BUTYRATE, *BACTEROIDES THETAIOTAOMICRON* AND *CAMPYLOBACTER JEJUNI* MODULATE THE EXPRESSION OF BETA-DEFENSINS, TOLL-LIKE RECEPTORS AND CYTOKINES IN CACO-2 CELLS

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Dedication

To my father,

By leading through example, your work ethic, drive, and continual humouring of my desire to talk science since I was young have been instrumental to providing me with the foundation to succeed. Thank you for only joking about understanding why lions eat their young, and for giving me the chance to forge my own path my own way.

To a Microbe,
'The best laid schemes o' microbes an' men gang aft agley.'

Abstract

Dietary fibre fermentation produces short-chain fatty acids, including butyrate (NaB). NaB increases histone tail acetylation within the chromatin of colonic cells, a process associated with increased gene expression. *Campylobacter jejuni* a prominent foodborne pathogen triggers inflammatory enteritis, whereas NaB and the commensal enteric bacterium *Bacteroides thetaiotaomicron* (*B. theta*) have documented anti-inflammatory properties. In this study I have developed a model system for studying the NaB-colonocyte-bacteria interaction and have used it to investigate responses to NaB, *C. jejuni* and *B. theta* using an in-house immunomodulatory gene array. NaB induced significant levels of transcription for nearly every immunomodulatory gene. Further, NaB and *B. theta* stimulate the expression of defensins and Toll-like receptors, while *C. jejuni* decreased both. This study provides mechanistic insights into the human colonocyte immune response and presents several gene targets as potential biomarkers for inflammatory expression responses *in vivo*.

Preface

The K12E5 gene expression results in Chapter 3 were presented as an abstract and poster at the 5th Annual ASM Beneficial Microbes Conference, Washington D.C., September 27-31, 2014.

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Appendices

List of Symbols, Abbreviations and Nomenclature

ACTB - actin
AE – absorptive enterocytes
AMP – antimicrobial peptide
AP-1 – activator protein-1
APC – antigen-presenting cell
B. theta (B. t) – *Bacteroides thetaiotaomicron*
BHIS - brain heart infusion media
bp – base pair
Buk pathway – butyrate kinase fermentation pathway
But pathway – butyryl-CoA: CoA-transferase pathway
C. j – *Campylobacter jejuni*
CCL –C-C- motif ligand
CD – Crohn's disease
cDNA - complementary DNA
CFU – colony forming unit
CoA – coenzyme-A
CpG – cytosine-phosphate-guanine
CXCL –C-X-C motif ligand
DC – dendritic cell
DF – dietary fibre
DGM – distal GIT microbiota
DMEM – Advanced Dulbecco's minimal essential medium
DMEM Complete+AB – supplemented DMEM with antibiotics
DMEM Complete-AB – supplemented DMEM without antibiotics
DMT – DNA methyltransferases
DPBS – Dulbecco's Phosphate Buffered Saline
ENA-78 – epithelial-derived neutrophil-activating peptide-78 (CXCL5)
GAPDH – glyceraldehyde 3-phosphhate dehydrogenase
GBS - Guillain-Barré syndrome
GC – goblet cell
GIT – gastrointestinal tract
Gpr - G protein-coupled receptor
GusB – glucuronidase β
H3K9 – histone 3 tail lysine 9
H3K9ac – acetylated histone 3 tail lysine 9
H3K9me – methylated histone 3 tail lysine 9
HAT – histone acetyl transferase
hAD-1 – human α defensin 1
hBD-1 – human β-defensin 1
hBD-2 – human β-defensin 2
hBD-3 – human β-defensin 3
HDAC – histone deacetylase
HDACi – histone deacetylase inhibitor
IBD – Inflammatory Bowel Disease
IBS – Irritable Bowel Syndrome
IEC – intestinal epithelial cell
IESC – intestinal epithelial stem cell
IFN-γ – interferon-gamma
IκB - NF-κB inhibitor protein
IL – interleukin
IL-8 – interleukin-8
IP-10 – interferon gamma induced protein 10 (CXCL10)
LA – loosely adherent
LPS – lipopolysaccharide
LS means – least squares means
MCP-1 – monocyte chemoattractant protein-1 (CCL2)
MEC – mucosal epithelial chemokine (CCL28)
MOI – multiplicity of infection
MS – multiple sclerosis
NaB – sodium butyrate
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NK – natural killer
NTC – no template control
PAMP – pathogen-associated molecular pattern
PBS – phosphate buffered saline
P.I. – post infection
PPAR-γ – peroxisome proliferator activated receptor-gamma
PpiB – peptidylprolyl isomerase B
PRR - pattern recognition receptors
PSA – polysaccharide A
PTM – posttranslational modification
PVDF – polyvinylidene fluoride
RA – rheumatoid arthritis
RT-qPCR – Real-time qPCR
SCFA – short-chain fatty acid
SDS – sodium dodecyl sulfate
TA – tightly adherent

TBS – tris buffered saline
TBST – TBS Tween 20
TCA – tricarboxylic acid cycle
TECK – thymus-expressed chemokine (CCL25)
TGF-β – transforming growth factor- β1
Th – T helper
TLR – Toll-like receptor
TNF – tumour necrosis factor
TYG - tryptone yeast extract glucose

“Let me tell you the secret that has led me to my goal. My strength lies solely in my tenacity.”

– Louis Pasteur

Chapter 1 Literature Review

1.1 Enteric Health

Human beings are born into a microbial world, sterile and immunologically naïve. From this moment, life becomes an ecological interaction, and their immune systems must rapidly develop in order to filter and eradicate biotic and abiotic threats[1]. The gastrointestinal tract (GIT) mucosal surface, estimated to span 30-40 square meters,[2] represents the human body's largest interface with its environment. Microbial communities that inhabit the GIT, collectively termed the microbiota, are significant for host development, metabolism, and immunity. The molecular mechanisms that govern these processes remain obscure, especially with respect to the role of diet.

Over a century ago, Russian scientist Élie Metchnikoff (1845-1916) conceptualized the role of the GIT in host physiology and pathology[3]. Metchnikoff suggested that the composition of the microbiota and its interaction with the host were vital components of host physiology. More recently, modern technologies such as intestinal (enteric) tissue cultures, gnotobiotic animal models, and next-generation sequencing have enabled researchers to elucidate a clearer picture of the dynamic relationship between the host GIT, microbiota, diet, microbial metabolites, and their implications for host health.

1.1.1 The Gastrointestinal Tract

The human GIT spans from the stomach to the anus (Figure 1.1A), and is lined by intestinal epithelial cells (IECs) (Figure 1.1B). IECs consist of four principle lineages: absorptive enterocytes (AEs), and three secretory cell types known as goblet cells (GCs; mucin producing), enteroendocrine cells (hormone producing), and Paneth cells (antimicrobial peptide producing) (Figure 1.1B). IECs form a monolayer that line structural surfaces and are anchored by a thin

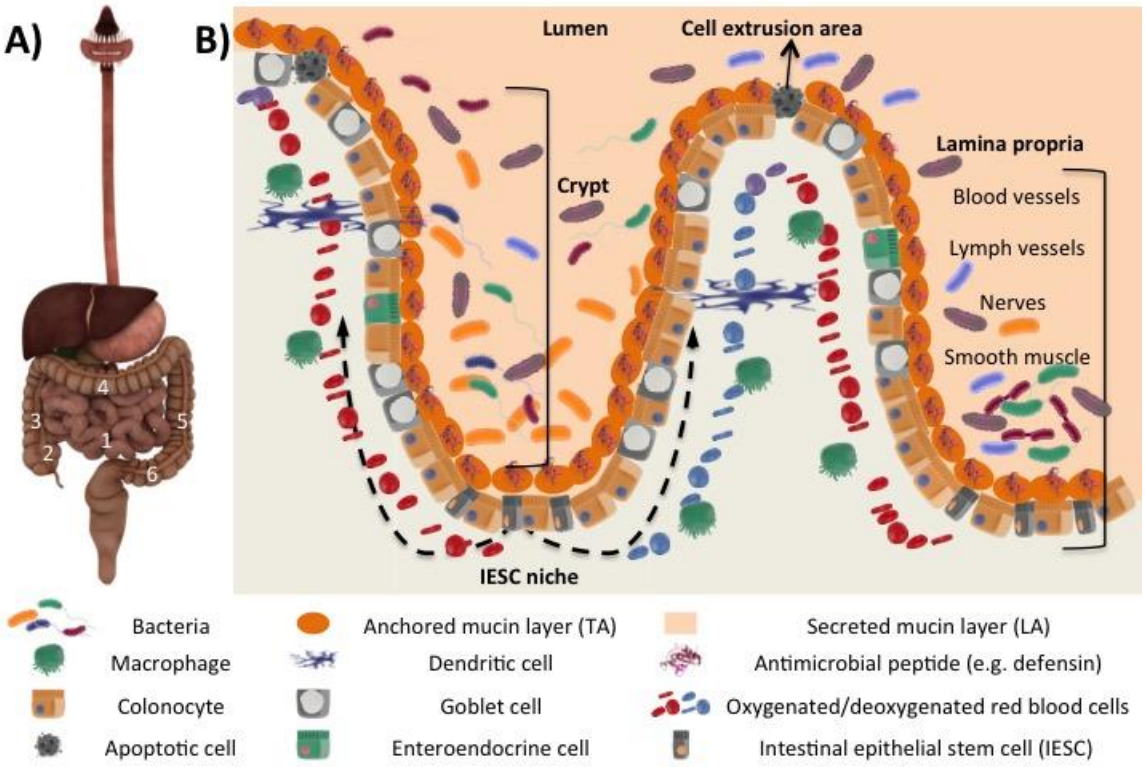

Figure 1.1 Human GIT anatomy and intestinal epithelial cell barrier.

