# Intramuscular immunization of mice with KatA (catalase) generates functional antibodies against *Campylobacter jejuni* invasion of gut epithelial cells *in vitro*

**by**

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**A thesis submitted in partial fulfillment**

**of the requirements for the degree of**

**Master of Science (MSc) in Biology**

**The Faculty of Graduate Studies**

**Laurentian University**

**Sudbury, Ontario, Canada**

## THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
**Laurentian Université/Université Laurentienne**
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis
Titre de la thèse: Intramuscular immunization of mice with KatA (catalase) generates functional antibodies against C. jejuni invasion of gut epithelial cells in vitro

Name of Candidate
Nom du candidat: Rickaby, Barbara

Degree
Diplôme: Master of Science

Department/Program
Département/Programme: Biology

Date of Defence
Date de la soutenance: January 19, 2015

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**ACCESSIBILITY CLAUSE AND PERMISSION TO USE**

**ABSTRACT**

The high incidence of *Campylobacter jejuni*-associated diarrhea, increase in the frequency of drug resistance in clinical isolates, and the recent association of *C. jejuni* infections and Guillain-Barré syndrome has heightened the need to develop effective anti-*Campylobacter* vaccines. Due to the risk of auto-immunity, vaccination using inactivated whole-cells or attenuated cells cannot be considered. Subunit vaccines however are a viable alternative and have been shown to be effective against similar pathogens. The main goal of this research is to test efficacy of the catalase A (KatA) protein as vaccine candidate against this pathogen. It was determined that KatA co-fractionates with both the soluble and insoluble cell fractions and a balanced IgG1/IgG2a response could be produced against it following immunization in mice. It was demonstrated that these antibodies can mediate complement-specific bactericidal activity and reduced adhesion and invasion of human epithelial (Caco-2) cells by *C. jejuni* using established *in vitro* assays.

**KEYWORDS**: *Campylobacter jejuni*, catalase, KatA, functional antibodies, vaccine,

**ACKNOWLEDGMENTS**

I would like to thank Dr. Francisco Diaz-Mitoma for inviting me to join his research team at the Advanced Medical Research Institute (AMRIC) and for having faith in me even when I doubted myself. His enthusiasm and eternal optimism for our research was essential for finishing the project. I would also like to recognize the guidance of Dr. Nelson Eng in helping make the project come to fruition. In addition, Dr. Reza Nokhbeh offered wise advice and helped me see the light at the end of the tunnel. Dr. Nokhbeh as well as Dr. Mazen Saleh provided help as my committee members and assisted with the completion of the project.

I would also like to thank AMRIC lab members Nya Fraleigh and Carly Buckner, for their guidance and instruction in lab techniques and thesis writing. In addition, Misty-Lee Carlson was a tremendous help in all matters administrative. Finally, I would like to thank my parents Doug and Carol for assisting me with my many, many years of post-secondary education which would never have been possible without their support. I dedicate this thesis to them with my love.

Thank you all!

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| | |
|---|---|
| APC | Antigen presenting cells |
| BCR | B cell receptor |
| CARD | Caspase-recruitment domain |
| CD | Cluster of differentiation |
| CDT | Cytolethal distending toxin |
| CFU | Colony forming units |
| Cia | Campylobacter invasion antigen |
| CPS | Capsular polysaccharides |
| CT | Cholera toxin |
| CTL | Cytotoxic lymphocytes |
| CWC | Campylobacter whole-cell (vaccine) |
| DC | Dendritic cell |
| DHEA | Dehydroepiandrosterone |
| d.nm. | Diameter - nanometers |
| DT | Diphtheria toxin |
| DTH | Delayed-type hypersensitivity |
| ER | Endoplasmic reticulum |
| FAE | Follicle-associated epithelium |
| GALT | Gut-associated lymphoid tissue |
| GB | Gnotobiotic animals |
| GBS | Guillain-Barré syndrome |

| | |
|---|---|
| HAMPS | Host-associated molecular patterns |
| hfa | Human flora (gut) |
| IBD | Inflammatory bowel disease |
| IEC | Intestinal epithelial cells |
| IEP | Isoelectric point |
| IL | Interleukin |
| ISCOM | Immune stimulating complex |
| Ig | Immunoglobulin |
| KatA | Catalase (*C. jejuni*) |
| LOS | Lipooligosaccharide |
| LPS | Lipopolysaccharide |
| LRR | Leucine rich repeat |
| LT | Heat-labile enterotoxin |
| M | Microfold cell |
| mAb | Monoclonal antibodies |
| MAC | Membrane attack complex |
| MALT | Mucosal-associated lymphoid tissue |
| MAPK | Mitogen-activated protein kinase |
| MASP | Mannan-binding lectin-associated serine protease |
| MBL | Mannose-binding lectin |
| MBP | Maltose-binding protein |
| Mbp | Megabase pair |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| MPL | Monophosphoryl lipid A |
| mV | Milli-volts |
| MWD | Molecular weight distributions |
| NF | Nuclear factor |
| NK | Natural killer (cell) |
| NLR | Nod-like receptor |
| OD | Optical density |
| $O_2$ | Oxygen |
| PAMP | Pathogen-associated molecular patterns |
| PML | Polymorphonuclear leukocytes |
| PRR | Pathogen recognition receptor |
| PSD | Particle size distribution |
| PYD | Pyrin domain |
| ROS | Reactive oxygen species |
| T3SS | Type III secretion system |
| TCR | T cell receptor |
| Th | T helper cell |
| TGF | Transforming growth factor |
| TIR | Toll/IL-1 receptor |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| V | Variable (antibody structure) |

# 1.0 INTRODUCTION

## *1.1 Campylobacter jejuni*

Diarrhoeal disease alone claims a toll of 5 million children per year (1). Consequently, there is a tremendous need for vaccines to combat the enteric pathogens causing these illnesses. Common pathogens that cause infections in the gastrointestinal tract include *Vibrio cholerae, Helicobacter pylori, Campylobacter jejuni, Salmonella spp., Clostridium difficile* and enterotoxigenic *Escherichia coli* (2). All of these infections continue to represent a challenge for the development of vaccines that can either prevent the pathogen from colonizing, or penetrating the mucosal epithelium, and/or blocking the binding of microbial toxins or neutralizing them (2).

*Campylobacter jejuni* is the most frequently reported causative agent of bacterial gastroenteritis in humans. *Campylobacter jejuni* cells are spirally curved Gram-negative rods with tapering ends. They possess a flagellum at one, or both, ends thus possessing a high degree of motility (3). They have a small genome of approximately 1.6-1.7 megabase pairs (Mbp) of AT-rich DNA (70%) (4). Infection occurs most often as a result of contact with poultry and through consumption of contaminated meat products, notably poultry (5; 6), pork (7), and beef (8), as *Campylobacter* species exist as a commensal flora in many domestic animals (9). In addition, the consumption of contaminated drinking water (10) and raw milk (11) have also been linked to large outbreaks of campylobacteriosis. Infected individuals often present with a broad range of symptoms including abdominal pain, bloody diarrhea, and fever while a histological examination of intestinal tissues reveals crypt abscesses, ulcerations, and elevated numbers of immune cells in the colon (12). Bacteraemia is detected in less than 1% of patients with *Campylobacter* enteritis, and occurs most often in patients with a severely compromised immune system (13; 14; 15).

Although the vast majority of infections are self-limiting, post-infectious complications include inflammatory bowel disease (IBD) (16), reactive arthritis (17), Miller-Fisher-Syndrome (18) and Guillain-Barré syndrome (GBS) (19). *Campylobacter jejuni* is also reported to be the second most common bacterial cause of traveler's diarrhea, after *Vibrio cholerae,* among visitors in endemic regions (20; 21) and is thought to be one of the leading causes of mortality for young children in the developing world (22). An estimated 2-10 million cases of campylobacteriosis occur each year in the United States (23). The total annual costs from infection, calculated from both medical and productivity loses, is thought to be up to 8 billion US dollars annually (24). Campylobacteriosis is often treated with antibiotics and shortens the duration of diarrheal disease experienced by a mean of 1.3 days (25). Considerable antibiotic resistance has developed over the past decade, as a consequence to this over usage, to both fluoroquinolones (26) and macrolides (27) further necessitating the development of a prophylactic treatment.

The development of a *Campylobacter* vaccine has been impeded by a fundamental safety concern due to the association of Campylobacteriosis with GBS. GBS is a neurologic disease characterized by ascending paralysis that can lead to respiratory muscle compromise and death (19). It is the most common cause of acute neuromuscular paralysis in the developed world (19). The US centers for Disease Control and Prevention have estimated the incidence of *C. jejuni* infection to be 1000 cases for every 100,000 individuals in the population per year (28). It has also been estimated that 1 of every 1058 cases of *C. jejuni* infection are followed by GBS although the risk of developing GBS is increased if the patient was colonized by strain O:19 (19). *Campylobacter jejuni* lipopolysaccharides (LPS) and lipooligosaccharide (LOS) have been reported to be the causative triggers for GBS, as they resemble human gangliosides, and infection can trigger the generation of antibodies that attack the gangliosides on the myelin

sheath (29). Immunoglobulin G (IgG) antibodies to GQ1b and GT1a gangliosides are found in more than 90% of patients with this acute demyelinating disease (21). This finding has provided evidence to implicate these antibodies for damaging motor-nerve terminals in afflicted individuals (21). Because of this association, there are serious regulatory concerns regarding *C. jejuni* vaccine candidates (29). Researchers are now searching for novel antigens to replace a whole-cell vaccine strategy.

The high prevalence of campylobacteriosis poses a significant health burden and validates the need for a vaccine against this pathogen. The current lack of understanding of *Campylobacter jejuni*'s pathogenesis is largely due to the absence of a suitable animal model to represent human infection (30; 31). The animal model most frequently used to study human pathogens is the mouse, but murine models of *C. jejuni* infection have the disadvantages of sporadic colonization, the absence of clinical signs of disease, and a lack of intestinal immunopathology (30). This phenomenon is due in part to the colonization resistance exhibited by adult mice (older than 2 months) with a normal intestinal microflora (30). The colonization resistance against *Campylobacter* can be diminished by the administration of a quintuple antibiotic treatment (ampicillin, vancomycin, ciprofloxacin, imipenem and metronidazole) to generate gnotobiotic (GB) animals (32). These animals can be subsequently populated with typical human flora (hfa) while both GB, and hfa animals, display the characteristic inflammatory responses seen in human campylobacteriosis (33). Other models, using ferrets (34) or rhesus monkeys (35; 36), are believed to accurately mimic the human course of disease. Their applications, however, are limited by practicality as housing these species requires specialized personal and an extensive animal care facility (31). Other studies have utilized chickens (37; 38), a natural host of *C. jejuni*, although the generalizability to human campylobacteriosis is not well established (31).

### 1.1.1 Combating Reactive Oxygen Species (ROS)

*Campylobacter jejuni* is a microaerophilic bacterium and is damaged by the normal atmospheric level of oxygen ($O_2$) and requires $O_2$ in the range of (2-10%) for optimal growth (39). During aerobic metabolism, *Campylobacte*r is exposed to several reactive oxygen species (ROS) including superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide ($H_2O_2$) generated during the reduction of molecular oxygen (40). ROS species damage DNA and cause the peroxidation of lipids (40; 41). *Campylobacter jejuni* is also exposed to ROS produced by the host immune system, primarily by polymorphonuclear leukocytes (PMLs), whose oxidative burst is cytotoxic for bacteria (41). *C. jejuni*, however, possess a number of proteins and enzymes that are thought to protect it against the damaging effects of oxidative stress (42). The roles of SodB (superoxide dismutase), KatA (catalase), AhpC (alkylhydroxyperoxidase), Cft (ferritin), and FdxA (ferredoxin) in oxidative stress defense mechanisms have been partially characterized (43). In *C. jejuni*, the oxidative stress defense mechanisms are regulated by the peroxide-sensing regulator Per-R (44).

### 1.1.2 Epithelial Translocation

In an immunologically naive host, *C. jejuni* colonizes the mucous linings of the small and large intestines. The flagellum is composed of two closely related proteins FlaA and FlaB. This flagellum, combined with the spiral shape of the bacterium, confers the advanced motility that allows *Campylobacter* to burrow deep into the mucous (45). Because of this remarkable adaptation, it had once been speculated that adhesion to the host epithelia was not necessary and the bacterium remained in the intestine by colonizing the mucous (46). More recently, several adhesion proteins have been identified, notably CadF and FlpA, that permit binding to the

extracellular matrix by attaching to fibronectin (47; 48). In response to the host environment, *C. jejuni* begins the synthesis of *Campylobacter*-invasion antigens (Cia), which are exported from the bacterium's flagellar type III secretion system (T3SS), to facilitated host invasion (49). Internalization of the bacteria requires host cell signalling as well as rearrangement of the host cell cytoskeleton (50; 51). There is evidence these bacteria may also cross the epithelia via a paracellular route through tight junctions (3). Intracellular *C. jejuni* are able to induce the loosening of connections between adjoining epithelial cells thus facilitating the translocating across the enterocytes of the intestinal barrier (52).

It has been suggested that *Campylobacter sp*. preferentially associate with the microfold (M) cells, to facilitate their invasion (53). M cells are a component of the mucosal-associated lymphoid tissue (MALT), located in follicle-associated epithelium (FAE), and are designed for the sampling and inward transportation of luminal material (54). The host response to infection includes the expression of pro-inflammatory cytokines that are dependent on nuclear factor κβ (NF-κβ ) and mitogen-activated protein kinase (MAPK) signalling pathways resulting in the recruitment of neutrophils and macrophages to site of infection (55; 56). The resulting inflammation exacerbates local tissue damage resulting in a loss of epithelial integrity. The compromised tissue leaks fluid containing blood, proteins, and inflammatory cells into the lumen generating the characteristic bloodied diarrhea (45).

The immune cells within the intestinal mucosa, such as dendritic cells (DCs) and other phacocytes, provide the primary defense against *C. jejuni*. DCs play an important role in attracting moncytes, macrophages, natural killer (NK) cells, and especially T cells during the initial stages of infection (57). PMLs, predominantly neutrophils, infiltrate the intestinal epithelia and readily phagocytise complement-opsonized *C. jejuni* and kill them efficiently following a

ROS burst (3). In contrast to phagocytosis by PMLs, opsonisation by antibody or complement is not required for efficient uptake by macrophages (58). After host invasion, *C. jejuni* appear to be largely confined to membrane-bound vacuoles but have also been observed in the cytoplasm *in vivo* (59). The bacteria have been observed to quickly invade Hep-2 (originating from human laryngeal epidermoid carcinoma) and INT407 cells (human embryonic intestinal cell line) after a 1-2 hour exposure *in vitro* (60). At this early stage, Konkel *et al*. (1991) observed no lysosomal host response and the bacteria still possessed spiral morphologies. At later stages, a strong lysosomal response was evident and lysosomes, containing endosomes, surrounded the bacteria. By this point of invasion, the bacteria have generally changed from their spiral morphology into a coccal form (61). In an *in vivo* invasion, there is a reduction in the number of viable bacteria after the first 6 hours with few viable cells remaining by 36 hours (61). There is little evidence for intracellular multiplication of *C. jejuni* although vacuoles containing multiple bacteria have been observed within INT407 cells (60).

