determine if IL-1-β acts in an autocrine-like manner to stimulate swine intestinal epithelial cells to produce an acute phase protein, complement C3. In a secondary experiment, the IPEC-1 cells could be pretreated with anti-IL-1-β antibody or IL-1Ra (IL-1 receptor antagonist) to reverse C3 production, if any.

In summary, we have generated data from this dissertation project that has provided some evidence and understanding of the research defining host immune responses to concurrent *T. suis* and *C. jejuni* infections. We demonstrated *in vivo* that *T. suis* plays a major contributing role in mediating changes in cytokine expression during concurrent *T. suis* and *C. jejuni* infection. Using our swine infectious disease model system, we began to define host local immune responses expressed during this concurrent

infection. *In vitro*, we showed that undifferentiated, crypt-like intestinal pig cells (IPEC-1) can serve as a suitable *in vitro* model for studying *C. jejuni*-enterocyte interactions and host enteric immune responses elicited by *C. jejuni* during infection. We discovered that *C. jejuni* can stimulate intestinal pig epithelial cells (IPEC-1 cells) to produce IL-1-β and TNF-α, and we found that these intestinal pig epithelial cells constitutively secrete IL-8. Hence, these cytokine responses are indicative of an innate inflammatory immune response that may be responsible in some part for eliminating bacteria from the host *in vivo*. In addition, we found that proinflammatory cytokines, such as IL-8, IL-1-β, or TNF-α, do not appear to directly effect *C. jejuni* interactions with host cells (particularly adherence, invasion, or stimulating changes in the transepithelial electrical resistance (TER) of IPEC-1 cells). Collectively, these data have provided some evidence for *T. suis* contributing significantly to cytokine dysregulation seen during concurrent infection, and these immunomodulatory effects likely promote *C. jejuni* infection of the intestinal mucosal epithelium by limiting the natural host resistance to bacterial infection.

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