A) A schematic of the human GIT. The small intestine (1) extends from the pyloric sphincter to the cecum; the colon (2) extends from the cecum to the rectum. The four sections of the colon are the ascending (proximal) (3), traverse (4), descending (distal) (5), and sigmoid (pelvic) (6) colon. B) The structure of the colonic mucosa. The intestinal epithelial stem cell (IESC) niche, containing epithelial, stromal, and haematopoietic cells are responsible for the continuous renewal of the epithelial layer by crypt-resident IESCs at the base of the crypt. The dashed arrows mark the path that differentiated IECs (except for Paneth cells) migrate up the crypt-villus axis to the cell extrusion site for cellular renewal. Secretory GCs and AEs secrete mucus and defensins to prevent bacterial attachment to the epithelial surface. Enteroendocrine cells represent a link between the central and enteric neuroendocrine systems through hormone secretion. GCs and IECs mediate the transport of luminal antigens and live bacteria across the epithelial barrier to the lamina propria where dendritic cells (DCs) and intestinal-resident macrophages can sample the lumen through transepithelial dendrites. The bracket represents the lamina propria containing blood vessels, lymph vessels, nerves, and smooth muscle. Adapted from [4].

layer of connective tissue underneath, called the lamina propria (Figure 1.1B). This monolayer is a physical barrier that segregates the microbiota from internal tissues, communicates with both beneficial and pathogenic microbes, and influences the maturation of surrounding immune cells. As part of this system, AEs and GCs secrete anchored glycoprotein mucins that form the tightly adherent (TA) mucus layer, and soluble loosely adherent (LA) mucus throughout the GIT as shown in Figure 1.1B[5].

During disease, IECs actively influence the inflammatory response through the production of cytokines and chemokines such as interleukin-8 (IL-8), interferon-γ induced protein 10 (IP-10), and epithelial-derived neutrophil-activating peptide-78 (ENA-78). These cytokines recruit eosinophils, neutrophils, and effector T cells, as well as promote their maturation and activation once they migrate to damage sites[6-8]. AEs also serve as antigen-presenting cells (APCs) in a manner similar to DCs that direct T and B cell differentiation[9].

1.1.2 The Human GIT Microbiota

The human genome lacks the enzymes required for digesting plant structural polysaccharides (e.g. celluloses, hemicelluloses, and pectins shown in Figure 1.2A-C) and other components of dietary fibre (DF) such as resistant starch (Figure 1.2D)[10]. Therefore, vertebrates rely on a symbiosis with members of the distal GIT microbiota (DGM) to metabolize DF. In Figure 1.2E, a common distal GIT microbe, *Bacteroides thetaiotaomicron* (*B. theta*) ferments DF into short-chain fatty acids (SCFAs), a nutrient for its host. Thus, the DGM has been called the "hidden metabolic organ"[11]. Within healthy individuals the bacterial fraction of the DGM is primarily composed of Bacteroidetes (17-60%) and Firmicutes (35-80%)[12, 13] with Actinobacteria, Proteobacteria, and Euryarchaeota also present[14, 15]. The GIT houses the greatest biodiversity (~1,000 species) and abundance (100 trillion individual microbes) found in the human body[16, 17].

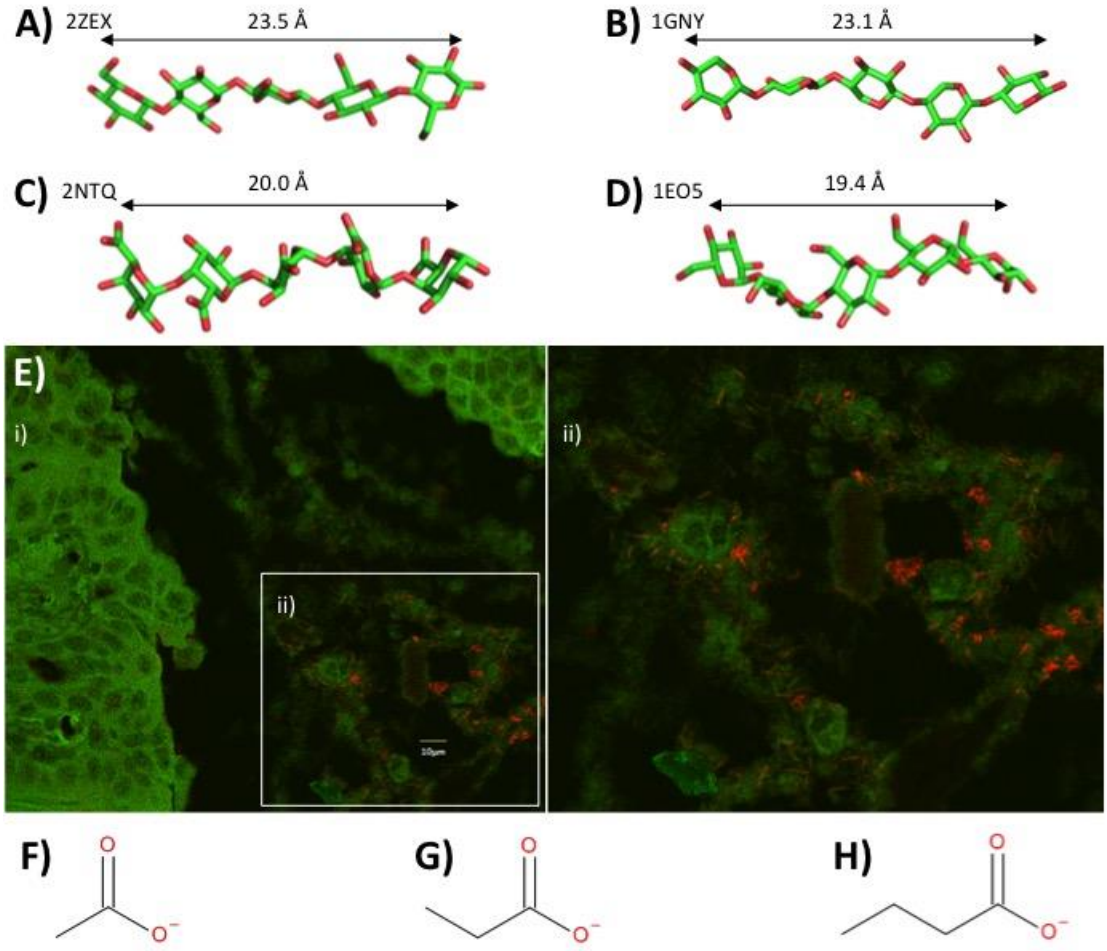

Figure 1.2 Substrates and by-products of dietary fibre fermentation by the DGM.
A) Cellulose: β-1, 4 linked homopolysaccharide of D-glucose (PDB ID: 2ZEX[18]); **B)** Xylan: β-1, 4 linked homopolysaccharide of D-xylose (PDB ID: 1GNY[19]); **C)** Homogalacturonan: α-1, 4 linked homopolysaccharide of D-galacturonic acid (PDB ID: 2NTQ[20]); **D)** Amylose, α-1, 4 linked homopolysaccharide of D-glucose (PDB ID: 1EO5[21]). The length (Å) of a pentaoligosaccharide demonstrates stereochemical differences between different DF glycans. **Ei)** A confocal micrograph (60x) of mouse intestinal sections (Appendix 3) showing EUB338 probed bacteria (red) and auto-fluorescing cells and plant material (green) with a 3x zoomed in inset (**Eii**). The ruler indicates 10 µm. The deprotonated forms of common short-chain fatty acids: **F)** acetate; **G)** propionate, and **H)** butyrate.

DF influences the composition of the DGM through favouring species capable of metabolizing complex carbohydrates ingested by the host. Key members of the DGM modulate several essential host functions[22] and can play beneficial (e.g. *B. theta*) or harmful (e.g. *Campylobacter jejuni*) roles in the maintenance of enteric health. For example, beyond its role in complex carbohydrate metabolism[23], *B. theta* has documented anti-inflammatory activity to offset excessive pro-inflammation[24]. *C. jejuni,* in contrast, is a transient member of the microbiota and one of North America's most prevalent causes of foodborne bacterial enteritis in human beings[25-28]. Campylobacteriosis, the disease state caused by *C. jejuni*, is generally self-limiting; however, afflicted patients may develop chronic post-infectious complications including Irritable Bowel Syndrome (IBS), Inflammatory Bowel Disease (IBD), Guillain-Barré syndrome (GBS), reactive arthritis, and immunoproliferative small intestinal disease[29-33].

Campylobacteriosis was estimated to affect 0.86% of the Canadian population in 2006, with Southern Alberta showing elevated levels at 1.7% of the population becoming infected by *C. jejuni*[34]. Using the 0.86% infection rate with Canada's population in 2015, the annual impact on the economy in terms of lost productivity per year due to *Campylobacter* spp. is ~$163,000,000 (assuming an average of 3 missed days of work at 7.5 hours per day of an average hourly salary of $23.67 per hour). Given the growing body of evidence supporting the role of DF in maintaining enteric health and the link between chronic disease and enteric bacteria[35], elucidating how DF and DGM bacteria such as *B. theta* influence the physiology of IECs and promote host defences to pathogens such as *C. jejuni* are strategic areas of research.