### 1.2 Acquired Immunity

The primary principle of vaccination is to administer a killed or attenuated form of a pathogen, that does not itself cause disease, that will instruct the body how to produce an effective immune response for when the pathogen is encountered again (62). Long-term protection requires the persistence of antibodies induced by the vaccine and the generation of immune memory cells capable of a rapid, and productive, reactivation (63). The first exposure to an antigen, either by infection or vaccination, leads to the activation of naive B lymphocytes and their subsequent differentiation into antibody-secreting plasma cells and memory cells. A secondary immune response is elicited when the same antigen stimulates these memory cells leading to a more rapid

proliferation and differentiation and the production of a greater quantity of specific antibodies then produced in the primary response (62).

Most prophylactic vaccines work by stimulating the production of high-affinity neutralizing antibodies (64). Therefore, effective vaccines against microorganisms must induce memory B cell formation and these events occur primarily through the activation T helper cells. Other antigen-specific T cells, including cytotoxic T cells, perform important effector functions, such as the targeted removal of host cells infected by intracellular pathogens and provide support for macrophages in their removal of extracellular pathogens (65). Whole-cell killed or live-attenuated bacterial vaccines are often the first formulation considered as they elicit both the innate and adaptive immune responses as the true pathogen would (66). These vaccines, however, may not always be safe to use depending on the pathogen and the target population. Before examining current developments in *C. jejuni* vaccines, the mechanisms in the development of protective immunity will be reviewed.

### 1.2.1 Antigen Recognition

The innate immune system recognizes molecular structures that are characteristic specific to microbes. These elements are referred to as pathogen-associated molecular patterns (PAMPs) and are expressed differentially by different classes of microbes (e.g. Gram-negative bacteria, Gram-positive bacteria, viruses, fungi). Most host cell phenotypes express pathogen-recognition receptors (PRRs) constitutively although phagocytes and DCs contain the most numerous and diverse receptors (67). Toll-like receptors (TLRs) are integral membrane glycoproteins capable of recognizing a variety of microbial PAMPS. The cytoplasmic portion of the TLR is similar to that of interleukin (IL-1) receptor family, referred to as Toll/IL-receptor (TIR), and is responsible

for the initiation of intracellular signalling (68). TLRs contain leucine-rich repeats (LRRs) in the extracellular domain capable of recognizing PAMPS (69). The successful activation of TLRs by microbes initiates signal transduction pathways which induces dendritic cell maturation and cytokine production (67). After ligand binding, TLRs dimerize and undergo a conformational change permitting the recruitment of a TIR-domain-containing adaptor molecule. To date, the TLR family consists of 13 members; 9 of which are functional in humans (Table 1) (67). TLRs expressed on the plasma membrane can recognize PAMPs in the extracellular environment. They may also be expressed internally on the endosomal membrane and detect the features of prokaryotic DNA and RNA. *C. jejuni* primarily activates TLR2 and TLR4 while TLR4 signalling is responsible for most of the inflammatory changes seen during infection (70; 71). In contrast, TLR2 signalling has a protective role and acts to promote mucosal integrity (72).

Bacteria and fungi are sometimes able to exploit the TLR system to evade host immune responses. Certain pathogens have modified forms of the normal TLR ligands, such as flagellin from *H. pylori* and *C. jejuni,* that TLR5 cannot recognize (73). Many individuals infected with *C. jejuni* are asymptomatic carriers (74; 75) and TLR5 evasion may be critical for establishing this carrier state which facilitates the persistence of the bacteria within human populations (73).

In addition to the membrane-bound TLRs described above, two major classes of PRRs are found in the cytoplasm. The first, NOD-like receptors (NLRs) are a family of over 20 different cytosolic proteins which bind PAMPs and recruit additional proteins to form signalling complexes and promote inflammation. NLRs, like TLRs, contain three domains: (1) a LRR domain involved in ligand recognition; (2) a central NOD domain acting as an ATP-dependent dimerization domain (NACHT); and (3) an N-terminal caspase recruitment effector domain (76).

Table 1. Mammalian Toll-like receptors, predominant cellular localization, and specificity.

| Toll-like receptor (TLR) | Location | Specificity |
|---|---|---|
| 1 | Cell membrane | Bacterial lipopeptides |
| 2 | Cell membrane | Bacterial lipopeptides and peptidoglycan |
| 3 | Endosome | dsRNA |
| 4 | Cell membrane | Lipopolysaccharide (LPS) |
| 5 | Cell membrane | Bacterial flagellin |
| 6 | Cell membrane | Bacterial lipopeptides |
| 7 | Endosome | ssRNA |
| 8 | Endosome | ssRNA |
| 9 | Endosome | CpG DNA |

Adapted from Uematsu & Akira (2006) (68).

There are three NLR subfamilies categorized by the effector domain utilized to initiate signalling. The caspase-recruitment domain (CARD) subfamily has two members: NOD1 and NOD2 which respond to bacterial cell wall peptidoglycans. NOD1 recognizes PAMPs derived from Gram-negative bacteria while NOD2 recognizes muramyl dipeptide found in both Gram-negative and Gram-positive bacteria (77). These peptides may arrive in the cytoplasm of the host cell most often released from intracellular bacteria. They may also be transported into the cell from an extracellular pathogen via type III and type IV bacteria secretion systems (62). These secretion systems are found in Gram-negative bacteria, such as *Salmonella* and *C. jejuni*, encoded within pathogenicity islands (78; 79). Consequently, NLR detection can occur by non-phagocytising cells such as epithelial cells. When a peptide binds the CARD effector domain, a conformational change occurs facilitating the formation of the NOD signalasome and subsequent activation of the transcription of inflammatory cytokines and chemokines important for the stimulation and recruitment of additional effector cells (78). NOD1 and NOD2 are important in the defense against bacterial pathogens in the gastrointestinal tract such as *C.jejuni* and *H. pylori.* The second subfamily contains 14 known members with a Pyrin effector domain (PYD), which is activated by the presence of microbial products such as flagellin, muramyl dipeptide, LPS and crystalline substances, such as aluminum hydroxide (62). Activation results in the assembly of the inflammasome complex that binds and activates the enzyme caspase-1 (80). Caspases are cysteine proteases that initiate cellular programs leading to inflammation (pro-inflammatory) or cell death (pro-apoptotic) (81). Caspase-1 cleaves and activates IL-1β, an important pro-inflammatory mediator produced at sites of tissue injury to recruit other immune cells. (82).

### 1.2.2 Dendritic Cells

Dendritic cells have been described as professional antigen-presenting cells and are the primary source of antigen transport into the lymphatic system (83). Dendritic cells are strategically located at the common sites of entry of microbes to the epithelia of the skin, gastrointestinal, and respiratory tracts, and in lymphoid organs and provide the primary defense against enteropathogens (57). In addition, they link the innate and adaptive immune responses to microbial pathogens by producing, and responding to, inflammatory cytokines (84). Dendritic cells are key initiators of adaptive immunity and are distributed throughout the body in their immature form (85). They actively sample the environment in peripheral tissues by endocytosis and macropinocytosis (86). Immature DCs are highly phagocytic, and upon encountering a PAMP, undergo a process of maturation resulting in the increased expression of major histocompatibility complex (MHC) molecules (87), costimulators (88), Th polarising cytokines (89), and migration to the lymph nodes to prime naive T-cells (90).

DCs express high numbers of pathogen-recognition receptors (PRRs) and most members of the TLR family to facilitate pathogen recognition. Bacteria encountered are internalized, by receptor mediated endocytosis, into phagosomes. The phagosome will undergo maturation and fuse with both endosomes and lysosomes. These vesicular interactions deliver host molecules with antimicrobial properties including generators of toxic reactive oxygen species (discussed later), proteases, thiol reductases and proton pumps (91). The resulting peptides from the protease digestion associate with MHC-II molecules present within the phagosome (92). External signals direct the DC to regional draining lymph nodes and initiates antigen processing and presentation (93).

Dendritic cells activated by *C. jejuni* express high levels of MHC molecules with bound peptide and upregulate co-stimulatory molecules such as cluster of differentiation (CD) CD40, CD80, and CD86 (94). The *C. jejuni* infection of DCs initiates expression of NF-κB leading to production of IL-1β, IL-6, IL-8, IL-10, IL-12, interferon (IFN) gamma and tumour necrosis factor (TNF) alpha (95). In addition, DCs begin expressing a chemokine receptor CCR7 specific for two chemokines (CCL19 and CCL21) allowing for migration to the T cell zones of lymph nodes (90). Naive T cells also express CCR7 and migrate to the same regions of the lymph nodes antigen bearing DCs are concentrated. The recognition of MHC class II by the T cell receptor (TCR), along with adequate co-stimulation, results in the clonal expansion and differentiation of naive CD4+ T cells into an effector subtype of helper T cells (Th) (96). The Th cells that have been activated are induced to proliferate, express the CD40 ligand, secrete cytokines, and migrate towards B-cells in the follicle (62).

Most antigens from tissue sites are transported to lymph nodes by afferent lymphatic vesicles that drain into the subscapular sinus of the nodes. Soluble antigens (smaller than 70 kDa) may reach the B cell zone through conduits that extend throughout the follicle and interact directly with B cells (62). Large antigens may be captured by macrophages, in the subscapular sinus, or by dendritic cells, in the medullary region, that actively filter particulate antigen (97). The antigen is then transported into follicles where it can activate B cells. In all cases, the antigen presented has not been processed by antigen presenting cells (APCs) and in an intact, naive conformation (62). B cell activation is initiated when an antigen binds the B cell receptor (BCR). Although protein antigens, being monovalent, typically do not provide strong enough signals to induce B cell proliferation and differentiation, they can activate B cells and initiate a number of events. Activation of the BCR by antigen initiates coordinated assembly of the signalosome and

triggers a variety of cellular response including modifications to gene expression, reorganization of the cytoskeleton, and internalization of the antigen (98). Following antigen endocytosis, it is processed inside endosomes and presented in complex with a MHC class II molecule (99). This peptide is then presented by the B cell to a helper T cell activated when recognized an identical peptide presented by a dendritic cell (Fig. 1).

### 1.2.3 T helper cells

The CD40 receptor is constitutively expressed on B cells and is crucial for the initiation of antibody class switching. The CD40 ligand is upregulated on activated T cells and recognizes the CD40 receptor and provides a signal necessary for B cell survival (100). This binding induces a conformational change initiating an enzyme cascade which terminates with the activation of transcription factors which stimulate B cell proliferation and increase secretion of IgG (101). In addition to CD40L on Th cells activating B cells, Th cells also secrete cytokines that contribute to B cell responses and control the class of antibody produced. This process is referred to as isotype switching and provides B cells with the capacity to produce antibodies that perform distinct effector functions in the defence against various infectious agents.

INF-γ is the main inducer of the production of IgG2a, an essential opsonin and effective activator of complement mediated lysis (102). Viruses and many bacteria activate the Th1 subtype and initiate responses resulting in the enhancement of several cytotoxic mechanisms. Th1 cells produce the cytokine IFN-γ which promotes the activation of macrophage and results in increased killing of intracellular pathogens (103). This occurs through increased expression of macrophage receptors for IgG2a and antibody-dependent phagocytosis (102). IFN-γ is also believed to be a major component of delayed type hypersensitivity reactions (DTH) (104). IFN-γ

initiates the inflammatory reaction by attracting T cells which secrete cytokines to recruit monocytes and granulocytes to the site of infection (104).

The IL-4 cytokine is produced by Th2 and leads to the production of high antibody IgG1 levels and IgE antibodies. In addition, Th2 cells produce IL-3, an activator of mucosal mast cell proliferation (102), and IL-5 a stimulator of eosinophils (105). Consequently, Th2 activation can lead to the activation of allergic responses which is considered an adverse event following vaccination. Th1 and Th2 cells have been found to cross-regulate each other with respect to IgG and IgE production (103); Th2 cytokines (IL-4 and IL-10) have been shown to inhibit proinflammatory cytokines from macrophages (IL-1 and INF-γ) while IFN-γ enhances macrophage activation (106). Th17 cells produce the cytokine IL-17 which can induce neutrophil rich inflammation.

Several theories are believed to influence the Th1/Th2 phenotype generated. The PAMP hypothesis postulates that interaction of host PRRs with PAMPs is the most important factor (85). A second hypothesis, referred to as the cytokine milieu, proposes that the cytokines secreted by activated T-cells in microenvironment promote differentiation of Th cells (85). During the course of an actual *C. jejuni* infection, dendritic cells generate a cytokine milieu that promotes the expansion of Th1, Th17 and the Th1/Th17 double-positive T cells (107). This occurrence reflects the importance of the production IFN-γ, IL-22, and IL-17 in enabling functional immune responses against *C. jejuni* infection (107).