1.1.3 Dietary Fibre

DF can be defined as the portion of plant foods that are recalcitrant to human digestive enzymes, including polysaccharides, such as plant structural polysaccharides and resistant

starches (Figure 1.2 A-D)[36]. These substrates are fermented by the microbiota into short-chain fatty acids (SCFAs) that provide energy[37] to the host and regulate host responses[38]. DF is delineated into two portions; soluble fibre or viscous fermentable fibres, such as homogalacturonan fermented in the colon; and insoluble fibre, such as wheat bran that is largely responsible for fecal bulking and is fermented to some extent in the colon as well[39, 40].

In 430 BC, Hippocrates first described the laxative effects of coarse wheat in contrast to refined wheat[41]. J.H. Kellogg later determined that bran promoted laxation, increased stool bulk, and prevented disease[41]. More recently, DF has been linked to the prevention of many Western disorders, including diabetes, cardiovascular disease, colonic cancer, and obesity[40-42]. However, despite a rich and detailed research history, the majority of health claims associated with DF intake remain to be substantiated through experimental science.

1.1.4 Fermentation

Colonic bacterial metabolism is performed under anaerobic conditions, and therefore, energy extracted from DF is harvested through a fermentative process. The DGM is a stratified community with specialized members that have evolved to fill metabolic niches[43]. DF complexity and degrees of mastication,[44] alkaline pH, digesta transit time through the distal ileum/colon, anaerobic conditions, peristalsis, and the enteric mucus layer all can influence DGM community structure[28, 45, 46].

Acetate, propionate, and butyrate (Figure 1.2F-H) are the principle fermentation products in the colon,[47] and are produced in the order of acetate > propionate $\geq$ butyrate with a molar ratio of approximately 3:1:1[48-50]. Total SCFA in the proximal colon (Figure 1.1A3) is estimated to be between 70-140 mM,[50, 51] falling to 20-70mM in the distal colon[50]. Butyrate, the deprotonated form of butyric acid often ionically associated with sodium (Na) to form sodium

butyrate (NaB), is the primary energy source for colonocytes, while acetate and propionate (the deprotonated forms of acetic acid and propionic acid) have distal roles in liver, muscle, kidney, heart, and brain gluconeogenesis[47, 52]. SCFAs 1-6 carbons in length are transported into the epithelium both passively[53] and actively by monocarboxylate transporters such as SLC5a8[54, 55]. Low SCFA production and perturbations to these levels have been implicated in enteric diseases, including IBD and cancer[56, 57]. Therefore, increased SCFA production through the consumption of foods that promote bacterial fermentation is beneficial for preventing these diseases[58].

NaB has been shown to play an important role in maintaining barrier function within the enteric epithelium. Two mutually exclusive metabolic pathways produce NaB in bacteria (Figure 1.3). The NaB kinase (*buk*) pathway, often utilized by saccharolytic soil-associated bacteria, utilizes a phosphotransbutyrylase and NaB kinase pathway[59]. Alternatively, the butyryl-coenzyme A (CoA): acetate-CoA transferase (*but*) pathway, predominant in the DGM, uses cross feeding of exogenous acetate from species such as *B. theta* to feed a butyryl-CoA: acetyl-CoA transferase enzyme to produce NaB[60]. Thus, butyrogenic bacteria within the DGM represent a functional group in contrast to a discreet phylogenetic group within the DGM.

DF by the DGM is of further importance due to the ability of fermentation by-products, such as NaB, to influence host immunity. Several bacteria have been shown to be able to activate the activator protein-1 (AP-1) pathway through SCFA production[61]. The AP-1 signalling pathway is important for contributing to this process by regulating cell proliferation, differentiation, transformation, cell migration, and apoptosis[62, 63]. NaB has also been shown to induce Caco-2 cell apoptosis mediated by a caspase-3 mitochondrial pathway[64]. NaB therefore helps to promote epithelial cell turnover and barrier maintenance, and prevents the propagation of transformed colonocytes[65].

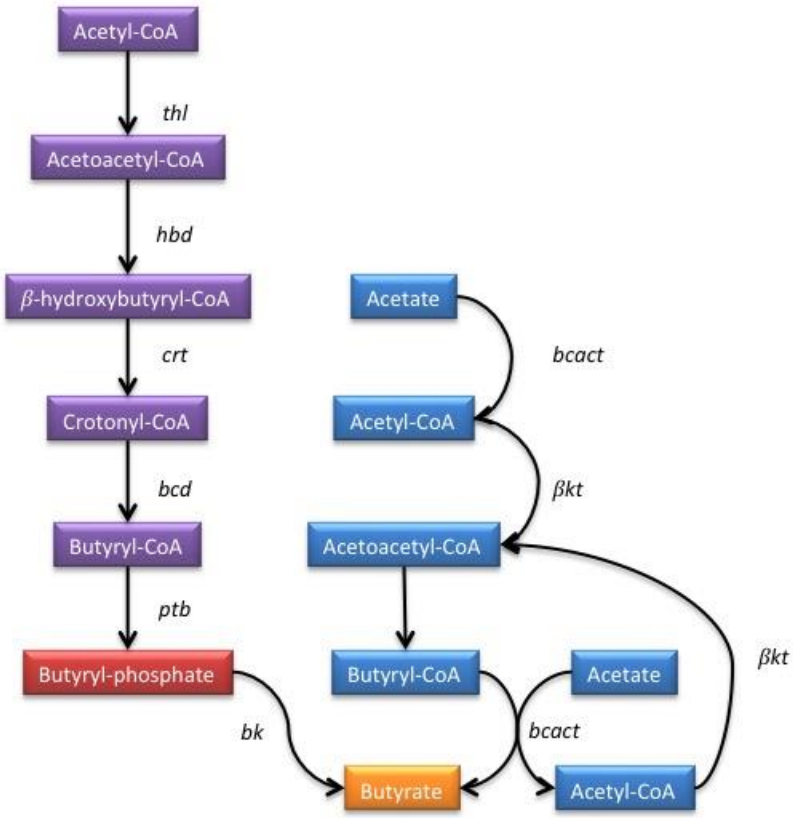

Figure 1.3 Butyrate formation pathways.

The NaB kinase (bk) pathway (purple) includes enzymes: *thl*, thiolase; *hbd*, β-hydroxybutyryl-CoA dehydrogenase; *crt*, enoyl-CoA hydratase (crotonase); *bcd*, butyryl-CoA dehydrogenase; *ptb*, phosphotransbutryrylase; *bk*, NaB kinase. The butyryl-CoA: acetyl-CoA transferase pathway (blue) utilizes butyryl-CoA: acetyl-CoA transferase (*bcact*) and β-ketothiolase (*βkt*) to make use of two exogenous acetate molecules to form NaB.

1.2 The DGM and Host Immunity

1.2.1 Bacterial Modulators of Host Immunity

Many foodborne microbes, including pathogens, are considered transient members of the DGM. Transient species can differentially affect the immune system, potentially being innocuous, beneficial, or pathogenic. These species have not coevolved with host species to the same extent. Transient species cannot compete with indigenous luminal DGM for nutrients, and therefore have not established the same strategies to sequestrate on the luminal side of the epithelium[66]. Instead, transient species must persist and proliferate in the presence of host and microbiota defences, subverting and manipulating host immunity to evade attack. In this regard, many acute intestinal pathogens have adapted strategies to forcefully colonize the intestine; however, this often induces a strong pro-inflammatory immune response from the host targeted at clearing out the incitant[67].

The presence of commensal bacteria provides an immunostimulatory effect for the host by stimulating the general recruitment of immune cells to the mucosa, as well as for the generation and maturation of organized GIT-associated lymphoid tissues[68]. Further, commensal bacteria can stimulate protective epithelial barrier functions for the enteric lining, such as modulating the mucus production feedback system and defensin secretion[28, 69]. These mechanisms for stimulating host protection include both direct and indirect responses to bacteria and bacterial fermentation by-products. Some beneficial members of the DGM also secrete immunomodulins (e.g. NaB) to influence the type and intensity of host immune responses. Commensal bacterial immunomodulation is often more subtle than that of the transient bacterial pathogens as they do not cause overt changes in the health status of the host. Instead, they assist with the maintenance and regulation of host homeostasis.

With known correlations between NaB and immunomodulation, epithelial barrier function, and host health, further investigation into substantiating these correlations *in vitro* and *in vivo* and their mechanism of action is warranted. Presently, an *in vitro* model system to investigate the role(s) of NaB in the context of modulating host immunity within the bacteria colonized distal GIT is lacking. For such a model, Caco-2 cells represent an appropriate IEC line for investigating host cell relationships with bacteria that possess beneficial (e.g. *B. theta*) and pathogenic (e.g. *C. jejuni*) qualities.

Previously, the impacts of *C. jejuni*[70], *B. theta*[24] and NaB[71] on IEC immune gene expression have clearly shown differential immunomodulatory potential. To date, a multi-faceted pro-inflammatory response of Caco-2 cells in response to *C. jejuni* has not been well characterized beyond the cytokines IL-6[72], IL-8[73], MCP-1, and IP-10[70] and the antigen sensing receptors TLR2, TLR4, TLR5, and TLR9[27, 72]. It has been shown that NaB facilitates IL-8 responses to lipopolysaccharide (LPS) which otherwise failed to induce secretion in a priming action[74]. Since *B. theta* is capable of metabolizing DF and has been reported to attenuate inflammation by regulating the nuclear cytoplasmic shuttling of transcription machinery[24], I have chosen to investigate its immunomodulating effect on *C. jejuni* infection by measuring the differential changes to immunomodulatory genes in Caco-2 cells in concert with changes in NaB concentration.

1.2.1.1 Immunomodulatory Activity of *B. theta*

B. theta is a Gram-negative anaerobic bacterium, belonging to the *Bacteroides* genus in Phylum Bacteroidetes. It is primarily considered a human commensal of the GIT; however, under circumstances of perturbed host defense, it has been reported to become a pathogen[75]. *B. theta* possesses a vast repertoire of genes involved in modifying diet- and host-derived

polysaccharides (there are currently 88 identified polysaccharide utilization pathways within its genome)[76], and has emerged as a model organism for studying symbiotic bacteria-host relationships within the human GIT[77]. *B. theta* contributes to disease pathogenesis mitigation in pediatric Crohn's disease (CD) patients by consistently inducing IL-8 production in CD tissues[78]. It has been shown to inhibit the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulated immune markers (Appendix Table A4.1) by preventing nuclear translocation of RelA through PPAR-γ dimerization in Caco-2 cells[24]. This has an anti-inflammatory effect on the enteric immune system by retaining NF-κB within the cytosol, preventing inflammatory markers from being transcribed. In addition, a syntrophic relationship between the acetogenic *B. theta* and butyrogenic *Faecalibacterium prausnitzii* has been linked to mucin production in the colonic epithelium of a gnotobiotic rodent model[28].

Bacteroides are commonly associated with the human GIT. Many Bacteroides are known to confer positive health benefits to the host. The relationship between humans and Bacteroides is complex, and Bacteroides species can become harmful when they escape the GIT. Pathologies, such as bacteremia and abscess formation can occur[79]. *Bacteroides fragilis* itself only accounts for between 4 and 13% of the normal human fecal microbiota; however it is present in 63 to 80% of *Bacteroides* infections[80]. In contrast, the related *B. theta* accounts for between 15 and 29% of the fecal microbiota but is associated with only 13 to 17% of infection cases[80]. *B. fragilis* is capable of a high amount of within-strain phase and antigenic variation of surface components, indicating that it may be a more successful opportunistic pathogen than other related *Bacteroides* species. Investigating the properties of other *Bacteroides* species, such as *B. theta*, in immunomodulation of the host is an important avenue for future research.

1.2.1.1.1 Commensal Stimulation of Pro-inflammatory Responses

Many GIT commensal bacteria initiate pro-inflammatory responses and contribute to host health and well-being by stimulating and priming the immune system[81]. In contrast, other commensal bacteria inhibit or mitigate epithelial cell inflammatory responses by modulating immunity through TLRs or NOD-like receptor expression and signalling[82]. Others can suppress immune cascades by inhibiting NF-κB activation[24] or by increasing the secretion of anti-inflammatory cytokines, such as IL-10[83]. Collectively, *B. theta* acutely triggering NF-κB[24] and the ability of NaB to stimulate defensin (hBD-1[84] and hBD-3[85]), cytokine (TGF-β[71]) and chemokine (MCP-1[86], MEC[87] and IL-8[86]) transcription signifies that commensal bacteria may increase available transcripts for rapid response to a later stimulus to expedite a more robust response to rapidly eliminate enteric threats.

1.2.1.2 Pro-inflammatory Effects of *C. jejuni*

C. jejuni was chosen as a model organism in which to investigate the potential interactions between the innate immune response and Th1-polarized adaptive immune responses. *C. jejuni* is an appropriate model for studying human enteric acute inflammation because it is a prominent foodborne pathogen that is already known to induce a Th1-like immune response within Caco-2 cells[88-97]. IL-12β appears to be a key cytokine produced by APCs to trigger a Th1-directed response after 24 or 48 hours of *C. jejuni* incubation with DCs[98, 99]. *C. jejuni* is able to invade underlying tissues such as the lamina propria, can enter the bloodstream, and once in the blood stream may reach distinct organs around the body.

C. jejuni has been found to be ubiquitous in the aerobic environment, possessing regulatory systems capable of sensing and adapting to external stimuli, such as oxidative and aerobic (O_2) stress[100]. The heterogeneity of *C. jejuni* through its rapidly changing, flexible genome[101] has made studying the pathogenicity of this pathogen a challenge[102]. Recently,

advances have been made in understanding key factors associated with the virulence of *C. jejuni*, such as the cytolethal distending toxin (CDT)[103], the molecular mimicry process in GBS[104] and both Type III[105] and IV[106] secretion pathways.

Invading epithelial cells and CDT production are both important bacterial virulence mechanisms for inducing enterocolitis. Cellular invasion has the potential to result in injury to the epithelium. This can decrease the host's capacity to absorb nutrients, while CDT production *in vitro* promotes IECs interleukin-8 (IL-8) release that is important for the host mucosal inflammatory response induced by *C. jejuni*[107, 108]. CDT is capable of causing damage due to three factors: the catalytic CdtB subunit encoded by the *cdtB* gene possessing DNase I-like activity and the CdtA and CdtC binding proteins for delivering CdtB into target cells. Within the nucleus, CdtB induces genotoxic effects on host DNA that triggers DNA repair cascades, which can ultimately result in cell arrest and cell death[109].

Many individuals may be colonized by *C. jejuni* without exhibiting clinical symptoms even during outbreaks of the disease[26, 110]. Recently, there has been great interest in elucidating the mechanisms of *C. jejuni* pathogenesis. *C. jejuni* and *Campylobacter coli* both have modified flagella that are heavily glycosylated and change in glycan composition affects autoagglutination and microcolony formation on IECs[111]. Motility is a key component for the pathology of *C. jejuni* as its flagella are required for effective colonization of the small intestine and transit to the colon[112, 113]. The flagella are not only for motility and cell binding, they also act as a type III secretion system (T3SS) for the delivery of *Campylobacter* invasion antigens (Cia) proteins into the extracellular space or into the host cells[111, 114-117]. T3SS also induces protein secretion and the export of proteins across the inner and outer membranes without a periplasmic intermediate step. *C. jejuni* can also induce translocation of non-invasive bacteria across the enteric

epithelium leading to cellular inflammation[118]. If *C. jejuni* infection diminishes host tolerance to commensal microbes, there are implications for chronic inflammation long after *C. jejuni* has been cleared from the host. For example, *C. jejuni* is the causative agent of GBS, a post infectious autoimmune-mediated neuropathy[104]. Developing GBS is thought to be related to the sialylated lipooligosaccharides (LOS) on the cell surface of *C. jejuni* that closely mimic the gangliosides on peripheral nerves[119]. The invasion-associated marker (*vir*B11) gene is another virulence gene linked with *Campylobacter* spp. invasiveness. It has been shown *in vitro* that this gene in *C. jejuni* strains is associated preferentially with both adherence and invasion[120]. *C. jejuni* also possesses serine proteases (high temperature resistant protein A; HtrA) that play an active role in transmigration across the intestinal epithelium[121]. HtrA can be secreted into cell culture supernatant *in vitro*[122], and cleaves the major adheren junction protein, E-cadherin on epithelial cells[123]. *C. jejuni* also possesses a functional type VI secretion system (T6SS)[124] generally found in Proteobacteria. The T6SS promotes pathogenicity and helps to adapt to fluctuating environments[125]. Importantly, the T6SS aids in host cell adherence and invasion[124].

Previously, NaB has been shown to suppress TNF induced degradation of IκB in HT-29 cells leading to NF-κB suppression[126]. This result was also observed in human monocytes[127]. Chang *et al* showed that NaB did not have an effect on TNF expression in response to LPS stimulation of bone marrow derived macrophages[53]. To date, *C. jejuni*-TNF research has focused on TNF expression by DCs, in contrast to IECs such as Caco-2 cells[128, 129]. Further investigation is required to understand how IECs are affected by *C. jejuni*.

1.3 Host Responses

Multiple regulatory mechanisms have evolved to ensure that host responses to the DGM are context-specific and appropriately weighted. This balance is achieved through DGM composition

influencing the immune system by producing immunomodulatory molecules (i.e. immunomodulins) such as NaB[130]. Therefore, regulated production of immunomodulins by the DGM influences the health and maintenance of the GIT epithelium of such as cell turnover, cell differentiation, mucin production from AEs and GCs, and coordinating a large portion of the immune response within the GIT, partly through epigenetic means[65, 86, 131, 132]. While the immune system is viewed as an organ protecting the host against pathogens, the mucosal immune system is a specialized branch located where most pathogens invade. Therefore, maintaining a balanced interaction between the mucosal immune system and DGM, and the processes that help shape their responses (i.e. fermentation of DF) is key for a healthy enteric environment[133].

1.3.1 The Mucosal Immune System

The enteric mucosal immune system regulates host inflammation and innate immunity, and is responsible for preventing enteric pathogens and foreign material from penetrating the epithelium and for responding when the barrier has been compromised[134]. The first layers of protection against enteric infection are the LA and TA mucus layers that help to contain the microbiota within the luminal space[135]. Mucus producing GCs and IECs produce various mucin proteins decorated with various sugars to form the two layers. Paneth cells and IECs release antimicrobial peptides (AMPs) (Figure 1.1B), while immunoglobulin A (IgA) is continuously released from crypts following stimulation of dendritic cells[136].