### 1.2.4 Plasma Cells

Some of the progeny of the B cells that proliferate in response to antigen and T cell simulation will become antibody-secreting plasma cells. Long-lived plasma cells are generated in T-

dependant germinal center responses (Fig. 1). This process is dependent on the initiation of high levels of Blimp-1 and interferon regulatory factor 4 (IRF4) (108). T cells secrete the cytokines that promote Blimp-1 expression, including IL-2, IL-6, and IL-21, through activation of the STAT3 transcription factor (109). The plasma cells, generated in the germinal centers, acquire the ability to travel to the bone marrow or local mucosa-associated lymphoid tissues (MALT) through the expression of CXCR4 (110). A proportion of plasma cells will remain in the spleen, initially in close association with mature DCs and later dispersing into red pulp (100). The stromal cells in the bone marrow, and the DCs in the spleen, secrete two related cytokines, BAFF and APRIL (111). The binding of these cytokines to the plasma cell receptors allows the cell to survive for long periods, often as long as the lifespan of the host. Memory B cells also express the antiapoptotic protein Bcl-2 which contributes to the long lifespan of these cells (112). The bone marrow becomes the major site of antibody production approximately 2-3 weeks after encountering a T-cell dependent antigen (113). Plasma cells may continue to secrete antibodies for months or even years after the antigen is no longer present (113). These antibodies can provide immediate protection if the antigen is encountered again. It is estimated that almost half the antibody in the blood of a healthy adult is produced by long-lived plasma cells and is specific for antigens encountered in the past (62). The ability of antibodies to neutralize pathogens is dependent on high-affinity and high-avidity interactions. Antibodies are highly specific for antigens can distinguish between two linear protein determinants differing by only a single amino acid substitution (62). The B cells in the repertoire that express the most specific antigen receptors are primed for proliferation. Continued antigen exposure drives rounds of B cell receptor diversification, selection, and proliferation until receptors reach high affinity (known as

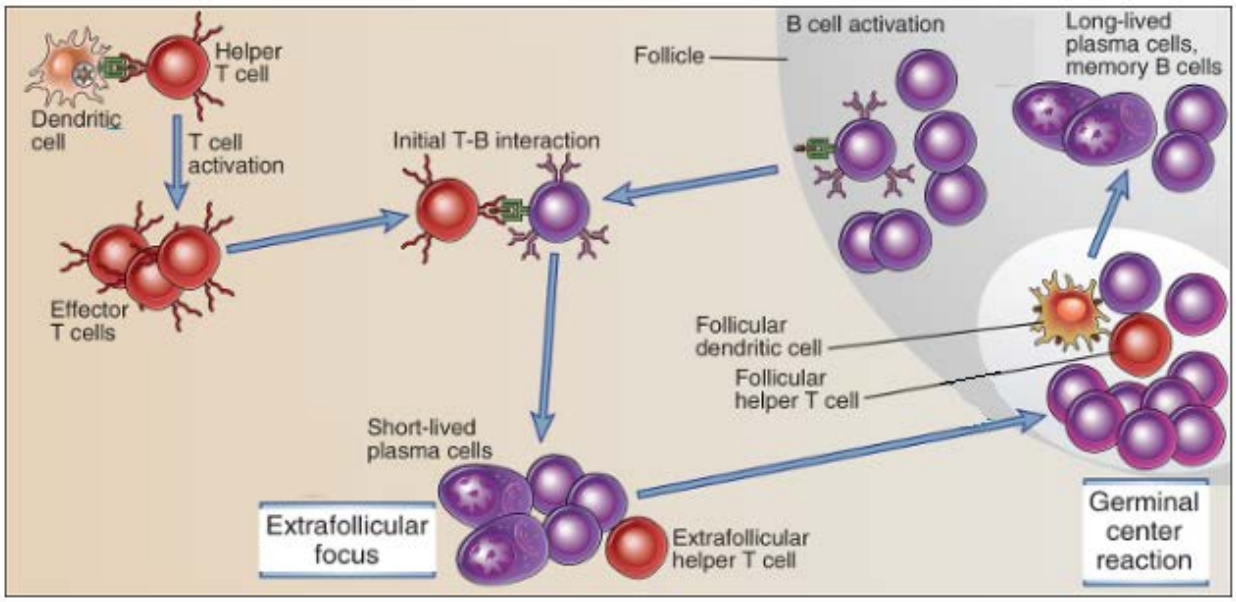



**Figure 1**. Helper T cell dependent activation of B cells in response to protein antigens. Adapted from Abbas, Lichtman, and Pillai, (2012) (62).

affinity maturation) (114). The result of this process is that B cells producing higher affinity antibodies are preferentially selected and become the dominant cells in the population (114).

Antibodies are large molecules, consisting of paired heavy and light polypeptide chains. These form a variable (V) antigen-binding region (known as $V_H$ and $V_L$ for the heavy and light chains respectively), as well as a constant (C) region (115). Antibodies are classified as isotypes, depending the type of heavy chain they possess, as either IgA, IgG1-IgG4, IgD, IgE or IgM. Each isotype has a unique capacity to reach different sites of infection and to recruit different effector mechanisms (65). CD4+ activated T cells in lymphoid tissue release cytokines which drive the class switch and differentiation of B cells (116). Although this is a very complex and dynamic process, the cytokines identified to direct isotype switching are listed in Table 2 (below).

IgG is the principal isotype in blood and extracellular fluid (117). IgG opsonises pathogens to facilitate engulfment by phagocytes and activates the complement system (discussed below). In contrast, IgA is a less potent opsonin and a weak activator of complement. IgA is the major isotype in mucosal secretions and functions on epithelial surfaces where complement are phagocytes are absent. Therefore, the function of IgA is thought to be as a neutralizing antibody (118).

**Table 2**. Class-Switch Recombination (CSR); cytokines which direct immunoglobulin isotypes.

| Ig Isotype | Dominant cytokine |
|---|---|
| IgA | TGF- β (119; 120) |
| IgG1 | IL-4 (121; 122) |
| IgG2a | IFN- γ (121) |
| IgE | IL-4 (122) |

### 1.2.5 Antibody Function

The main function of antibodies is to neutralize and eliminate infectious microbes and microbial toxins. Antibodies produced in the lymph nodes, spleen, and bone marrow may enter the blood and circulate throughout the body. Humoral immunity in the gut is dominated by the production of secretory IgA (sIgA) and sIgM in the gut-associated lymphoid tissues (GALT) (1).

There may be some contribution from serum-derived or locally produced monomeric IgA and IgG which can reach the mucosal surface rapidly by paracellular diffusion through the epithelium (123) and transported across the mucosal epithelium into the lumen (Fig. 2). Activated B cells that undergo isotype switching into IgA producing cells in the gut-associated lymphoid tissue (GALT) and mesenteric lymph node become resident plasma cells in the lamina propria. The sIgA antibodies secreted on to the mucosal surfaces in the intestine are essential for preventing bacterial infection by binding and neutralizing microbes (124). These antibodies are thought to inhibit adhesive reactions by interfering with the ability of the microbes to interact with cellular receptors by causing steric hindrance. In some cases allosteric effects may occur from the binding of even very few antibodies. Alternatively, their binding may induce a conformational change in microbial surface molecules preventing interactions with cellular receptors (62).

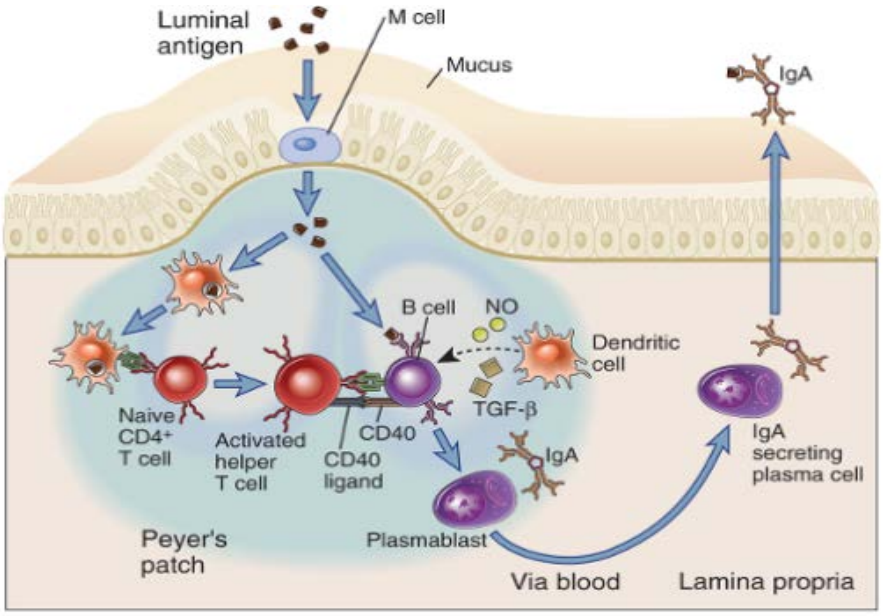



**Figure 2**. The production of high-affinity IgA antibodies against an antigen via a T cell dependent activation of B cells. The resulting secretion of high-affinity IgA can result in the neutralization of enteric pathogens. Adapted from Abbas, Lichtman and Pillai (2012) (62).

### 1.3 Adjuvants

Adjuvants are substances that can improve an antigen-induced immune response by protecting the antigen, modulating cytokine release, activating CD8+ cytotoxic lymphocytes (CTLs) responses, or delivering the antigen to target tissue (125). Purified antigens are not usually strongly immunogenic and most acellular vaccines require the addition of an adjuvant in order to be effective (126). They enhance vaccine effectiveness by stimulating the innate immune responses at the sites of antigen exposure. Many adjuvants in experimental use are microbial products, such as killed bacteria and LPS, which can engage cellular TLRs.

Dendritic cells are principle mediators of responses to TLR agonists (127) and activation stimulates the migration of DCs to lymph nodes where T cells are located. Adjuvants are highly effective immune stimulators and as a result can cause damaging amounts of localized inflammation. As a consequence, a tremendous amount of effort has been devoted to the development of safe and effective vaccine adjuvants in humans (128). LPS is a component of the cell wall of Gram-negative bacteria and it is a potent adjuvant and TLR4 activator (129). LPS, however, is too toxic for clinical applications as even the injection of a small amount of LPS can cause systemic inflammation (130). A derivative of LPS, monophosphoryl lipid A (MPL) has been shown to retain the immunostimulating capacity but with decreased toxicity (127). This adjuvant, a derivative from *Salmonella minnesota*, is widely used experimentally and has been also tested in human clinical trials (131). Alum, composed of aluminum salts, remains the gold standard vaccine adjuvant as has been used for over 80 years due to its role in clinical applications. Aluminum is thought to enhance the mucosal immune response by localizing antigenic material in the interstitial fluid and extending the duration of antigen presentation to immune cells (127). A deposit is formed at the site of injection causing localized tissue damage

and increasing recruitment of APCs and antigen phagocytosis (132). In addition, alum taken up by macrophages has been shown to increase the duration of their survival (133). Alum signals through NLR NOD-2 and, as a result, is an effective inducer of humoral immunity and Th2-biased CD4+ T cell responses (134). Other experimentally used adjuvants include lipopolysaccharide-polyribonucleotide complexes, imiquimod, muramyl dipeptide and analogues, nonionic block copolymers, saponin immune stimulating complexes (ISCOMS), the oral mucosal adjuvant cholera enterotoxin (CT), and dehydroepiandrosterone (DHEA) (135).

### 1.4 *Campylobacter jejuni* Vaccine Research

At present, there is no commercial vaccine available against *C. jejuni* although some benefit from whole-cell vaccines has been established. Infection of adult volunteers with live *C. jejuni* milk-borne strain 81-176 by Black *et al*. (1988) demonstrated the development of jejunal fluid sIgA decreased the likelihood of illness at secondary exposure (136). A second clinical challenge study was recently published by Tribble *et al*. (2010) (27) vaccinating people with the same strain. The rates of systemic and mucosal response were comparable to rates observed after a natural exposure to the pathogen (137). Individuals were challenged one-year later and demonstrated a greater magnitude of IgA and IgM serological responses, and more rapid anamnestic fecal IgA, than naive participants. Before the study began, the LOS of the strain was characterized to determine if molecular mimicry was possible and initial results came back negative (138). Unfortunately, following the study a genetic analysis of the LOS core revealed that mimicry was in fact possible (139). It was determined that the vaccination had in fact posed a risk to the participants although no incidents of GBS were reported (139). A new strain of *C. jejuni* CG8421 has recently been identified and does not possess a risk of GBS for humans (140). Healthy adult volunteers were challenged with this live strain, by Kirkpatrick *et al*. (2013), and

were rechallenged after three months (141). Immune responses were the most robust following the first exposure where seroconversion, a minimum four-fold increase in antibodies, was present in 67% of participants. IgA levels returned to baseline within three months and neither IgA or IgG achieved seroconversion at rechallenge. A two-fold or greater increase in IFN-γ was observed following the first challenge with levels falling close to baseline by three months. Unfortunately, participants experienced diarrheal disease clinically similar from that of the primary infection following the rechallenge. The lack of immunity provides further evidence that long-lived protection is unlikely after a single exposure to live whole-cell *C. jejuni*.

### 1.4.1 Subunit Vaccine Strategies

Although attenuated vaccines elicit long-lasting immunity, safety issues including the administration of potentially harmful bacterial components, and the potential for replication or reversion to a pathogenic form, are of concern as vaccines are normally administered to healthy individuals (142). In the case of *C. jejuni*, the risks of eliciting an auto-immune response prohibit a whole cell vaccination strategy. For this reason, the use of a subunit vaccine approach is often warranted in which only the microbial components required to produce an appropriate immune response are administered. Although subunit vaccines have many desirable qualities, their ability to stimulate potent immune responses is much weaker than traditional whole-cell preparations (143).

Recent work involving conjugate vaccines has been published combining capsular polysaccharides (CPS) from *C. jejuni* with protein carrier. Monteiro *et al*. (2009) conjugated CPS with diphtheria toxin (DT) mutant $CRM_{197}$ and subcutaneously injected the non-human primate *Aoutus nancymaae* (144). Post-immunization serum collection revealed significant increases in

IgG, and IgM, but no increase in IgA. The animals were later challenged with $6 \times 10^{11}$ colony forming units (CFU) of *C. jejuni* and observed for symptoms of illness. They reported that no animals experienced diarrheal symptoms but necropsy revealed intestinal colonization by the bacteria. Bertolo *et al*. (2013) produced similar antibody levels with their CPS conjugate, CPS and an un-named protein, but the functionality of the antibodies in preventing infection is yet to be tested (145). The principle obstacle with these vaccine designs is the considerable structural variation of CPS between serogroups (146). This diversity makes the design of a CPS vaccine capable of providing a broad range of immunity quite challenging. Additional vaccines have also been targeted against *C. jejuni* flagellum due to the important role it plays in invasion. Lee *et al*. (1999) immunized mice with a recombinant FlaA protein fused to the maltose-binding protein (MBP) of *Escherichia coli* (147). Mice were immunized intranasally with and without the mutant *E. coli* heat-labile enterotoxin (LT). Their formulation was effective in eliciting antigen-specific serum IgG and responses were enhanced when LT was co-administered. Significant levels of sIgA were also detected in the intestine. Animals were challenged orally with between $8x10^{8}$ – $8\ x10^{10}$ CFU and protection against colonization ranged from 71.4% to 100% depending on CFU concentration. Although this formulation appears to provide good levels of protection in the murine model, it may be problematic in humans as portions of the *C. jejuni* flagella are highly conserved with other bacterial flagellins and may generate immune responses against normal gut microflora (148). Baqar *et al*. (2008) attempted to circumvent this issue by targeting the secreted non-flagellar proteins that are associated with virulence. FlaC, FspA1, and FspA2 are secreted Cia proteins required for the invasion of intestinal epithelial cells (IECs) (149). Each protein was administered individually intranasally to mice and all three were confirmed to be immunogenic. FspA1 induced the highest levels of serum IgG and fecal IgA, and provided the greatest percent

protection (63.8%) against 3 x$10^9$ CFU when combined with LT. Although promising results, increased levels of protection are still desired and FspA1 is yet to demonstrate efficacy in higher animals.

Other research has focused on vaccine development against a virulence determinant of *C. jejuni*; cytolethal distending toxin (CDT). Advances in proteomics have permitted the design of pentapeptides, the smallest determinant functions as an antigen (150), which contain domains against CDT while being absent from the human proteome (151). Although intelligent in design, this vaccine strategy for *C. jejuni* has not been tested *in vivo*.