The enteric lining is decorated with various pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) that recognize various pathogen associated molecular patterns (PAMPs). Once the PAMP ligands bind to the PRRs, signal transduction cascades are triggered to prompt various immune responses and subsequent release of highly specific factors involved in the immune response[137]. These response factors, typically cytokines and chemokines, are

released within the lamina propria, below the epithelial lining, and can activate lymphocytes[138]. This provides the host immune system with a mechanism to respond to beneficial and potentially harmful microbes. The immune response has two main subsets: the innate and the adaptive that are not mutually exclusive of one another, but together work to establish immunity.

1.3.1.1 Innate Immunity

Innate immunity is important for fast-acting broad-spectrum host responses. In conjunction with the physical barrier of the epithelium and chemical barrier of antimicrobials (defensins such as β-defensin 1 and β-defensin 3) produced by the epithelium, innate immunity serves to mitigate infectious challenges by clearing pathogens, determining the localization and extent of the invasion, and facilitating the adaptive immune response. IECs are the first host cells that enteric pathogens physically interact with (Figures 1.1B). Importantly, AEs act as sentinels for the immune system, and play key roles in detecting and orchestrating immune responses to different pathogens[4]. Conserved microbial structural PAMPs, such as flagellin and LPS, are recognized by pattern recognition receptors (PRRs) such as TLRs (e.g. TLR2, TLR5, and TLR9) or nucleotide-binding oligomerization domain receptors (NOD-like receptors). Enterocytes typically respond to PAMP signals by triggering inflammatory or cell death pathways[139, 140].

1.3.1.1.1 Defensins

Vertebrate and invertebrate hosts secrete a range of AMPs, including defensins. In the case of humans, defensins are secreted into the mucus layer and serve as regulators of the enteric microbial ecology[141] by acting as immunomodulators[142] and bacterial cytotoxins (Figure 1.1B)[143]. Once their concentration approaches μg mL^{-1}, defensins play important roles in the innate immune system protecting mucosal surfaces from infection against Gram-positive and Gram-

negative bacteria, mycobacteria, enveloped viruses, and fungi[144, 145]. These host defence peptides are secreted primarily from specialized Paneth cells and to a lesser extent IECs. Defensins belong to one of three subfamilies: α-, β-, Θ-defensins. The three subfamilies mostly differ in size, disulphide bonding, precursor structure, and sites of their expression[146]. The α-defensins are commonly found in the small intestine due to the increased density of Paneth cells[147, 148]. β-defensins such as human β-defensin 1 (hBD-1) are primarily found in the large intestine, but can be found in the small intestine as well as they are expressed most by epithelial cells[149]. Under balanced conditions in the GIT, epithelial cells prevent microbial invasion by saturating the TA mucus layer with defensins such as the constitutively expressed hBD-1, and selectively modulating the composition of the DGM (Figure 1.1B)[150]. Constitutive expression of hBD-1 suggests that it plays a role in immune surveillance in a healthy host, and it is recognized as the most important AMP in epithelial cells[151-153]. During inflammation or infection, additional defensins and AMPs can be induced, such as human β-defensin 3 (hBD-3)[154].

Similar to hBD-1, hBD-3 possesses bactericidal activity against Gram-positive and Gram-negative bacteria, including multi-drug resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* and reportedly against *Burkholderia cepacia*[155, 156]. Transmission electron microscopy has shown that hBD-3 has the ability to induce morphological changes, similar to perforation of the peripheral cell wall, within about 30 minutes of interacting with *S. aureus*[155]. When incubated with hBD-3, *C. jejuni* showed a thinning of the cell wall with the formation of membrane-enclosed blebs leading to the subsequent loss of cytoplasmic contents[151]. Further, hBD-3 has been shown to act as a both a stimulant and an attenuator of chemokine and cytokine responses[157], although this dichotomy is not entirely understood. This relationship

provides a linkage between innate and adaptive immunity[157-160]. Currently there is a high level of interest in developing hBD-3 for possible pharmaceutical applications[161].

The majority of AMPs act by permeabilizing the bacterial membrane through several different types of mechanisms, including membrane depolarization, and the creation of physical holes in membranes[162-166]. The effect of hBD-3 on *Bacteroides* sp. seems to rely on the presence of oxygen[167]. Since most commensal bacteria are anaerobic and most pathogens are aerobic, the host can control *Bacteroides* populations when under attack to mitigate commensal invasion.

Research has primarily focused on defensins of the small intestine. Currently, the effect and induction of large intestinal defensins is less well understood. Further, the induction and activation of defensins can depend on the presence of select members of the microbiota and their metabolic potentials[168]. These findings emphasize that feedback loops exist between the host and its microbial symbionts.

1.3.1.1.2 Toll-Like Receptors

The TLRs responsible for the onset of an acute inflammatory response are critical antecedents for the maturation of adaptive immunity, particularly for the induction of a Th1 cell response[169, 170]. So, the genetic and developmental variation in the expression of microbial PRRs may affect how the host is predisposed to infections. Similarly, mechanisms that alter the signal transduction pathways downstream of TLR activation are important. TLR expression is nearly ubiquitous in human immune cells, driving the innate and adaptive immune mechanisms. In the absence of inflammation, TLRs are expressed at low levels on the epithelial surface and their expression is highest in the crypts to ensure the stem cell niches are protected[171]. This pattern is TLR dependent as some families can be expressed on the surface of apical and basolateral membranes of polarized IECs.

TLR mediated inflammatory molecule production is a double-edged sword. Proper maintenance of TLR signal transduction is required because while pro-inflammatory cytokines trigger the recruitment and the activation of APCs at infection sites; they also favour inflammatory tissue damage[172]. Further, uncontrolled inflammatory cytokine induction is not desirable for maintaining a state of local homeostasis with the commensal DGM. In this regard, commensal bacteria can also actively participate in TLR signalling regulation. *Bacteroidetes* is a dominant taxa of the human GIT microbiota, and *B. theta* can induce the expression of peroxisome proliferator-activated receptor-gamma (PPARγ), an intracellular negative regulator that can circumstantially inhibit TLR signalling in IECs. PPARγ promotes the export of the p65 subunit of NF-κB from the nucleus, and prevents pro-inflammatory gene expression[24]. Alternatively, non-virulent *Salmonella* strains can inhibit the NF-κB pathway by blocking the degradation of IκBα, in turn promoting attenuation of inflammatory responses triggered by pro-inflammatory stimuli[173].

1.3.1.1.2.1 Toll-like receptor 2 (TLR2)

TLR2 is a membrane-anchored receptor is generally expressed on the surfaces of IECs. It is primarily responsible for host responses against Gram-positive bacteria and yeast by regulating NF-κB stimulation[174]. TLR2 is mainly expressed in crypt cells, as a means of protecting the enteric stem cells[175]. It has been shown to detect *C. jejuni*'s surface polysaccharides and trigger IL-6 secretion by IECs[72]. Other cytokines triggered by TLR2 signalling include TNF and various interleukins (IL-1a, IL-1B, IL-8, and IL-12) that begin to participate in a Th1 response.

1.3.1.1.2.2 Toll-like receptor 5 (TLR5)

TLR5 recognizes the bacterial virulence factor flagellin (flagella)[176] and mobilizes NF-κB, which then activates many inflammatory related target genes[177]. TLR5 is mainly expressed in the

colon[175], and expressed on the basolateral surface in T84 cells, thus sensing flagellin only when microbes cross the intestinal epithelial barrier during active invasion[178]. TLR5 localization is cell dependent as HT-29 cells express TLR5 on the apical surface, whereas Caco-2 cells express TLR5 both basolaterally and apically[179-181]. TLR5 plays a role in the tolerance of the commensal DGM as well as in limiting pathogenicity of potentially dangerous microbes through a TLR5 dependent induction of anti-flagellin antibodies that prevents commensal bacterial association with the enteric mucosa by limiting bacterial motility[182, 183].

1.3.1.1.2.3 Toll-like receptor 9 (TLR9)

TLR9 is involved in the detection of bacterial DNA[174]. Nucleotide sequences containing unmethylated CpG dinucleotides are found much more frequently in prokaryotic DNA than in vertebrate DNA. Both apical and basolateral sides of polarized human colonic epithelial cell lines have been shown to express TLR9. Interestingly, the location of TLR9 alters the response[184]. When stimulated on the basolateral surface, TLR9 activates the NF-κB pathway. In contrast, apical stimulation prevents NF-κB activation from conferring tolerance to the chronic TLR challenges presented by hosting the commensal DGM[184]. TLR9 activation at either the apical or basolateral sides of IECs may induce a variety of different cytokines depending on whether NF-κB pathways are triggered[184]. Some species are able to avoid detection by TLR9 by maintaining a heavily AT rich genome, such as *C. jejuni*[185].

By monitoring the expression of different PRRs, insight will be provided into how the DF fermentation by-product NaB, *B. theta*, and *C. jejuni* influence could influence host immune signal transduction pathways. To date, a xenobiotic microbial metabolite is the only microbial metabolite shown to induce expression of a TLR (TLR4[186]), showing the potential for modulation.

1.3.1.2 Adaptive Immunity

Since enteric microbes have been shown to influence the immune system long after initial exposure it is important to understand immune processes beyond the innate immune system. Once a pathogen is recognized by the innate immune system, constantly circulating memory B cells in the blood stream become matured with the receptors for the pathogen for quick resolution of subsequent infection after antigen presentation by a variety of cell types such as IECs[187]. This adaptive response occurs several days after infection, but is a stronger response than the innate response, given that B and T cells are utilized to resolve infection.

T cells come in two main forms, the cytotoxic T cells and helper T cells. Cytotoxic T cells differentiate from various CD8 mediated signals, and permit apoptosis to destroy engulfed cells[188], while CD4 signals mediate helper T cells as the other T cell forms[189]. Helper T cells have three main groups: Th1, Th2, and Th17 (Figure 1.4). During infection, T cell responses are orchestrated by secreted molecules (e.g. cytokines) based on the presence of bacteria near the epithelial lining influencing the differentiation of naïve T helper cells (Th0) into specific helper T cells: Th1, Th2, Th17, and Treg as shown in Figure 1.4. Different cytokines promote IECs, macrophages and dendritic cells within and below the epithelial lining to produce other cytokines that influence T cells to further produce cytokines and chemokines that attract specialized immune cells[190].

Cytokines released from T helper cells have the ability to up-regulate or down-regulate other T cell responses, and some act on a number or T cells. Generally, a Th1 response is promoted by IFN-γ, IL-8, TNF, and IL-12β. IFN-γ also inhibits a Th2 anti-inflammatory response that functions to promote B cells to produce antibodies and white blood cell differentiation into mast cells and eosinophils. Th2 cells use IL-4, Il-5, IL-9, and IL-13 to induce IgE, mast cell and eosinophil production. Th2 cells also produce IL-10 that inhibits IFN-γ enabling the two

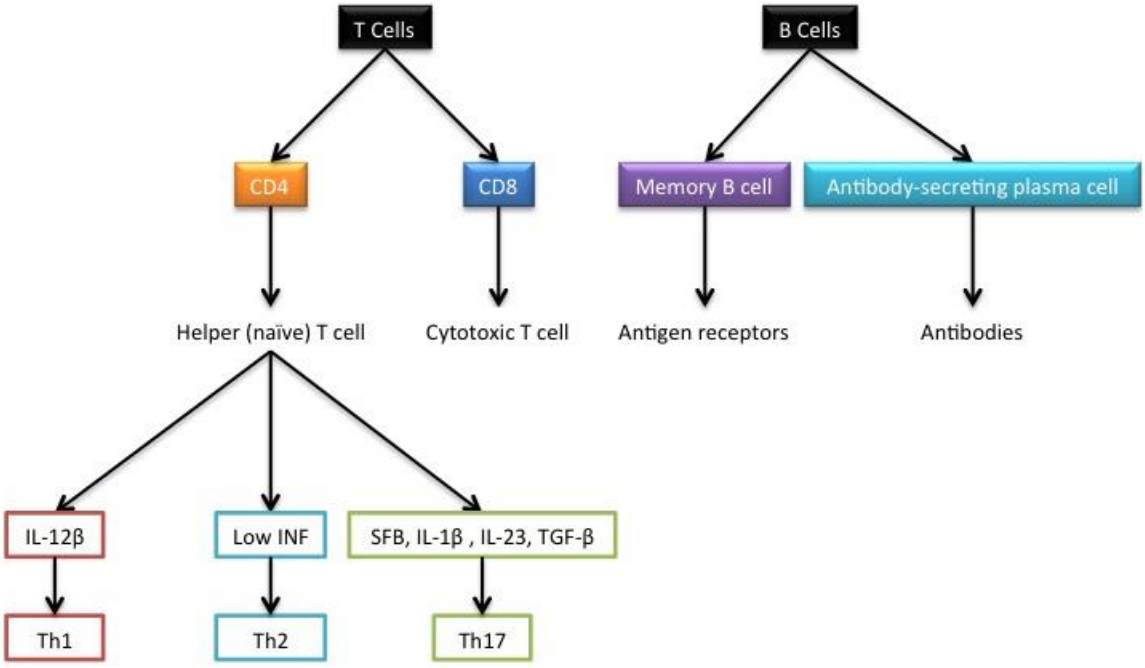

Figure 1.4 B and T cell maturation.

Mature B and T cells form the various subsets of the adaptive immune response. Cytokines play crucial roles in the differentiation and effector functions of Th1, Th2, and Th17 cells. Upon activation triggered by antigen-presenting cells, naïve CD4 cells can differentiate into distinct T helper lineages. Each lineage expresses unique cytokine receptors that can respond to cytokines produced by sentinel cells. Upon antigen presentation, naïve B cells can develop into memory B cells or into terminal, antibody-secreting plasma cells.

responses to be relatively exclusive; however it is more constructive to think of them in a regulatory manner. Generally, when IFN-γ is low, the Th2 response will be functioning, and when external factors trigger IFN-γ, then the Th2 response is downregulated and the Th1 response is upregulated. Segmented filamentous bacteria usually initiate the Th17 response; however there are other triggers such as IL-1, IL-23 and TGF-β. Lastly, naïve helper T cells differentiate into Treg cells in the presence of retinoic acid, IL-10 and TGF-β (released by DCs in response to immunomodulatory signals received from either TLR or bacterial products). The Treg pathway is self-promoting by producing more TGF-β and IL-10 to regulate pro-inflammatory responses.

1.3.1.2.1 Cytokines

Cytokines are a group of small proteins secreted from cells for intercellular signalling and communication. Cytokines can be divided into functional classes, including primarily lymphocyte growth factors, pro-inflammatory or anti-inflammatory molecules, and other cytokines that polarize the immune response to antigen.

The cytokine designation includes a variety of types: interleukins (e.g. IL-12β), tumour necrosis factor, transforming growth factors (e.g. TGF-β), chemokines, interferons, lymphokines, and colony-stimulating factors. Cytokines are produced by a number of cells, including immune cells such as macrophages, B lymphocytes, T lymphocytes, and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells; and a given cytokine may be produced by more than one cell type. Cytokines act through receptors, and are essential to the immune system. By modulating the balance between humoural and cell-based immune responses, they regulate the maturation, growth, and responsiveness of particular cell populations. Many cytokines have multiple and sometimes unrelated functions depending on the target cell or the presence/

absence of other cytokines; partly explaining why many infections possess broadly similar cytokine profiles (T helper cell subsets), but their clinical presentations are quite different.

1.3.1.2.1.1 Interleukin-12β (IL-12β)

IL-12β stimulates interferon-gamma (IFN-γ) production in T and natural killer (NK) cells[191], and enhances the naïve $CD4^+$ T cells development into Th1 type cells in the Th1 response[192]. IL-12β also bridges the innate and adaptive immune system by maturing DCs in the lamina propria to migrate to the draining lymph nodes to activate naïve T cells[193]. Thus, IL-12β is important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to an intracellular pathogen. It is important to this project because it is regulated by NF-κB (Appendix Table 4.1), and NaB has been shown to decrease IL-12β in an anti-inflammatory manner, in human monocytes[127].

1.3.1.2.1.2 Tumour Necrosis Factor (TNF)

TNF (formerly TNFα) is an adipokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Primarily TNF is produced by activated macrophages; however many other types of cells can produce it[194]. TNF primarily regulates immune cells, but it has an exceptionally vast spectrum of bioactivities with most cells showing some form of TNF responsiveness. TNF levels must be fine-tuned as high levels induce shock-like symptoms, and prolonged exposure of low concentrations can result in wasting syndrome (cachexia)[195]. Dysregulation of TNF has been implicated in a variety of diseases, including IBD[196] and autoimmunity[197]. TNF is of clinical relevance to this project being involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis[198], lipid metabolism[199], and coagulation[200].

1.3.1.2.1.3 Transforming Growth Factor (TGF-β)

A potent cytokine, TGF-β has diverse effects regulating hemopoietic cells. Primarily known for maintaining tolerance through the regulation of lymphocyte proliferation, differentiation and survival with several other functions in most cells[201], TGF-β is implicated in both the initiation and resolution of inflammatory responses by influencing chemotaxis, activation, and survival of lymphocytes, NK cells, DCs, macrophages, mast cells, and granulocytes[202]. TGF-β has different regulatory activity depending on the cell differentiation state and the presence of other inflammatory cytokines and costimulatory molecules. TGF-β is also important in inhibiting the development of immunopathology to self or benign antigens without diminishing immune responses to pathogens[201]. Excess TGF-β in lesions is associated with unresolved inflammation and fibrosis[203]. TGF-β has clinical relevance with roles in immunity[204], cancer[201, 205], bronchial asthma[206], and insulin-dependent (type 1) diabetes mellitus[207].

1.3.1.2.2 Chemokines

Chemokines are small proteins that constitute a large family of peptides structurally similar to cytokines, whose main function is to regulate cell trafficking and therefore have important roles in coordinating the adaptive immune system. There are four subfamilies separated by the number and location of the cysteine residues at the N-terminus of the molecules and are named CC, CXC, CX_3m and C[208]. Chemokines are produced in response to signals such as pro-inflammatory cytokines where they can recruit monocytes (e.g. CCL2[209, 210]), thymocytes (e.g. CCL25), IgA B cells (e.g. CCL28)[211], neutrophils (e.g. CXCL8[212]) and DCs (e.g. CXCL10). After induction, cells expressing the chemokine receptors are directed to migrate along the chemical ligand gradient known as the chemokine gradient. This allows cells to enter damage sites from low to high local concentrations of chemokines[213]. Additional chemokines have activating functions, such as CXCL5 on neutrophils[214].