### 1.5 KatA (Catalase) Protein

The role of the catalase protein of *C. jejuni* (KatA) was discussed briefly above. KatA is a homotetrameric protein approximately 55 kDa in size. The protein is considered a typical catalase family member as it lacks peroxidise activity. Catalase appears to be primarily a cytosolic protein, however, data from investigations using the related species *Helicobacter pylori*, has indicated that the protein may have a surface localization as well (152; 153; 154). Harris *et al*. (2002) demonstrated that *H. pylori* was resistant to high concentrations of hydrogen peroxide (100 mM) using an *in vitro* tolerance assay. They compared the survival of KatA-deficient mutants and discovered these bacteria were unable to survive the harsh incubation (155). It was subsequently demonstrated *in vivo* that mice immunized with the KatA-deficient mutant cleared the infection significantly more than those immunized with the wild-type strain (156). Palyada *et al.* (2009) confirmed the necessity of the KatA protein for *C. jejuni* colonization i*n vivo* (42). They examined the ability of the *C. jejuni* strain NCTC 11168 *ΔkatA* mutant to colonize the cecum of one-day old pathogen-free baby chickens. They determined that

mutants were not able to establish a colony (the number of mutants per gram of cecum was below detection limit) compared to the wild-type strain which had $2.0x10^7$ CFU per gram of cecum. The *katA* gene has been sequenced and codes for 508 amino acids (157). It has been suggested to be a potential vaccine candidate for a variety of enteric bacteria that require the KatA protein for colonization (155). Although many bacteria express catalase, they do not necessarily share homology; *C. jejuni* KatA is only 56% homologous to KatA from *Helicobacter* species, but is 99% conserved with other sequenced *C. jejuni* strains (158). The conserved sequence identity between *C. jejuni* strains made KatA a prospective candidate for our research on *C. jejuni* vaccine development. It has not yet been determined if humans can generate antibodies against this protein and a primary goal of this thesis was to determine KatA potential for vaccine development.

### 1.6 Rationale Behind the Project

One of the major concerns about the development of a vaccine against *C. jejuni* revolves around inadvertently causing the autoimmune dysfunction, Guillain-Barré syndrome. As a consequence, more traditional vaccination strategies, such as using whole-killed or live attenuated bacteria, are not feasible in these circumstances. The best vaccination strategy is therefore to select a strongly antigenic epitope against *C. jejuni* and attempt to elicit a robust immune response against it. The catalase of *C. jejuni* (KatA) has been proposed to be a vaccine candidate against this enteric pathogen.

### 1.6.1 Aims

1) To determine if KatA is associated with the bacterial cell membrane.
2) To determine if KatA is a potent antigen suitable for vaccine development.
3) To determine if the antibodies generated against KatA, following the vaccination, have bactericidal properties and/or prevent invasion and adhesion of *C. jejuni* to human IECs.

### 1.6.2 Hypothesis

Catalase from *Campylobacter jejuni* will induce robust immune responses when administered *in vivo*. The antibodies produced will also be functional and will prevent *C. jejuni* from infecting immortalized human epithelial cells. The primary goal of this research was to validate KatA as a potential vaccine target and to further the development of a safe vaccine against campylobacteriosis.

## 2.0 MATERIALS AND METHODOLOGY

### 2.1 *Campylobacter jejuni* Culture Conditions

Frozen *C. jejuni* strain 11168 was obtained from NCTC (Salisbury, UK). Ten microlitres (µL) of the bacterial suspension was streaked on Mueller-Hinton (MH) (Difco, New Jersey, USA) agar plates and incubated for 24-48 hours under microaerobic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) at 37°C in an Invivo$_2$ 500 Hypoxia Workstation (Ruskinn Technology Ltd, Pencoed, UK). A single colony was selected and inoculated in 25 mL MH broth medium and incubated for 24 hours. A total of 25 uL of this broth culture was then transferred to 250 mL sterile MH broth medium and incubated as above until the desired cell density was achieved.

### 2.2 Bacterial Growth Curve

Following the initial 24-hour growth period, 1 mL of MH broth culture was removed and transferred to a disposable clear cuvette and the optical density (OD) was measured at 600 nm using a portable spectrophotometer (Cell Density Meter 40, Fisher Scientific). Prior to measuring the culture broth, the cell density meter was calibrated using sterile MH broth. The measurement was repeated at time(s) 0, 1, 2, 6, 10, 16, 24, 30, 48, 54, 72 hours. A second aliquot of 100 µL was removed and diluted serially in sterile PBS to achieve a final dilution of $10^{-3}$,$10^{-4}$, and $10^{-5}$ at time 0-9 hours, $10^{-4}$, $10^{-5}$, and $10^{-6}$ from time 10-24 hours, and $10^{-5}$, $10^{-6}$ and $10^{-7}$ at time 24-72 hours. A 22 µL aliquot of diluted bacteria was then transferred to 11 mL of warm MH agar (0.7% agar), mixed thoroughly, and poured on to a 25 mL MH agar plate (1.5% agarose). The plates were incubated for 48 hours under microaerobic conditions, as described above, and the number of colonies were counted. The number of colonies at each time point was multiplied by the dilution factor to determine the population density as a function of OD. The growth pattern

was also plotted as a function of time and as a function of OD. Protocol based on work described by Al Rashid (1998) (159).

### 2.3 Determination of Protein Concentration

Protein concentration in bacterial fractions, and purified antibodies, was determined using a Thermo-Scientific (Canada) microplate protein assay kit (Cat#23225) containing bicinchoninic acid as the detection reagent. Standards were prepared by diluting bovine serum albumin (BSA) to 100 µg/mL in 0.15M NaCl to create standards of 1000, 1500, 750, 500, 250, 125, 25 µg/mL and 0 µg/mL. The BCA working reagents were combined at a ratio 50 parts reagent A with 1 part reagent B as per manufacturer's instructions. 25 µL of each standard or sample was transferred into a microplate well followed by the addition of 200 µL of working reagent. The mixture was incubated at room temperature for 5 minute before the absorbance was read at 562 nm in a microplate reader (Synergy H4 Hybrid Reader, Biotek). In this colorimetric assay, the standards are the point of comparison for the protein concentration in the samples. This protocol is a dye-binding assay in which a differential colour change occurs in relation to the concentration of protein. Sample OD was determined following subtraction of the OD of the blank (0 µg/mL).

### 2.4 Measurement of Particle Size Distribution and Zeta Potential

The protein size distribution was measured using dynamic light scattering at 25°C with a Zetasizer (Malvern Instruments, UK). Briefly, 5 µL of KatA protein suspension (50 mM Tris, 0.2% SDS, 0.1% Tween 20) was diluted in 50 µL of dd$H_20$ (1:11 dilution) and added to a quartz low-volume cuvette (Hellma Analytics, Germany). The intensity of the Helium-Neon laser (633nm) light scattered by the sample preparation (Phase analysis Light Scattering) was

measured to generate a particle-size distribution (PSD) profile. For the zeta potential measurement, 20 µl of sample was diluted in 980 µL of $ddH_20$ and transferred to a disposable capillary cell (Malvern Instruments, Cat#DTS1070). Three sets of measurements, comprised of 10 unique measurements each, were conducted. Data was analyzed using Zetasizer Software version 6.32 (Malvern Instruments).

### 2.5 *Campylobacter jejuni* Fractionation to Determine KatA Localization

One-hundred millilitres of *C. jejuni* strain 11168 were grown in a 250 mL flask to an $OD_{600}$ of 1.5-2.5. They were then concentrated by centrifugation for 5 minutes at 3500 RPM and resuspended in 2 mL PBS (pH 7.4). The cells were then lysed by sonication using 6 cycles of four 5-second bursts with a 1-minute time interval between where the solution was placed on ice. The cell extract was ultracentrifuged at 100,000 xg for 1-hour at 4°C to separate the soluble and insoluble fractions. The insoluble fraction (pellet) was resuspended in 1 mL PBS and transferred to a 1.5 mL microcentrifuge tube and spun at 10,000 xg for 10 minutes. The wash and centrifugation steps were repeated twice. The supernatant fraction was concentrated using Amicon 3 K (3000 kDa cut-off) tubes until a final volume of 1 mL was achieved. Protocol based on work described by Blaser *et al.* (1983) (160).

### 2.6 Antigen Purification of KatA Antibodies

The KatA polyclonal antibodies were further purified using KatA protein. Nitrocellulose paper (BioRad, Mississauga, Canada) was cut into 1 cm squares and placed in each well of a 24-well tissue culture plate (Sarstedt, Montreal, Canada). 14 µg of purified KatA protein from A. Stintzi (refer to Appendix II: collaborator methodologies) was then spotted on each square and allowed to air dry in the shortest time possible to limit oxidation. The membrane was then blocked with

300 µl of 5% skim milk (Bioshop, Toronto, Canada) in PBS. The plate was placed on a rocking platform for 1 hour at 25°C. The membrane was washed 3 times in PBS and probed with the polyclonal serum from immunized mice from A. Stinzi (refer to Appendix II: collaborator methodologies) diluted in 1:5 in PBS and incubated overnight at 4°C. Membranes were then washed again 3 times in PBS for 5-minutes each at room temperature. The bound antibodies were eluted by the addition of 150 µl 0.1 M glycine (BioShop) buffer pH 2.5. The eluate was then neutralized with 15 µl 1 M Tris-HCl pH 8.0. The final solution was stored at -20°C.

### 2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Immulon 2HB microtiter plates (Fisher Scientific, ON, CA) were coated overnight at 4ºC with recombinant KatA protein at 5.0 µg/ml in carbonate coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) and 100 µl of the antigen/coating buffer mixture was added to each well of the plate. Plates were washed 6X with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST). Diluted mouse serum samples were added to each plate at 100 µl/well and allowed to incubate at 4ºC overnight. Plates were washed again with TBST. Biotinylated goat-anti mouse IgG, IgG1 and IgG2a antibodies (Invitrogen, ON, CA) were diluted 1/10000 and then added to each plate at 100 µl/well and were subsequently incubated for 1 h at room temperature. Plates were washed and alkaline phosphatase conjugated with streptavidin (Cedarlane Laboratories, Hornby, ON, CA) was diluted 1/5000 and 100 µl was added in each well followed by another 1 h incubation at room temperature. Plates were washed 6X times in double distilled water ($ddH_2O$). Di(Tris) p-nitrophenyl phosphate (PNPP) (Sigma) was diluted 1/100 in PNPP substrate buffer and 100 µl/well was added to each plate. The reaction was allowed to develop for 5-15 min, and optical density (OD) absorbance values at 405 nm in a Synergy H4 Microplate Reader (BioTek Instruments, VT, USA) were recorded. IgG ELISA results are reported as titers, which are the

reciprocal of the highest dilution that gave a positive OD reading. A positive titer was defined as an OD reading that was at least two times greater than the values for a negative sample. IgG1 and IgG2a ELISA results were reported as OD readings to determine if there was a shift in the quality of the immune responses. Sera from naïve, unimmunized mice were used as negative controls.

### 2.8 SDS-PAGE

Samples were incubated with 2β-mercaptoethanol (Merck, Germany) for 10 minutes at 100°C then separated by 12.5% acrylamide SDS-PAGE at 90 mV for 1.5-2.5 hours. Gels were stained with G250 Blue Coomassie stain (BioRad). A BLUeye prestained molecular weight marker containing standard proteins (FroggaBio, North York, Canada) was used. The gel was photographed in FluorChem FC3 imager (ProteinSimple, USA) to capture the relative molecular sizes of the sample proteins.

### 2.9 Western Blot

Preparations were processed by electrophoresis (SDS-PAGE, acrylamide 12.5%), transferred to nitrocellulose using a semi-dry transfer method and incubated overnight on a rocking platform in blocking buffer (5% skim milk in TBST) at 4°C. Blots were then incubated with the polyclonal antibody diluted in blocking buffer at room temperature for 1-2 hours. The primary antibodies used were as follows: (1) *C. jejuni* Kat A protein Westerns: polyclonal serum from rabbit against KatA protein provided by Dr. Alain Stintzi (University of Ottawa, ON, Canada) (diluted 1:5000); (2) purified high-affinity KatA antibodies (from above serum) (diluted 1:1000). After washing 3 times with TBST for 10 minutes on the rocking platform, secondary antibodies were added and incubated for 1-2 hours at room temperature. The secondary antibody used was donkey anti-

rabbit IgG-HRP linked whole antibodies (GE Healthcare, Cat#NA934) (diluted 1:10000). The membrane was placed in an activated ECL Western blotting detection reagent (GE Healthcare, Cat#RPN2209/8/9) for one-minute prior to film exposure. Blots were exposed to Amersham ECL Hyperfilm (GE Healthcare) and developed in a medical film processor SRX-101A (Konica Minolta, Japan).

### 2.10 Animals and Immunization

All animal model experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and was approved by the Animal Care Committee of Laurentian University. Forty-eight female BALB/c mice were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada). The mice were divided into six (6) groups of 8 mice each. The mice were 3-4 weeks old when they arrived and were housed at the Laurentian University Animal Care Facility (Sudbury, Ontario, Canada) under diurnal lighting (7 A.M. to 7 P.M.). Four mice were housed in each disposable plastic cage (Innovive, USA) food and water were provided *ad libitum*. The mice received the following formulations (Table 3): (A) PBS, (B) KatA protein from A. Stintzi (refer to Appendix II: collaborator methodologies), (C) KatA protein and alum, (D) KatA protein and monophosphoryl lipid A (MPL), (E) KatA protein, alum, and MPL, and (F) $10^8$ inactivated *Campylobacter jejuni* 11168 cells. *C. jejuni* was inactivated by a combination of heat and formalin inactivation. Half of the cells were inactivated at 60°C for 60 min, while the other half were inactivated by the addition of 0.02M formalin and incubated at room temperature for 1-hour. Inactivation was verified when cells were streaked on MH agar plates and no growth was observed over 48-hours under microaerobic conditions. All formulations were in a final volume of 50 µl that was delivered intramuscularly. The amount of KatA, alum and MPL in each of their respective

formulations was 5 μg, 100 μg, and 5 μg, respectively. The amounts of each component in each formulation are outlined in Table 3. The formulations were prepared fresh one day before immunization. The mice were immunized three weeks apart for a total of three immunizations. Any signs of adverse reactions to the immunizations were monitored. Mice were bled prior to immunization and one week after each subsequent immunization by facial vein techniques. One week after the final immunization, a final bleed was conducted by cardiac blood draw. These procedures were performed by Dr. Nelson Eng and Dr. Nitin Bhardwarj (Advanced Medical Research Institute of Canada, Sudbury, Canada: 2012).

**Table 3**. Mouse subject immunization schedule and vaccine details.

| Group | Vaccine Formulation (per dose) | Route of Immunization (t = 0, 3, 6 weeks) |
|---|---|---|
| A | PBS | IM* |
| B | 5 µg Kat A | IM |
| C | 5 µg Kat A, 100 µg of alum | IM |
| D | 5 µg Kat A, 5 µg MPL | IM |
| E | 5 µg Kat A, 100 µg alum, 5 µg MPL | IM |
| F | $10^8$ inactivated/killed *C. jejuni* in PBS | IM |

*IM = intramuscular injection

## 2.11 Bactericidal Assay

Serum was pooled from the eight animals in each immunization group. Each group of sera was then incubated at 56°C for 30 minutes to inactivate endogenous mouse complement. Subsequently, 50 μL of de-complemented sera, diluted tenfold in PBS, was mixed with 25 μL of a *C. jejuni* (strain NCTC 11168) suspension containing 5 x $10^4$ CFU/mL in a sterile 96-well flat bottom microtitre plate. The plate was incubated for 15 minutes under microaerobic conditions. Subsequently, 25 μL standard guinea pig complement (diluted 1/50 in PBS) was added and the plate was further incubated for one hour under the same conditions as above. One millilitre aliquots were then removed and diluted in molten MH agar (0.7% agar) and poured on top of MH agar (1.5% agar) plates. Plates were incubated at 37°C using the above conditions for 48 hours and the colonies on each plate were counted. Bactericidal activity was calculated as a percentage reduction in viable bacteria relative to respective control plates where no serum was added in the formula [CFU (bacteria + complement) - CFU (bacteria + complement + serum)] / CFU (bacteria + complement). Plating was done in duplicate on three different occasions. In a separate experiment done once, mouse complement was also used to compare activity.