1.3.1.2.2.1 CCL2, Monocyte Chemotactic Protein-1 (MCP-1)

MCP-1 is involved in a number of diseases, and regulates migration and infiltration of monocytes/macrophages, but not neutrophils or eosinophils[209, 210]. MCP-1 production is highly varied. Cell types including endothelial[215], epithelial[216] and monocytic[217] among others, can express MCP-1, either constitutively or after induction by cytokines or growth factors[209]. MCP-1 elicits its effects through its receptor CCR2. In contrast to MCP-1, CCR2 expression is relatively restricted to certain cell types. It has been reported that the two forms of CCR2 enable MCP-1 and CCR2 to perform both pro- and anti-inflammatory actions[209].

1.3.1.2.2.2 CCL25, Thymus Expressed Chemokine II (TECK)

TECK is believed to influence the development of T-cells[218], showing chemotactic activity for thymocytes, macrophages, and DCs[219]. TECK is primarily and constitutively expressed in the small intestine[220], but reports have shown that it is also expressed, to a lesser extent, in the colon[221]. The main receptor for TECK, CCR9, is specifically expressed on a subset of GIT-homing T cells expressing integrin α4β7, as well as on IgA-secreting cells from GIT organs[222, 223]. TECK also recruits $CCR9^+$ intraepithelial lymphocytes, in the thymus and small intestine[224]. CCL25/CCR9 interactions regulate inflammatory immune responses in the large intestinal mucosa by helping to balance different subsets of DCs.

1.3.1.2.2.3 CCL28, Mucosae-Associated Epithelial Chemokine (MEC)

MEC is thought to function as a homeostatic chemoattractant of subpopulations of both T cells and IgA B cells, and to mediate antimicrobial activity[211]. It has been shown to increase with enteric inflammation, and in response to NaB. Previous findings have shown that MEC may act to counter regulate colonic inflammation[87]. MEC is constitutively expressed in different mucosal sites, including salivary and mammary glands, the trachea, as well as in the colon, and to a lesser

extent, the small intestine[225]. MEC can chemoattract populations of CD4 and CD8 T cells that express the cognate receptor for MEC, CCR10[226].

1.3.1.2.2.4 CXCL5, Epithelial Cell Neutrophil Activating Protein-78 (ENA-78)

ENA-78 stimulates the chemotaxis of neutrophils possessing angiogenic properties, in response to the detection of pathogens such as *C. jejuni* in cooperation with IL-8[27]. IL-8 acts as a chemotactic factor directing neutrophils towards damage sites, and ENA-78 helps to activate the migrated neutrophils. *In vitro* studies suggest that DCs encounter and quickly internalize *C. jejuni* resulting in NF-κB activation and cytokine secretion[227]. DCs then mature after antigen presentation by neutrophils and IECs, and migrate to lymph nodes for T cell activation.
The pro-inflammatory stimuli, TNF and IL-1β stimulate ENA-78 expression[228]. Previously, infection of T84 cells led to a dose-dependent increase in ENA-78 and IL-8 mRNA expression in a flagellin-independent manner[229].

1.3.1.2.2.5 CXCL8, Interleukin 8 (IL-8)

IL-8 is a chemokine produced by macrophages and other cells including IECs[230]. Pro-inflammatory stimuli such as LPS, IL-1β, and TNF modulate IL-8 expression[231]. IL-8 induces chemotaxis in target cells, primarily neutrophils and to a lesser extent granulocytes[212]. All cells with TLRs involved in the innate immune response can secrete IL-8.

1.3.1.2.2.6 CXCL10, Interferon-γ induced protein-10 (IP-10)

IP-10 has been shown to have several roles, such as chemoattraction for monocytes/ macrophages, T cells, NK cells, and DCs, promotion of cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis[232]. IP-10 is secreted by several cell types in response to IFN-γ, including monocytes, IECs, and fibroblasts[232].

Reports have shown that IP-10 levels are increased in the serum and/or tissue in various autoimmune diseases such as RA[233], MS[234], and type 1 diabetes mellitus[235].

1.3.1.3 Inflammation

Inflammation is the host's physiological response to harmful stimuli. A sophisticated regulatory network orchestrates this response, involving the activation of cytokines, chemokines and other inflammatory response factors. The GIT is in a constant state of controlled (physiologic) inflammation regulated by the activation and repression of inflammatory response genes[236]. Different levels of inflammation are present depending on the incitant source and host response (e.g. antimicrobial defence, tissue repair). Essential to maintaining a steady state of inflammation is to ensure that the resident DGM does not elicit an aberrant and uncontrolled immune response[237]. Key to avoiding aberrant immune responses to the DGM is a layered defence composed of a stratified mucous layer (Figure 1.1B), a relatively impenetrable but highly responsive epithelium (i.e. colonocytes), and lamina propria populated with immune cells that actively participate in containing the DGM and limiting aberrant immune responses[16].

Inflammation associated with an influx of cytokines begins at a local site of damage or infection, and can spread through the body by way of the systemic circulation. Rubor (redness), calor (heat), dolor (pain), tumor (swelling or edema), and "function laesa" (loss of function) are the hallmarks of acute inflammation. When localized within the epithelia or other tissue, these responses increase blood flow, enable vascular leukocytes and plasma proteins to migrate to extravascular sites of injury, increase the local temperature (an advantageous situation for host defence against bacterial infections), and generate pain as a warning mechanism for the host about the local response. These responses often occur at the expense of local organ function, especially when tissue edema causes a rise in extravascular pressure and reduces tissue

perfusion. Compensatory repair processes are part of the immune response timeline normally, and are initiated soon after inflammation begins. In many cases, tissue and organ function is completely restored; however, in cases where inflammation is complicated and inflammation is severe or the primary etiological agent damages local tissues, healing may occur with fibrosis[238]. If the appropriate responses to inflammation and tissue injury in the GIT become excessive or become perpetuated, they can lead to distortion of tissue architecture and fibrosis[238]. Fibrosis in turn can result in persistent organ dysfunction[239].

NF-κB is a central transcription factor in the modulation of genes that control inflammation. NF-κB activation is dynamic, and relies on the phosphorylation-induced proteosomal degradation of NF-κB inhibitor proteins (IκBs) responsible for retaining the inactive NF-κB in the cytosol of unstimulated cells[240]. Various bacterial molecules and metabolic by-products are capable of inducing or suppressing NF-κB activation[69, 126, 241, 242]. The network of NF-κB-dependent transcription responsible for activating both pro- and anti-inflammatory mammalian genes remains to be determined; however genes controlled by NF-κB have been documented (Appendix Table A4.1). In this study, I will investigate the ability of Caco-2 cells to express hBD-1, hBD-3, hAD-1, TLR2, TLR5, TLR9, TGF-β, IL-12β, TNF, MCP-1, TECK, MEC, ENA-78, IL-8, and IP-10 mRNA.

1.3.2 Epigenetic Control of Mucosal Health and Immunity

1.3.2.1 Histone Modification

The human genome consists of approximately 2 meters of DNA that is housed in a nuclear compartment only 10 μm in diameter. DNA packaging must occur in an ordered fashion for physiological expression and silencing of differential genes. This process is achieved through interactions with histones and other scaffolding proteins to form chromatin. Chromatin folding

is a dynamic process that must respond to environmental, metabolic, and developmental stimuli. Post-translational modifications (PTMs) of histone N-terminal tails, such as acetylation, provide cells with a dynamic response system (Figure 1.5). The combination of histone PTMs and other factors define a regulatory network[243-245]. The existence of these modifications and the modules that recognize these PTMs led to the "histone code" hypothesis proposed by Strahl and Allis[243].

Epigenetic modification helps to coordinate the level of chromatin folding and packaging within the nucleus. The two varieties of chromatin are euchromatin and heterochromatin. Euchromatin is lightly packed DNA, rich in gene concentration, and is typically under active transcription. The gene regulatory proteins, including RNA polymerase complexes can bind with the DNA sequence due to the unfolded structure of the euchromatin, as well as the PTMs that favour transcription. In contrast, heterochromatin is tightly packed DNA, commonly found on the peripheral areas of the nucleus. Heterochromatin is found in two main states, and is responsible for gene regulation and protecting chromosomal integrity. Heterochromatin exists as a continuum between the two extremes of constitutive and facultative heterochromatin. Constitutive heterochromatin is generally inherited silenced sections of DNA with poor expression, whereas facultative heterochromatin is the result of genes silenced through mechanisms such as histone deacetylation[246]. Under certain developmental or environmental signals, it can lose its condensed structure to become transcriptionally active.