## 2.12 *Campylobacter jejuni* Adherence and Invasion Assays

*C. jejuni* strain NCTC 11168 was grown in MH media for 24-48 hours under the standard microaerophillic conditions (as previously described). The cells were centrifuged for 10 minutes at 120 x g and the pellet was re-suspended in a volume of Dulbeccos's modified Eagle's Medium supplemented with Fetal Bovine Serum (10%) and non-essential amino acids (1%) to provide a final OD reading of 0.1. Caco-2 cat#HTB-37 (ATCC, Manasses, VA) cells were seeded and grown to confluence (1.0 x $10^6$) cells in 24-well tissue culture plates. Serum from the immunized

mice was diluted in PBS (1:10, 1:50; 1:100; 1:200) and incubated with the bacteria for 1 hour at 37°C. The mixture was then incubated with the Caco-2 monolayer for 3 hours at 37°C and 5% $CO_2$ atmosphere.

### 2.12.1 *Adherence Assay*

The cells were then gently washed three-times with pre-warmed PBS and lysed via a 15-minute incubation with 0.1% Triton X-100 (Cornell, Philadelphia, PA) to release adherent and intracellular bacteria. The number of viable bacteria released was assessed by plating a 50-fold dilution on MH agar. Percentages of adherent bacteria compared to the controls. First, the survival rate of bacteria without serum, and second, the survival rate of bacteria incubated with serum from mice immunized with PBS. Three independent assays were performed each done in duplicate.

### 2.12.2 *Invasion Assay*

The gentamycin protection assay used in this study was the same as that described by Louwen *et al.* (2008) (161) and applied by Habib *et al.* (2009) (162). Following the serum incubation with the Caco-2 monolayer (described above) the cells were incubated for another 2 h in 1 mL of DMEM containing gentamicin (480 µg $mL^{-1}$) to kill any extracellular bacteria. Following the incubation, the monolayer was washed and lysed as above.

## 2.13 Statistical Analysis

All comparisons were made using a one-way ANOVA with a post-hoc Tukey's multiple comparison test. Results were analyzed using Prism software version 5.0 (Graphpad, USA). Results were considered to be significant if $p \leq 0.05$.

# 3.0 RESULTS

## 3.1 *Campylobacter jejuni* Growth Curve

*Campylobacter jejuni* strain 11168 produced a predictable and reproducible pattern of growth MH media under microaerophillic conditions, (5% $O_2$, 10% $CO_2$, 85% $N_2$) and 37°C, over the 72 hour observational period (Fig.3). The period of exponential growth was determined to be from 6 to 36 hours following the commencement of the observational period (24 hours after broth was inoculated). The optical density (OD) of the broth translated reliably into a population density after the number of colony-forming units (CFUs) were counted. A linear function was applied to the region of exponential growth and was used to estimate the population of the broth based on the OD measured.

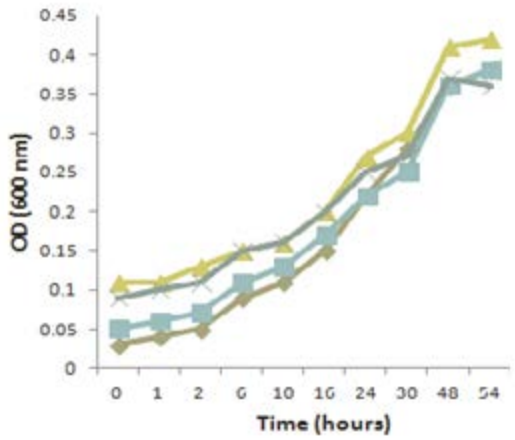
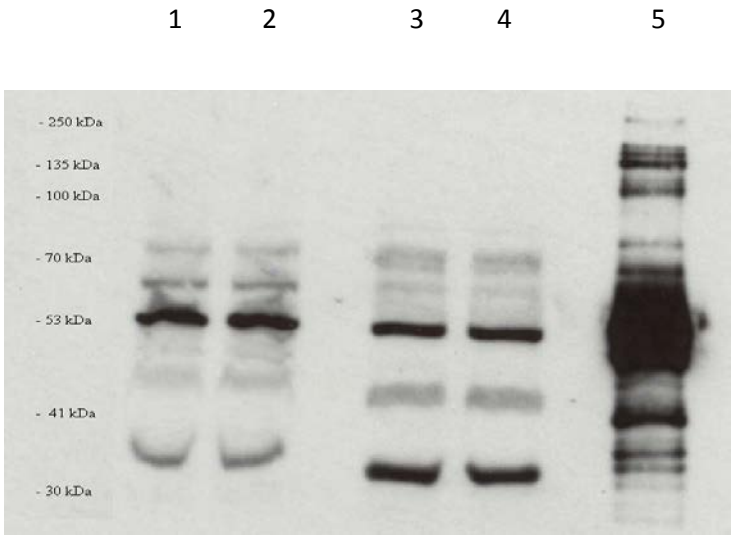



**Figure 3.** Bacterial growth of *C. jejuni* strain NCTC 11168 in MH medium under microaerobic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) monitored over 72 hours. Optical density (OD at 600 nm) as a function of growth time. Four unique experiments were performed.

### 3.1 KatA Antibody Specificity

The purified IgG antibodies received from our collaborator (A. Stintzi; refer to Appendix II) were derived from a mouse immunization with the KatA protein. The serum collected from the mice immunized by N. Eng and N. Bhardwarj (2012) (Sudbury, ON) was polyclonal in nature and produced high cross-reactivity with the assortment of *C. jejuni* proteins in our Western blots (Fig. 4A). The technique used in this thesis for isolating high-affinity antibodies from polyclonal serum for a specific protein was derived from Blaser *et al.* (1983) (160). This technique produced a significant increase in KatA protein specificity. Even with the additional purification, some cross-reactivity with other bacterial proteins was still evident validating the need for a method to more accurately detect this protein (Fig. 4B).

**A.**




**B.**

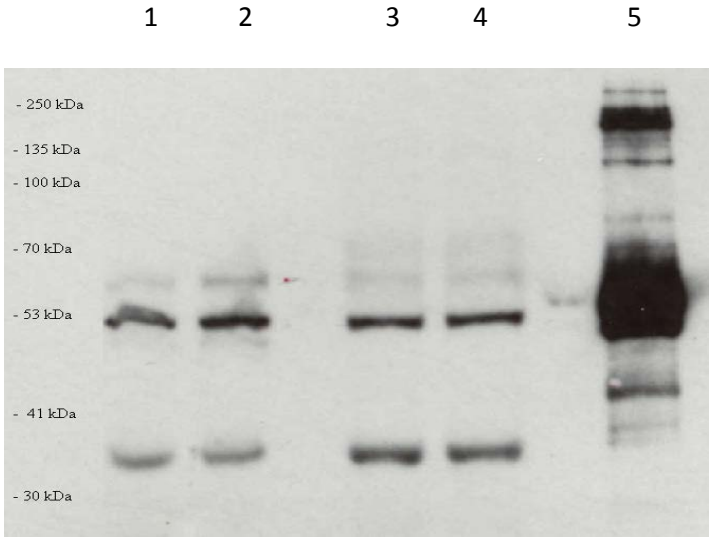



**Figure 4. Detection of KatA protein via Western blot with high affinity KatA antibodies and polyclonal IgG serum.** Duplicate Western blots from SDS-PAGE gels blotted using: (A) Antibodies purified with IgG magnetic beads and (B) high affinity KatA antibodies following additional in-house purification. Lane 1 and 2: duplicates of pelleted fraction; Lane 3 and 4: duplicates of supernatant fractions; Lane 5: purified KatA. Thirty-minute exposure to high-sensitivity film.

### 3.2 Localization of the KatA protein

In order for a vaccine against *C. jejuni* to be effective, the antibodies generated would have to bind epitopes on the bacterial membrane and prevent colonization in the small intestine. Thus, KatA would need to translocate to the membrane from the cytoplasm for antigen-specific antibodies to bind. While evidence has shown that this occurs with KatA in *H. pylori* (152; 153; 154), the present study investigated the possibility of a similar localization in *C. jejuni*. After sonication of *C. jejuni,* and separation of insoluble and soluble fractions by ultracentrifugation, the amount of protein in each fraction was assessed using a Bradford assay. It was determined that the soluble fraction contained 1.18 mg/mL of protein while the insoluble fraction had 1.14 mg/mL. Western blotting indicated that KatA is present in both fractions, providing some evidence that KatA may transiently localize to the membrane (Fig. 4 and Fig. 5). This suggests that a vaccine using KatA to prevent colonization may be possible. The detected bands were confirmed to be *C. jejuni* KatA by mass spectrometry.

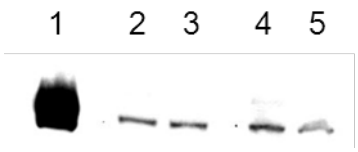
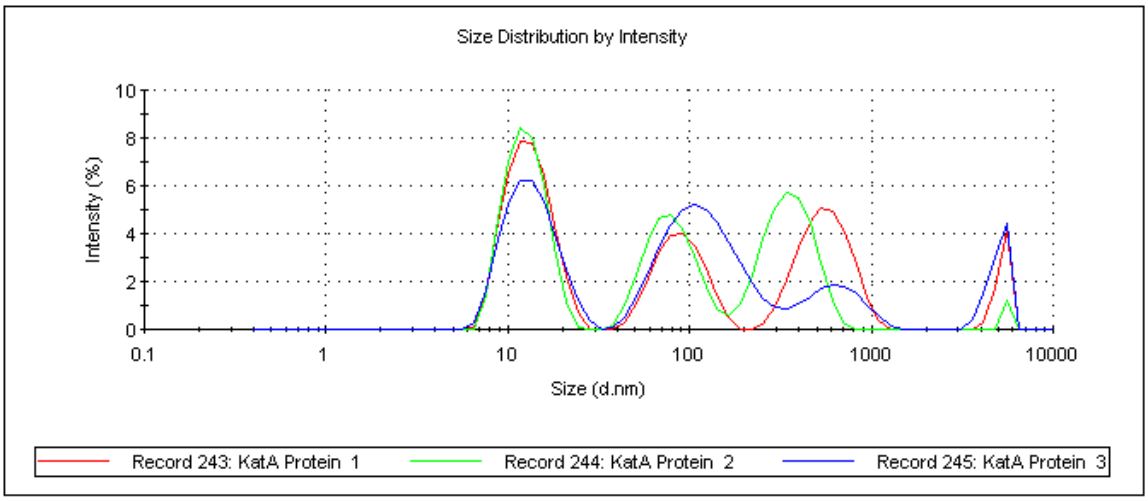



**Figure 5. KatA is localized both in cytoplasmic and membrane fractions of *Campylobacter jejuni*.** Polyclonal anti-KatA antibody was purified by antigen-affinity chromatography and was used to determine the presence of KatA in both supernatant and pelleted fractions of *C. jejuni* lysate. Lane 1: Purified KatA; Lane 2 and 3: duplicates of supernatant fractions; Lane 4 and 5: duplicates of pelleted fraction. The major band in lanes 2-5 were identified as KatA by mass spectrometry analysis. 15-minute exposure to high sensitivity film.

### 3.3 Physiochemical Evaluation of KatA

The average particle size distribution (PSD) for the KatA protein received from A. Stintzi (refer to Appendix II: Collaborator methodologies) was determined to be 108.3 nm. Three distinct peaks were detected with 41.6% of particles at average diameter 6.67 nm, 29.7% at 286.9 nm, and 22.6% at 45.49 nm. The polydispersity index PI, a measure of the width of molecular weight distributions (MWD), was recorded at 0.40. The PI describes how diverse the PSD range is (PDI<0.1 is monodisperse PDI>0.1 is multimodal) (163). A score of 0.40 reflects the presence of multiple, distinct distributions with no overlap between them. The surface charge of the particle was -4.58 mV indicating only poor predicted stability at ambient temperatures.

***A***




***B***

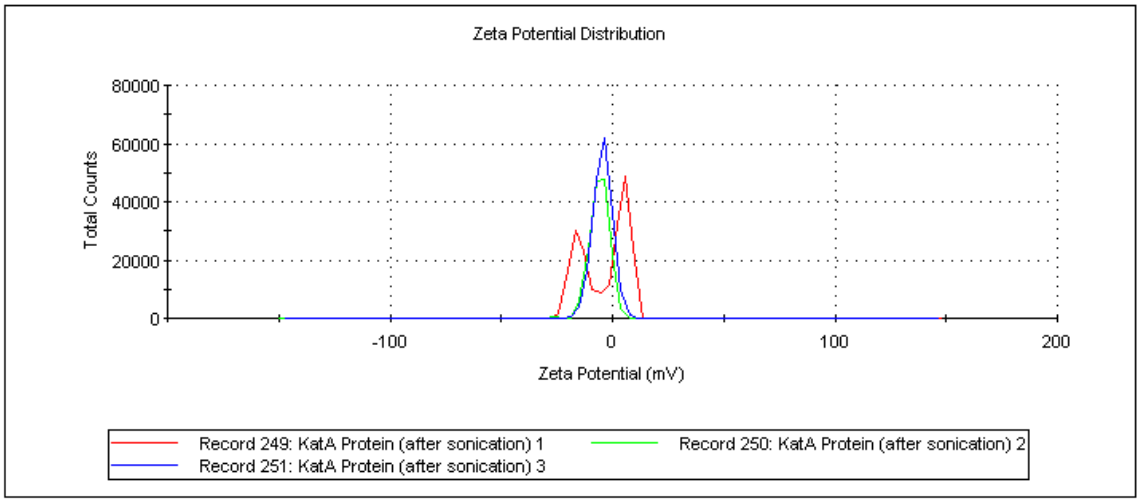



**Figure 6**. **Analysis of physical properties of the KatA protein at ambient temperature**. (A) Particle size distribution (PSD) in diameter nanometers (d.nm.). (B) Average particle surface charge = -4.58 milli-Volts (mV).

### 3.4 Antigenicity of the KatA Protein

The immunogenicity of KatA from *C. jejuni* (A. Stintzi) was evaluated following the intramuscular injection of BALB/c mice. Sera were collected from all mice one week post-3$^{rd}$ immunization (refer to Table 3: Immunization details), and was used for total IgG ELISA. Sera from mice immunized with KatA alone had mean anti-KatA IgG end point titers of $1.9x10^6$ (Fig. 7). Mean ELISA titers were comparable, $3.9x10^6$ and $3.8x10^6$ in mice immunized with KatA in conjunction with alum and MPL respectively (Fig. 7, $p < 0.05$ compared to the PBS group). Mice immunized with inactivated *C. jejuni* had mean end point ELISA titers of $2.5x10^5$. The highest titers ($5.6x10^6$) were observed in sera from mice immunized with KatA and a combination of both adjuvants (alum and MPL) (Fig. 7), which was significant ($p < 0.05$) compared to titers induced by KatA alone, PBS, and killed bacteria. These results suggest that *C. jejuni* KatA is immunogenic, and humoral responses can be enhanced with the addition of adjuvants.

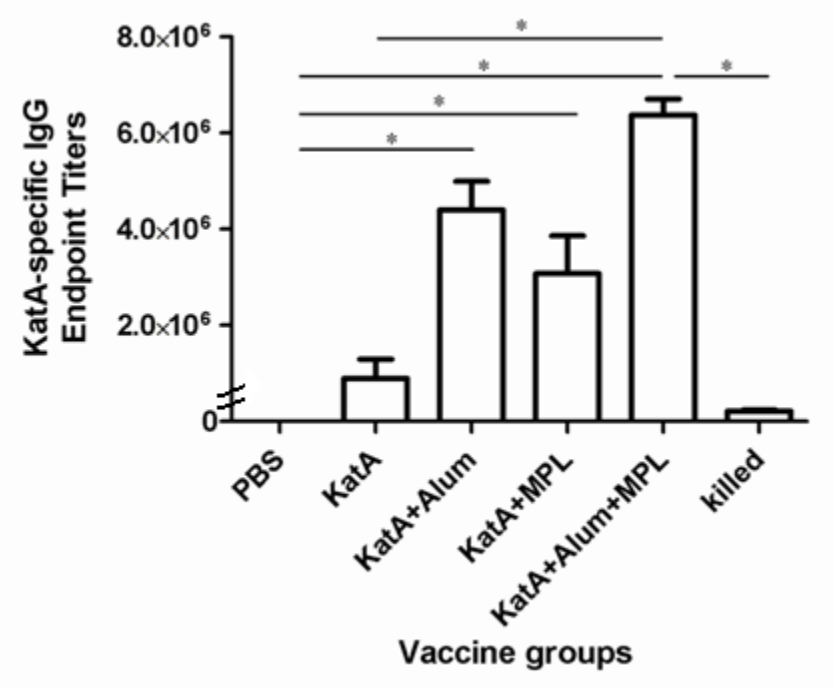



**Figure 7. *C. jejuni* KatA is immunogenic and immune responses are enhanced with the addition of adjuvants.** Total IgG in mouse serum one week after three intramuscular immunizations was enumerated for mice administered with PBS (negative control), KatA, KatA+alum, KatA+MPL, KatA+alum+MPL or inactivated *C. jejuni*. Values represent the mean endpoint titers within each group of mice (n = 8). Error bars shown represent the standard error within each mouse group. * $p < 0.05$.

### 3.5 Adjuvant Modulation of KatA-Specific Immune Responses

One week post-3$^{rd}$ immunization, sera were collected from all mice and used for IgG isotype ELISA. Sera from mice immunized with KatA alone had primarily antibody mediated immune response as reflected by the increased level of Th2 response compared to that of Th1 (Fig. 8). Mice immunized with inactivated *C. jejuni* also had a Th2 bias suggestive of a predominant antibody mediated response (Fig. 8). Balanced Th1 and Th2 responses were observed in mice that received KatA in conjunction with both alum and MPL (Fig. 8).

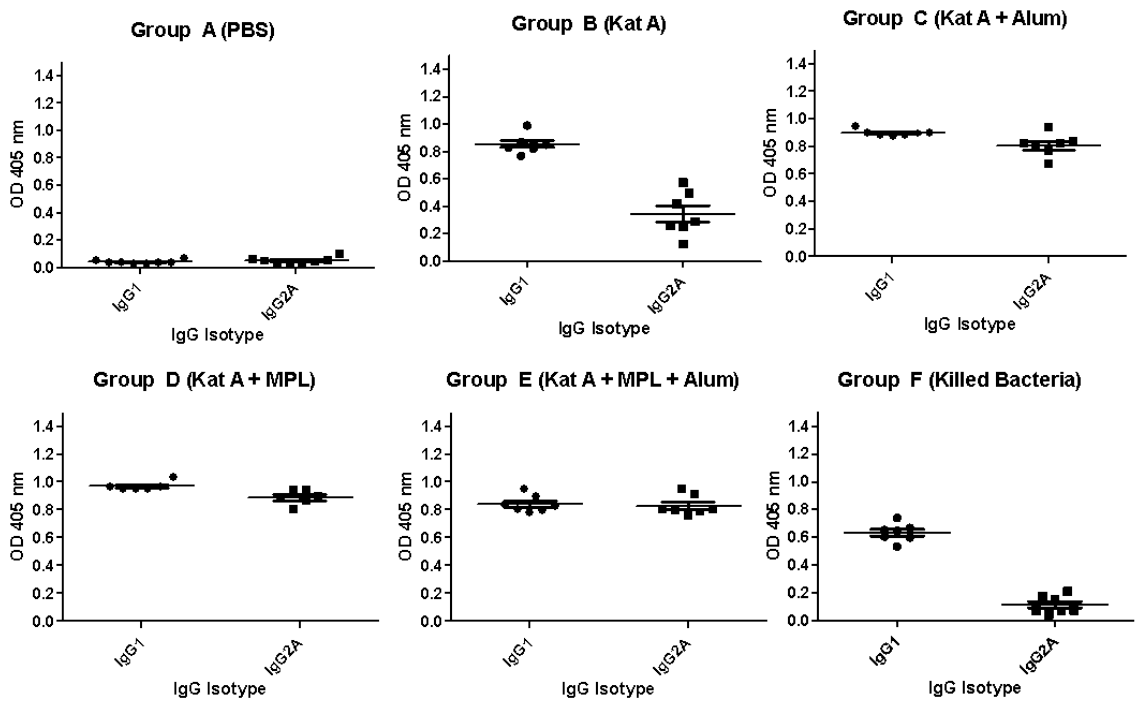



**Figure 8. The addition of alum or MPL to KatA induces a balanced IgG1/IgG2a ratio.** Mouse serum was evaluated for IgG1 and IgG2a subtypes and measured by $OD_{405}$ by KatA-specific ELISA. OD measurements are indicated for each mouse (n=8) in the scatterplot, where the line in each group represents the mean OD and error bars are indicative of standard error. Serum was analyzed from mice that were immunized with (A) PBS, (B) KatA, (C) KatA+alum, (D) KatA+MPL, (E) KatA+alum+MPL or (F) inactivated *C. jejuni*.

### 3.6 KatA Antibody Bactericidal Activity

To determine the functionality of the antibodies generated by mice induced by the formulations, a bactericidal assay was conducted to evaluate the ability of the antibodies to kill *C. jejuni* NCTC 11168. When bactericidal assays were conducted with guinea pig complement, serum from mice immunized with KatA alone showed a reduction of 11.3 ± 2.9 (SEM)%, while a 13.6 ± 2.2% decrease in bacterial counts was observed in serum from mice immunized with KatA and both alum and MPL (Fig. 9). As a control, immune serum from mice immunized with inactivated bacteria reduced counts by 24.6 ± 3.5%, which was significantly different from the reduction shown by KatA-induced serum (Fig. 9, $p < 0.1$). Interestingly, using mouse complement, serum from mice immunized with KatA alone and KatA+alum+MPL showed a bacterial reduction of 7.2% and 19.7% respectively (data not shown).

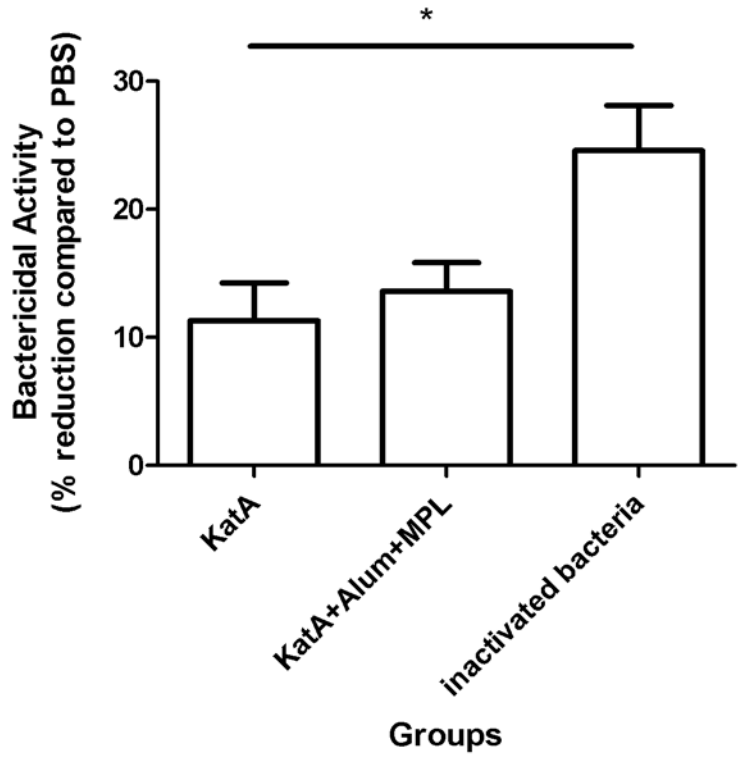



**Figure 9. Bactericidal activity of KatA antibodies generated following an intramuscular immunization in mice.** Bactericidal activity was conducted by counting the number of *C. jejuni* NCTC 11168 colony forming units (CFU) at t = 0 and t = 1 hours using guinea pig complement. Reduction in bacterial counts was expressed as a percentage normalized against the group of mice that was immunized with PBS. *$p<0.1$. Two unique experiments were conducted each with samples examined in triplicate.

### 3.7 Adhesion and Invasion Assays

In addition to bactericidal activity, antibodies may also be considered functional if they prevent the attachment or invasion of *C. jejuni* to epithelial cells in the small intestine (62). Serum (diluted 1:10, 1:50, and 1:100) from mice that were immunized with PBS, inactivated whole bacteria, KatA alone, and KatA with alum and MPL were incubated with *C. jejuni* and then added to Caco-2 human epithelial cells *in vitro*. At the 1:10 dilution, when normalized against serum from mice immunized with PBS, there was a 54.4 ± 2.4% CFU decrease in adhesion of *C. jejuni* to Caco2 cells using serum raised against whole bacteria, 43.5 ± 1.3% with KatA and alum/MPL, and 36.6 ± 2.5% decrease in CFU in serum raised against KatA alone (Fig. 10A, $p < 0.05$). A significant dose effect was observed ($p<0.05$) with the percent decrease in CFU diminishing in the assays using serum diluted at 1:50 (Fig. 10B) and 1:100 (data not shown).

In the invasion assay, at 1:10 dilution, there were no significant differences between the percent CFU decrease using serum from mice immunized with whole cell bacteria (53.5 ± 3.1%), KatA/alum/MPL (42.7 ± 4.9%), and KatA alone (40.4 ± 2.4%); however, all three groups showed significant reduction in bacterial invasion relative to the PBS control. This result indicates that all three sera equally interfered with the ability of *C. jejuni* to invade Caco-2 cells (Fig. 10C, $p > 0.05$). The addition of alum/MPL to KatA did not further decrease the number of bacteria invading Caco-2 cells compared to antisera raised against KatA alone. There was no significant difference between groups when the sera was diluted to 1:50 (Fig. 10D) and 1:100 (data not shown), but the percent CFU decreases across all groups were lower compared to the 1:10 dilution. In both cases, these results suggest that antibodies from sera induced by KatA affect the ability of *C. jejuni* to colonize *in vitro*.

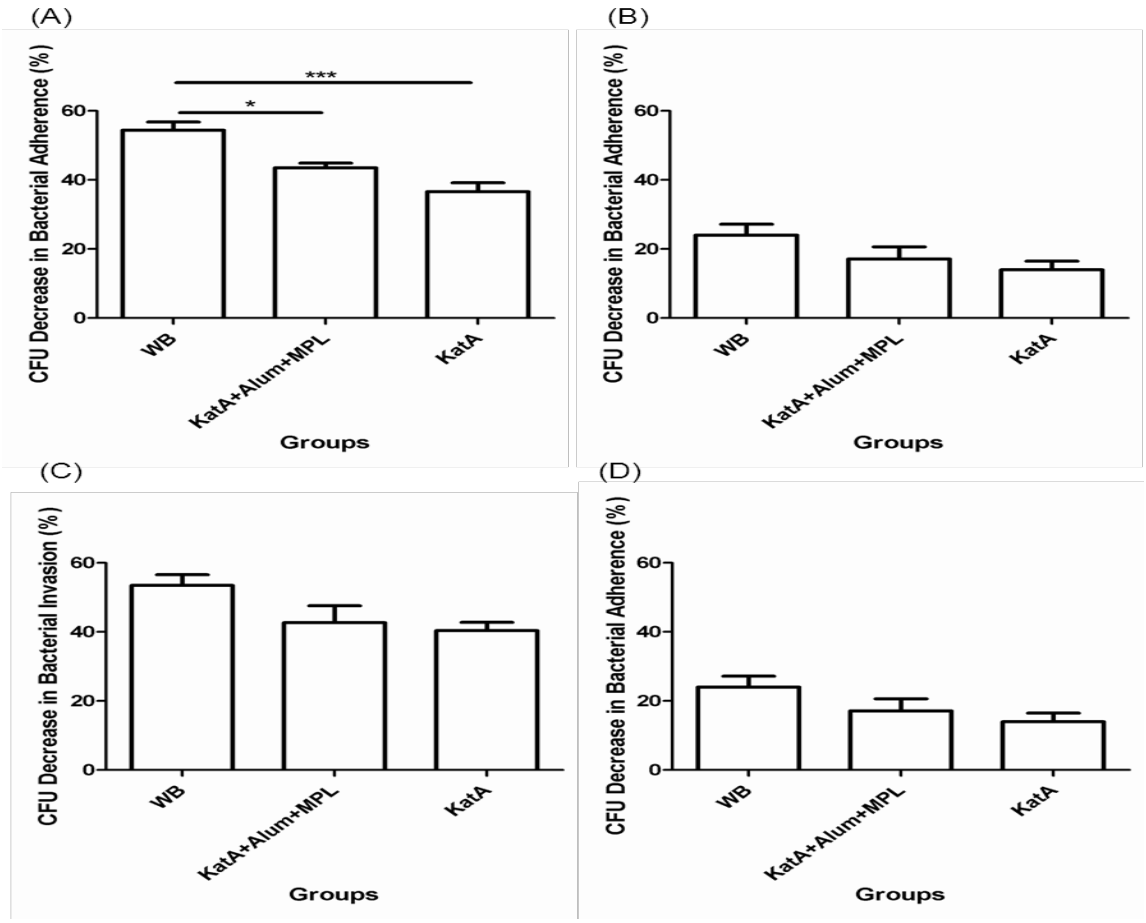



**Figure 10. The effect of KatA antibodies in preventing the adhesion and invasion of *C. jejuni* to human epithelial cells (Caco-2) *in vitro*.** Sera from mice immunized with KatA, KatA+Alum+MPL, or whole killed *C. jejuni* (WB) were diluted 1:10 (A) and 1:50 (B) and incubated with *C. jejuni* followed by addition to Caco-2 cells. Bacterial adherence (CFU counts) was enumerated following a 48-hour incubation under microaerobic conditions. A one-way ANOVA was performed with a Tukey's multiple comparison test to assess for statistically significant differences. *C. jejuni* invasion using 1:10 (C) and 1:50 (D) serum dilutions was determined using a gentamicin protection assay. The percent decrease of bacterial invasion (CFU/plate) for mice immunized with KatA, KatA+Alum+MPL, or whole killed *C. jejuni* (WB) was normalized relative to mice immunized with PBS. *$p<0.05$; ***$p<0.0005$.

# 4.0 DISCUSSION

## 4.1 *Campylobacter. jejuni* Observation

Despite the bacteria's reported *in vitro* culture fragility (164), the bacteria were easily cultured in the lab under microaerophillic conditions. As the culture aged, an increasing number of coccoid shaped bacteria were observed. This corresponds to work by Beck *et al.* (1983) who discovered that the age of the culture might influence the phenotypic properties of *C. jejuni*. As the bacteria age, their morphology transforms from the typical spiral shaped appearance to a coccoid form. The coccoid form dominates in older cultures and is believed to represent a degenerate state produced by unfavourable growth conditions (165). The bacteria were examined using a light microscope (400x – 1000x) to confirm the absence of coccoid forms, before usage in *in vitro* assays, to simulate *in vivo* invasion as much as possible.

## 4.2 KatA Protein Characterization

Proteins are dynamic structures and often exist in a state of flux between the native and partially unfolded intermediates (62). The size of a protein is an important physical characteristic that provides useful information including the presence of monomers, dimers and trimers, changes in conformation, aggregation state, and denaturation (166). The PSD analysis revealed the presence of several distributions of protein sizes (Fig. 6A) in contrast to an expected singular distribution. Proteins that may be denatured, unfolded, and aggregated into clusters, is undesirable from the immunization perspective as the resulting antibodies generated will not be of high-affinity for the native form presented by the bacteria (62). Consequently, the state of the protein may have reduced the effectiveness of the immunization as many antibodies are conformation-dependent (166).

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability (167). The greater the surface charge of a particle, the greater the force of repulsion between them smaller the probability of aggregation occurring (167). The zeta potential of KatA at physiological pH (≈7.0) was determined to be -4.58 mV. This indicates that under physiological conditions, the protein is near its isoelectric point (IEP); the pH of a protein solution at which the net charge or zeta potential of protein is zero (168). Unfortunately, at the IEP of protein, its structure is more hydrophobic, more compact and less stable due to absence of inter-particle repulsive forces (169). This may explain the apparent lack of stability and presence of larger size particles found in the PSD analysis.

**4.3 Cellular Localization of the KatA Protein**

The technique utilized in this work to isolate high-affinity KatA antibodies from a polyclonal IgG sera produced a visible increase in KatA protein specificity. Even with the additional purification, some cross-reactivity with other bacterial proteins was still evident (Fig. 4B) validating the need for a method to more accurately detect this protein. Monoclonal antibodies (mAbs) are efficient analytic tools for detection, screening and characterization of biomolecules, and have wide application as diagnostics (62). mAbs have been successfully used to identify serotype specificity but none are commercially available for the KatA protein. The development of a mAb for KatA would allow for more accurate protein detection and quantification through densitometric analysis.

Results from the bacterial fragmentation experiment indicated that KatA is present, in approximately equal amounts in both the soluble and insoluble partitions (Fig. 5). These findings provide some evidence that KatA may be able to induce the production of functional antibodies

that act on the bacterial surface but further investigation is warranted. In addition, the bacteria utilized in these experiments were not in their harsh host environment, but rather floating contently in their nutrient-rich broth. Other research has indicated that the expression of KatA is upregulated and transport to the surface occurs only after the bacteria have encountered a host (152). Consequently, this experiment does not reflect how KatA is presented when the infectious process is actually occurring. It may be of benefit to repeat the experiment using bacteria that have been primed for invasion by first having them encounter host cells. Following this priming, the KatA expression pattern will be more reflective of that *in vivo* and thus will provide more information concerning the localization of KatA during invasion.

In these experiments, the localization of KatA to the membrane was not confirmed definitively. The separation procedure divided the cellular contents into soluble (cytoplamsic contents) and insoluble (inner and outer cell membranes). The presence of KatA detected in the insoluble fraction does not discern which membrane, or at what ratio, the protein is associated.

Interestingly, two separate proteomic analyses by Jang *et al*. (2014) (170) and Elmi *et al*. (2012) (171) reported the identities of all proteins found within outer membrane vesicles (OMV) secreted by *C. jejuni*. OMVs are nano-sized vesicles (10-500 nm) (172) secreted by Gram-negative bacteria. They contain a sampling of outer membrane contents including LPS, OMPs, periplasmic proteins, virulence factors and occasionally cytoplasmic proteins (173). The presence of KatA was not reported in either analysis which does not support the idea that it has an outer-membrane localization. However, the bacteria used in these analyses were not activated by the presence of host cells thus the OMVs examined were not reflective of protein expression and localization that occurs during *in vivo* invasion.

The outstanding question of KatA localization could be resolved quickly if a monoclonal antibody was available. Monoclonal antibodies can be successfully conjugated to fluorochromes such as fluorescein, biotin, Texas Red, and phycobiliprotein (174). Conjugation of fluorescein isothiocyanate (FITC) to purified antibody is an extremely valuable technique for identifying surface molecules using either fluorescence microscopy or flow cytometry (174). Thus, fluorescent mAbs would highlight the presence of KatA at the cell surface under various conditions (i.e. unstimulated versus stimulated by epithelial cells). Alternatively, the outer membranes of *C. jejuni* could be extracted, as with other Gram-negative bacteria, using a mild detergent and separated using an SDS-PAGE in a similar fashion to that demonstrated by Acevedo *et al.* (2009) (175). The high-affinity KatA antibody preparation, used in the present study could be used to determine if the protein was absent. A positive result, however, could only be confirmed following protein band analysis using mass-spectroscopy due to antibody potential for cross-reaction. Again, the identification process would be simpler and more accurate if a mAb was available.

### 4.4 Antigenicity of the KatA Protein

The second major goal of this thesis was to determine if the KatA protein could stimulate antibody production. Large quantities of IgG were detected in serum one-week following the third IM vaccination of mice. As expected, the addition of adjuvants MPL and alum stimulated even higher titers of anti-KatA antibodies, especially when used in combination.

The administration of the KatA protein produced a dominant IgG1 response as injected soluble antigen favours Th2 responses (176). The administration of killed bacteria also produced a dominant IgG1 response as expected. The addition of MPL produced increased IgG2a as

expected through the predicted engagement of TLR4 (177). MPL has been shown to direct Th1 responses and is currently the adjuvant used in Melacine®, a licensed cancer vaccine (178). Recently, the combination of L-tyrosine and MPL® enhanced murine antigen specific IgG2 antibody responses without enhancing antigen specific IgE responses (179). For this reason, MPL is now being considered for use as an adjuvant in clinical allergy vaccinations (179). Therefore, the continued use of MPL in further *C. jejuni* vaccine development may be warranted to counter balance IgE levels, should it be elicited by the KatA protein.

The addition of alum increased the levels of IgG2a which was an unexpected result as alum normally promotes a Th2 biased response (180). Finally, the trifecta of KatA, MPL, and Alum produced approximately equal amounts of IgG1 and IgG2a. While there are several theories that exist as to how alum functions as an adjuvant including acting as a depot, and allowing extended contact with the antigen and the APC, there is another theory that suggests that alum is not actually driving Th2 differentiation but instead is suppressing Th1 responses (180). Brewer *et al.* (1996) observed that administration of an antigen with alum, in the absence of IL-4, generated concomittant T cell responses resulting in the production of both IgG1 and IgG2a isotypes (181).

Th2 responses normally occur when IL-4 stimulates transcription factor STAT6 which controls expression of the master Th2 regulatory transcription factor, GATA-3 (182). IL-2 has also been identified to play a critical role in this process throught the activation of STAT5 leading to transctiption of IL-4 (183). Th2 differentiation has also been proposed to proceed through an IL-4 independent mechanisms when peptides are presented at low concentrations (184). TCR activation under these conditions stimulates increases in GATA3 mRNA and induces IL-2 production activating STAT5 (185). The fact that IL-4 knockout mice can still stimulate Th2 is

believed to be due to this signal pathway redundancy (136). Therefore, IL-4 is not required under certain circumstances to initiate Th2 differentiation.

The suppression of Th1 by alum would allow for the Th2 differentiation to occur uninterrupted as IFN-γ would not be able to suppress the expression of GATA3. In the present work, the KatA protein adsorbed to alum was able to induce concomittant KatA specific Th1/Th2 responses. This preliminary data suggests that KatA induces an IL-4 independent Th2 response which would allow for the alum to lead to unbiased T cell responses. Consequently, isotype switching to both IgG1 and IgG2a would occur as observed in our experimental results. While we recognize that the immune system is complex, and multiple systems can influecen the reseponse, this work relies on the Th1/Th2 paradigm which does not take into account other T helper subtypes.

The achievement of a balanced Th1/Th2 response is desirable for a *C. jejuni* vaccine as Campylobacter-specific IgA and IFN-γ are associated with resistance to clinical disease suggesting these components might be important markers of protective immunity (136; 186). Initially, it would be desirable to prevent infection through the secretion of high-affinity anti-*Campylobacter* antibodies. If the first line fails, and infection occurs, then enhanced cell-mediated immunity via macrophage would be desirable.

### 4.5 Functional Antibody Assessments

The antibodies produced in the immunization had some bactericidal properties. Our bactericidal assay demonstrated only partial killing of *C. jejuni* using serum from mice immunized with whole killed *Campylobacter*, KatA alone or KatA+alum+MPL. None of the immunized groups demonstrated a true bactericidal activity as industry standards require cell survival to be less than

50% (187). The killing of the bacteria in this assay is believed to occur through the activation of complement cascade (reviewed in appendix 1) and subsequent insertion of the MAC leading to cell lysis (188). Serum from the animals immunized with whole bacteria induced the greatest amount of cell death as to be expected. Parkhill et al. (2009) analyzed the genome and predicted that approximately 20 genes code for constitutively expressed OMPs (189). Several of these proteins can be expected to be immunogenic and the other components of the cell surface, such as LOS and LPS have been determined to produce antibodies in mice. Therefore, following the IM administration, potentially dozens of antibodies were generated against the bacteria's surface thus increasing the likelihood of complement assembly.

Despite the advantages of the whole-killed bacteria administrations, the KatA immunization groups performed considerably well. The addition of adjuvants to the KatA protein did not significantly enhance bacterial killing. IgG1 is the IgG subtype with the highest affinity for complement proteins and is therefore the most critical for the activation of the classical pathway (62). The KatA protein alone generated high quantities of the IgG1 isotypes and the addition of MPL and Alum did not increase levels of IgG1 and enhanced IgG2a which has an extremely low affinity for complement (62). Thus, it makes sense for the KatA adjuvanted group to produce only slightly more bactericidal activity than the KatA protein alone. Interestingly, serum from the mice immunized with PBS displayed bactericidal activity suggesting that bacterial killing was also occurred due to alternative and lectin complement pathway activation. Consequently, data was normalized to subtract this baseline endogenous activity. This assay was not conducted as a simulation of a real invasion process as the bactericidal activity of serum is effective only against Gram-negative bacteria which have entered the bloodstream (190). Rates of bacteraemia are very low and detected in < 1% of patients with Campylobacter enteritis, occurring most often

in patients with a severely compromised immune system (15). These results do, however, provide evidence that the KatA antibodies generated are capable of binding to the bacterial surface.

### 4.6 Adhesion and Invasion Assays

It is not enough to report the production of antibodies; they must also be functional in order for an antigen to be useful in vaccine development (191). Most vaccines are validated using a challenge experiment in an animal model to determine vaccine efficacy (192). This is often done challenging the subject following the initial immunizations, by administering an elevated dose of the live pathogen, and examining whether the disease state occurs (192). Because of the lack of reliable animal models to simulate campylobacteriosis, cell culture models of bacteria with the epithelium are valuable for defining bacteria-host interactions. These models are used because they are thought to mimic the same bacteria-host interaction that occurs in the natural disease. The ability of *C. jejuni* to enter, survive, and replicate in mammalian cells has been widely studied using *in vitro* models (193; 59; 194; 195; 196; 197). Studies have shown that *C. jejuni* harvested from earlier phases of growth exhibit greater adherence to human epithelial cells than those from older colonies (>72 h) (60). Bacterial growth temperature is a second factor which influences adhesion to epithelial cells and adhesion was determined to be most efficient at 37°C (60). Because of this, all of our experiments were conducted with bacterial colonies in the log phase of growth (36-48 hours post-inoculum) in a microaerobic chamber set to 37°C. Results from our data suggest that KatA antibodies can physically prevent *C. jejuni* from attaching and invading host epithelial cells as shown by the *in vitro* studies using Caco-2 cells. Caco-2 cells were selected as they can partially differentiate in culture to produce microvilli, form tight junctions between cells, and express apical surface enzymes characteristic in *in vivo* enterocytes

(45). Confluent cultures, as used in the assay, exhibited the dome morphology characteristic of mature enterocytes indicating that the monolayer was functionally polarized (198). The polarity of IEC is important determinant of cell responses to microbes and essential for a realistic simulation of *in vivo* invasion (199). Interestingly, immunizing mice with KatA alone generated serum capable of decreasing the number of bacteria that could attach/invade Caco-2 cells. Mice immunized with whole bacteria, which included other potent immunopotentiators such as LPS, performed only marginally better than the serum from the animals immunized with KatA (Fig. 10).

It is important to note the limitations of the *in vitro* assays used in these experiments. First, the reported adhesion of *C. jejuni* with intestinal epithelial cells (IECs) is often reported to be less than 1% of the starting inoculum (200) (ours ranged from 0.01-0.1%). This low level of adhesion does not correlate with the clinical presentation in humans (200). This phenomenon is likely due to atmospheric oxygen conditions required for the survival of the IECs. Although our assay incubations were performed under low oxygen conditions, there were several periods up to 30 minutes in duration where the plates were under atmospheric conditions which may have been harmful to the bacteria. The relevance of the adhesion and invasion assays can be called into questions as during the course of a real *C. jejuni* host invasion, sIgA would be the most critical antibody to prevent adhesion. IgA levels were not evaluated in this experiment as they were not expected to be significant following an IM administration. The purpose of this investigation was to simply verify if KatA would be immunogenic following immunization. The next step in the course of investigation would be to administer the KatA protein mucosally, either orally or intranasally, and evaluate the levels of sIgA produced at mucosal sites. The adhesion and

invasion assays could be repeated *in vitro* but conducting a live challenge in the immunized animal would be the best way to evaluate protective immunity.

If indeed KatA is not present at the cell surface, then it would appear that we have generated cross-reactive antibodies capable of effectively binding another surface protein. This in itself would be an important discovery as antibody binding to this protein(s) is effectively reducing the rates of adhesion and invasion. This is in fact possible as the western blot of fractionated bacteria revealed that, even with the high-affinity antibodies, three additional proteins were detected (Fig. 4). These proteins were also sent for mass-spectroscopy and the results revealed that they were not the KatA protein.

### 4.7 Conclusion

The high prevalence of *C. jejuni* infections, increases in antibiotic resistance, and the incidence and severity of post-infectious syndromes validates the need for a prophylactic vaccine. The development of such a vaccine has been hindered by the unique structure of *C. jejuni* LOS and the potential for molecular mimicry to generate auto-immune disorders. Thus, the most promising strategies involve the development of subunit vaccines. The data in the present experiment provides some support the candidacy of the KatA protein as a vaccine antigen but many fundamental questions remain unanswered. This is the first indication KatA from *C. jejuni* may be a useful vaccine antigen. Catalase is also believed highly conserved between *Helicobacter sp*., although such a comparison is yet to be reported for *Campylobacter sp*., the protein is fundamental for the pathogen invasion. Future studies will examine the efficacy of KatA in mucosal deliveries combined with potent mucosal antigens. Before any true conclusions can be drawn, KatA must demonstrate protective immunity against a live *C. jejuni* challenge**.**

This work increases the vaccine antigen knowledge base against *C. jejuni* and offers hope that a safe and effective vaccine can be produced.

## 5.0 REFERENCES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## APPENDIX 1: The Complement System

The complement system is a humoral proteolytic cascade based defense mechanism and is a crucial component of the innate immune response against pathogens. It is comprised of over 25 plasma proteins and cell surface receptors (201). Together, they function in concert to opsonise microbes, to promote the recruitment of phagocytes to the site of infection, and in some cases to directly kill the microbes via lysis (202). There are three main ways to activate the complement system: through antibodies (classical pathway), through large multimeric lectins (lectin pathway), and through a low-level inherent activation (alternative pathway) (Figure 3). All three pathways merge at the level of cleavage of the plasma protein C3 which leads to the formation of active fragments and the initiation of a proteolytic cascade involving other complement proteins (190). The classical pathway of complement activation can be initiated once an antibody has bound to the surface of a microbe. The plasma protein C1q binds to IgG and IgM but not IgA, IgD or IgE (203). C1q consists of 6 identical subunits with globular heads capable of binding the Fc region on Ig. Each C1q must simultaneously bind at least 2 Fc regions in order to become active. Thus, only multivalent antigens with multiple antibodies bound can activate complement. C1q lacks enzymatic activity but is associated with two serine protease proenzymes C1r and C1s (204). C1q will bind to the $C_H2$ domain of IgG, or the $C_H3$ domain of IgM, antibodies resulting in a conformational change leading to the autoactivation of C1r which cleaves and activates C1s (205). C4 is also cleaved by C1s into C4a and C4b exposing a binding site for C2. Upon binding, C2 is cleaved into C2a and C2b while C2a remains bound to C4 and functions to recruit and cleave C3 into C3a and C3b. C3b contains a highly reactive thioester domain that is exposed following clevage (206). The thioester domain can form covalent attachments, to the amino or hydroxyl groups of surface proteins or polysaccharides, but reaction with water prevents attachment from occurring far from the site of activation (207). The

association of the C4b2a complex with C3b forms a C5 convertase which initiates the terminal pathway.

The alternative pathway of activation proceeds without antibody binding and begins when the C3 complement protein is cleaved by the C3 convertase yielding two fragments: C3a and C3b. Low rates of cleavage occurs continuously in plasma resulting in constitutive activity and C3 binds to pathogen or host cell surfaces (208). The host, however, expresses several regulators, expressed predominantly on the cell surface, that suppress complement activation against self by downregulating central proteolytic activity (209). This pathway has been called 'reverse-recognition' as it is based on the recognition of host-associated molecular patterns (HAMPs) rather than PAMPS. As microbes lack the HAMP regulators, predominantly sialic acid, the complement pathways proceeds (210). A second plasma protein (factor B) can bind to C3b once it has attached to a cell surface. Factor B is then cleaved by a plasma serine protease (factor D) resulting in the C3bBb complex which functions as the alternative pathway C3 convertase. C3 convertase increases the cleavage and activation of C3 molecules amplifiying the complement cascade (211; 212).

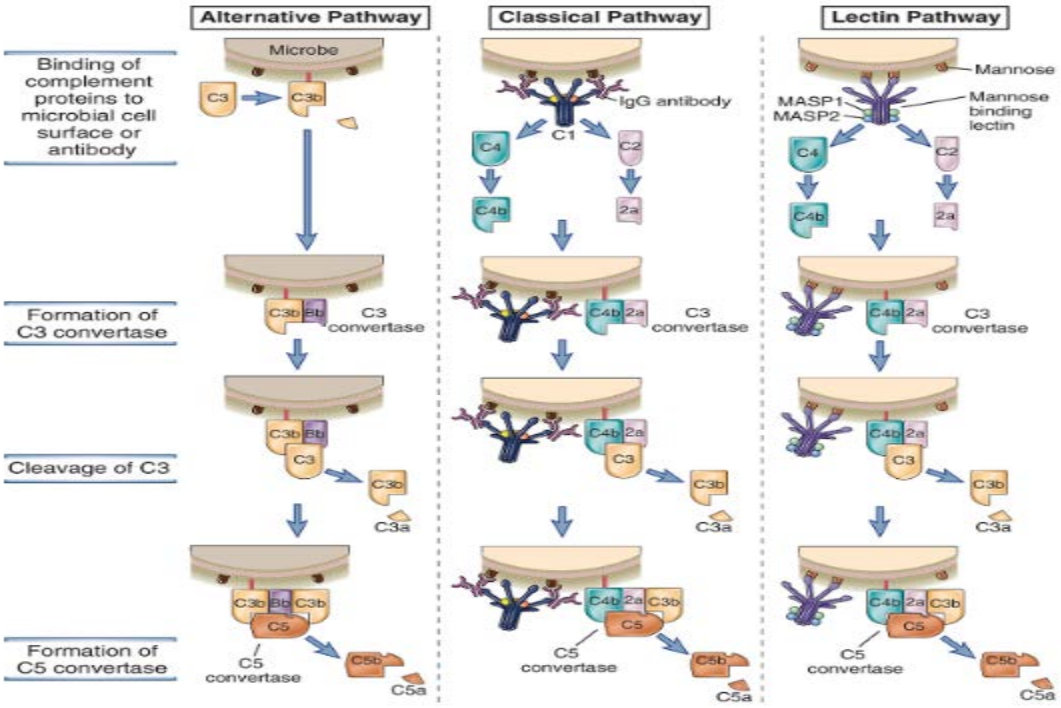


**Figure A1**. The steps of complement activation by the alternative, classical, or lectin pathways. Adapted from Abbas, Lichtman and Pillai (2012) (62)

The third pathway to complement activation, although similar to the classical pathway, is evolutionarily more ancient (213). It is triggered by mannose-binding lectin (MBL) and ficolins, plasma proteins which recognizes terminal mannose residues on microbial glycoproteins and glycolipids (214). Once MBL has bound to a microbe, two zymogens mannan-binding lectin associated serine protease (MASP1 and MASP2) are activated. MASP-2 cleaves both C4 and C2 while MASP-1 plays a role in transactivating MASP-2 (215). This initiates the same downstream proteolytic steps as in the classical and alternative pathways resulting in the cleavage of C5 into C5a and C5b by C5 convertase. C5b initiates the terminal pathway which leads to the assembly of the terminal components, C5,C6,C7,C8, and C9, which from the membrane attack complex (MAC). The MAC is a lipophilic complex which inserts itself in the bacterial cell membrane forming pores and ultimately residing in cell lysis (188). The complement system, however, does not play a major role in C. jejuni infection as rates of bacteraemia are very low and detected in < 1% of patients occurring most often in patients with a severely compromised immune system (15)

## APPENDIX II: COLLABORATOR METHODOLODIES

### KatA expression and purification

Overexpression of KatA (Fig. 1) was performed in *E. coli* BL21 cells using the protein expression vector pGST as described previously [26]. Briefly, the *C. jejuni* NCTC 11168 *katA* gene (Fig. 1) was PCR amplified using Pfx (Invitrogen, Burlington, ON) high fidelity polymerase and the KatA_*Nco*I (**GCCATGGCT**ATGAAAAAATTGACTAACGA) and KatA_*Not*I (**GCGGCCGC**TTAGTTTGCCACCAAAAGTGG) primers (restriction site outlined in bold). The amplified gene was cloned into the protein overexpression vector pGST using *Nco*I and *Not*I restriction sites, to give rise to pGST+KatA construct which was transformed into *E. coli* BL21 cells. Sequencing was performed to confirm the absence of polymerase-introduced mutations in the *katA* gene. Once *E. coli* was cultured as mentioned above, the cells were then pelleted, resuspended in 100 mM NaCl, 20 mM Tris pH 7.3 buffer containing protease inhibitor (Roche, Mississauga, ON), and the cell membranes were disrupted by sonication. Cell membranes and debris were removed by centrifugation at 13000 rpm for 15 min. The cell lysate containing the GST-KatA fusion protein was then affinity purified using glutathione sepharose 4B resin according to the manufacturer specifications (GE Healthcare, Baie d'Urfe, QC). Cleavage of the GST tag from KatA was performed on the resin by addition of TEV protease [26] and gentle shaking overnight at 4°C. The KatA protein was washed from the resin the following day using 100 mM NaCl, 20 mM Tris pH 7.3 buffer and concentrated using 30 kDa cut-off centrifugal filter unit (EMD Millipore, MA, USA). Concentrated KatA protein was further purified by size exclusion chromatography using the AKTA fast protein liquid chromatography (FPLC) system equipped with a Superdex-200 column (GE Healthcare) using

100 mM NaCl, 20 mM Tris pH 7.3 buffer as the filtration buffer and a flow rate of 0.4 mL/min. Purified protein was further concentrated to 2 mg/mL and stored at -20°C until use.

***Purification of KatA Antibodies from Sera***

Approximately 2.5 mg of KatA protein was used for antibody production by Immuno-Precise Antibodies Limited (Victoria, BC, Canada) using 2 rabbits. For each rabbit, a pre-immune bleed was performed prior to the primary immunization with the KatA antigen (0.5mg) using complete Freund's antigen. Over the course of the project, each rabbit received 3 additional boosts with KatA antigen (0.5 mg) using incomplete Freund's antigen followed by a terminal bleed and serum collection. Anti-KatA antiserum was stored at 20°C until use.

***Immunoprecipitation of KatA***

Immunoprecipitation experiments using wild-type C. jejuni, ΔCj1386, ΔkatA, and ΔCj1386 ΔCj1386 strains were performed using protein A-conjugated Dynabeads (Invitrogen). Strains were grown to mid-log phase (OD600 of 0.2) in MEM under microaerophilic conditions at 37°C prior to harvesting total soluble pro teins. Bacterial strains were spun at 6,000 rpm for 10 min, resuspended in 1ml PBS containing a bacterial protease inhibitor cocktail (Sigma) and 10 mg/ml lysozyme, and incubated on ice for 15 min. Cells were briefly sonicated once (five 5 pulses), followed by centrifugation at 13,000 rpm for 5 min at 4°C to remove membranes and cellular debris. Two hundred and fifty micrograms of anti-KatA antiserum diluted in 200 µl PBS-0.02% Tween 20 was incubated with 50 µl of Dynabeads for 1 h with end-over-end rotation at room temperature. The anti-KatA-bound Dynabeads were washed once with 500 µl PBS-0.02% Tween 20 before addition of 5 mg of protein lysate. The bead-lysate mixture was incubated overnight at 4°C with end-over-end rotation. The beads were washed 3 times with 200 µl of ice-chilled PBS,

and KatA was eluted from the beads twice in 100 µl soft-elution buffer (50 mM Tris, pH 8.0, 0.2% SDS, 0.1% Tween 20) with end-over-end rotation for 7 min at room temperature. Immunoprecipitation of KatA from each strain was visualized by SDS-PAGE run on a 10% denaturing gel followed by Coomassie blue staining. KatA protein concentration was determined for each immunoprecipitated sample by densitometry of the SDS-PAGE gel using Adobe Photo shop software (version 10.0). KatA protein content was normalized, and equal amounts of KatA (250 ng) from the wild-type NCTC11168, ΔCj1386, and ΔCj1386 ΔCj1386 strains were assayed for catalase activity using the method described above. Immunoprecipitate from the ΔKatA strain was used as a negative control for the catalase activity assay. Catalase activity assays were performed in quadruplicate, and statistical significance was determined using the Student t-test. P values of $<0.05$ were considered significant.

**Proteomics**

Proteomics analysis was performed at the OHRI Proteomics Core Facility (Ottawa, Canada).

**Protein Digest**

Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Nat Protocols 2006; 1(6):2856-60). The resulting peptide extracts were concentrated by vacufuge (Eppendorf) and resuspended in 0.1% formic acid (Fisher).

**LC-MS/MS**

Peptides were analyzed by LC-MS/MS (liquid chromatography – tandem mass spectrometry) on a system comprised of an UltiMate 3000 RSLC nano HPLC, LTQ Orbitrap XL hybrid mass spectrometer and nanospray ionization source (Thermo Scientific). The system was controlled

by Xcalibur software version 2.0.7 (Thermo Scientific). Peptides were loaded by autosampler onto a C18 CapTrap (Michrom) in 3% acetonitrile, 0.1% formic acid at a flow rate of 15 microlitres per minute for 5 minutes. Peptides were eluted over a 60 minute gradient of 3% - 45% acetonitrile at a flow rate of 300 nanolitres per minute through a 10-cm long column with integrated emitter tip (Picofrit PF360-75-15-N-5 from New Objective packed with Zorbax SB-C18, 5 micron from Agilent), and nanosprayed into the mass spectrometer. Nanoflow HPLC solvents contained 0.1% formic acid and 5% DMSO (Nature Methods 2013; 10(10):989). MS scans were acquired in FTMS mode at a resolution setting of 60,000. $MS^2$ scans were acquired in ion trap CID mode using data-dependent acquisition of the top 5 ions from each MS scan.

**Protein Identification Using Mascot**

MASCOT software version 2.4 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed MS/MS spectra were matched against *C. jejuni* sequences from SwissProt version 2013_05 and also against a database of common contaminants. Mass tolerance parameters were MS ±10 ppm and MS/MS ±0.6 Da. Enzyme specificity was set to 'Trypsin' with <=2 miscuts. Oxidation of methionine, protein N-terminal acetylation, and conversion of glutamine to pyro-glutamate were allowed as variable modifications. Carbamidomethylation of cysteine was set as a fixed modification.

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