Specific histone tail modifications have been associated with specific biological outcomes. For example, acetylation of histone tail 3 lysine 9 (H3K9) is a 'signal' for gene activation; however, tri-methylation of this same residue is a keystone marker for HP1 chromodomain recruitment and heterochromatin formation[247, 248]. H3K9 has been shown to

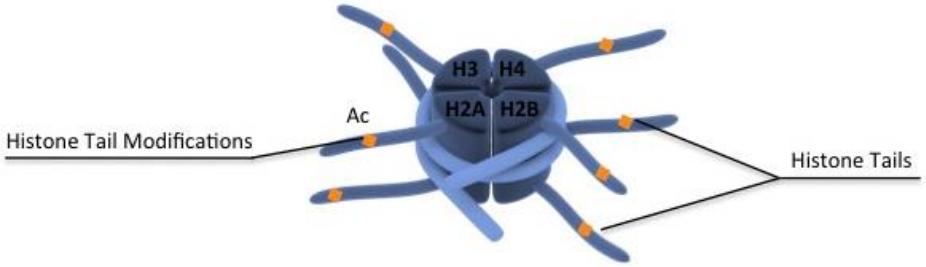

Figure 1.5 Histone tail modifications.

The N-terminal tails of histones can be post-translationally modified by acetylation to provide cells with dynamic response systems to alter the accessibility of DNA transcription factors. Histone 3-lysine 9 is a histone tail modification that acts as a signal for gene activation and bromodomain binding.

interact directly with bromodomains, modules that are commonly found within transcription factors (e.g. TAFII250[249]). It is becoming increasingly clear that many inflammatory disorders, including IBD and colonic cancer, have an epigenetic component contributing to the aetiology and progression of the disease[250, 251]. Currently, little is known about epigenetic pathways involved in the modulation of genes that regulate inflammation; however, it has been determined that the regulatory factors of the inflammatory process are controlled by distinct epigenetic mechanisms[252-254].

1.3.2.1.1 Histone Acetylation, Deacetylation, and Inhibition

Acetylated histone tails are generally associated with euchromatin characteristic of active genes. Acetylation neutralizes the positive charge on histones that alters histone structure and weakens histone-DNA interactions. Additionally, at particular amino acid residues of histones H3 and H4, acetylation serves as a binding site for transcription factors, such as NF-κB[255, 256]. HDACs are enzymes responsible for removing acetyl groups from histones,[257] and correspondingly, they are generally associated with transcriptionally repressed regions of DNA. Perturbing the activity of HDACs by inhibitors (HDACi) such as NaB[258] results in the hyperacetylation of histones and modulation of expression patterns in 5-10% of human genes[259]. Correspondingly, HDACis have anti-proliferative and anti-inflammatory effects both in *in vitro* and *in vivo* models of enteric inflammation[260] and are currently used in the treatment of several diseases. NaB has been shown to inhibit class I and class IIa HDACs[261]; the supporting evidence for NaB inhibiting class IIb (HDAC6 and HDAC10), however, is inconclusive[260]. This could be because NaB reduces histone deacetylation levels but does not inhibit HDACs directly[262]. NaB may also affect many other epigenetic-related enzymes, HDAC gene expression, and cytokines that affect TLRs by impairing transcription factor recruitment[263, 264].

SCFA levels in the proximal colon (Figure 1.1A3) are estimated to range from 70 to 140 mM[51, 265] in a 60: 20: 20 ratio of acetate: propionate: NaB [266]. These concentrations are several orders of magnitude higher than the concentrations commonly associated with cell culture[267, 268]. SCFA levels are inherently lower in cell culture because NaB is not used as an energy source in cancerous colonocytes as they rely on glucose as their primary energy source[269]. In turn, NaB accumulates and functions as an HDACi, just as it would *in vivo*, but this effect is observed at lower NaB concentrations. Thus, NaB concentrations ranging from 0-10 mM have been reported in cell culture with 5 mM regularly employed in HDACi investigations[74, 267, 270]. Although, NaB is a naturally occurring metabolic by-product within the colon and is commonly used for chemotherapy, the mechanism of NaB-mediated HDAC inhibition is unclear[271, 272]. In this regard, the effects of NaB on H3K9 acetylation and the modulation of specific genes that regulate inflammation within colonocytes require further investigation.

1.3.2.1.2 Butyrate Effects on the Expression of the Immune System

NaB influences the immune system in a number of additional ways. For example, it can be anti-inflammatory by suppressing NF-κB activation[126], inhibiting IFN-γ production[273] and up-regulating PPARγ[274]. NaB further exerts effects on the immune system by affecting immune cell migration (chemokines)[86], adhesion[275], cytokine expression[276, 277], and cellular processes: proliferation[276], activation[126] and apoptosis[278]. NaB is also implicated in innate immunity, and a role in regulating AMP expression is beginning to emerge[279-281].

NaB interacts with various G protein-coupled receptors (GPRs) such as Grp109a on the surface of IECs and submucosal leukocytes leading to altered gene expression, and NaB also has a role in NF-κB suppression[241, 277, 282, 283]. The immunosuppressive role of NaB in intestinal maintenance is in part related to its ability to block DC generation from bone marrow stem cells,

without affecting the generation of granulocytes[284]. This effect is in part dependent on the ability of NaB to promote histone acetylation and differential gene expression[284].

Currently, the relationship between NaB, H3K9, and regulation of immunomodulatory genes within colonic cells remains unclear, although, HDAC1-dependent Fas upregulation is thought to be involved[285]. NaB can also have a priming effect on IECs- enabling IECs to become activated by lipopolysaccharide and pro-inflammatory cytokines by increasing IL-8 mRNA at higher concentrations[286]. Indeed, the DGM has also been shown to affect host gene expression through chromatin modifications and other signal transduction pathways, beyond the activity of NaB. Histone modifications and chromatin remodelling during bacterial infections have been reported[287].

Upon microbial infection, host cells must undergo transcriptomic changes, activating genes involved in immunity, cell death, and motility to trigger an appropriate response[288]. Therefore it is not surprising that efficient pathogens have developed successful mechanisms to deregulate host expression, or that successful commensal species have developed mechanisms to maintain host expression. Bacteria have been shown to influence epigenetic factors upon infection[289], such as hijacking cellular signalling pathways that activate or repress transcription factors such as NF-κB or AP-1 in the cytosol.

1.4. The Caco-2 Intestinal Cell Model

The Caco-2 colonocyte cell line is commonly used as a carcinomic intestinal model cell line because it can differentiate and form high columnar epithelial cells[290]. Caco-2 cells express a combination of AE (colonocyte and enterocyte) phenotypes depending on the number of days post-passage. Before reaching confluence, Caco-2 cells express colonocyte function (Appendix Figure A1.2). Post-confluence, Caco-2 cells begin to express foetal ileal epithelial markers as a

polarized monolayer with cobblestone morphology[291]. AEs such as colonocytes have a foundational role in the mucosal immune response. So I propose that the model colonic Caco-2 cell line is appropriate for studying the epigenetic control of the immune system, as it exhibits differential gene expression induced by NaB, *B. theta* and *C. jejuni*.

1.5 Proposal

In order to examine the impacts of a common DF fermentation by-product NaB*,* and *B. theta* and *C. jejuni* on induced inflammation in colonocytes; and to establish a robust model system for evaluating inflammatory responses in culture, I will conduct a Caco-2 transcriptomic analysis using an in-house immunomodulatory gene array for Caco-2 cells. By comparing baseline expression levels of keystone immunomodulating and defence proteins to their expression levels in the presence of 5 mM NaB, *C. jejuni* (K12E5 and 81-176), and *B. theta* (complete factorial) I will seek to define potential biomarkers for detecting and prioritizing immune responses in Caco-2 cells. This knowledge will facilitate the identification of potential inflammatory biomarkers for animal model validation. Coupling these gene expression profiles to the acetylation patterns of H3K9, I will help to elucidate the effect of NaB on Caco-2 immune regulation, *B. theta* tolerance, and colonization resistance against *C. jejuni* infection.

The Caco-2 immune markers were chosen for their strategic roles in commensal bacterial tolerance, detection, as well as functional roles within innate and adaptive immunity. Defensins participate in regulating community structure by controlling the growth and location of commensals and threats to mucosal health. TLRs comprise a significant fraction of the host's ability to detect commensal and pathogenic bacteria. Cytokines are vital to the immune system signalling properly, particularly since IECs act as sentinels communicating danger to DCs and

other leukocytes through cytokine and chemokine production (such as the chemoattractive MCP-1 or IL-8).

1.5.1 Hypotheses

1) I hypothesize that NaB will induce expression of defensins and TLRs in Caco-2 cells.
2) I hypothesize that *B. theta* will promote defensin production against competing bacteria to modulate inflammatory cascades and down-regulate TLRs or pro-inflammatory cytokines. This effect may be attenuated in the absence of NaB, and induce pro-inflammatory-like responses.
3) I hypothesize that *C. jejuni* down-regulates defensin and TLR expression whilst inducing pro-inflammatory chemokine expression.
4) I hypothesize that together, *B. theta* and NaB will interact to elicit their effects in modulating the pro-inflammatory-like responses induced by *C. jejuni*.

1.5.2 Objectives

Using a Caco-2 colonocyte model:

1) Examine the impacts of NaB on inflammation responses in Caco-2 cells.
2) Determine the degree to which NaB and *B. theta* modulate immune responses triggered by *C. jejuni*
3) Identify biomarkers for subsequent validation in animal models

The following chapters will present the experimental methods (Chapter 2) and experimental results (Chapter 3) for this study. In Chapter 4 these findings will be discussed within the context of the literature. An overall summary and promising directions for future investigation will be presented in Chapter 5.

Chapter 2 Materials and Methods

2.1 Caco-2 Intestinal Model

Caco-2 human colon carcinomic enterocytes were used (cell passages 21 to 30). Caco-2 cells (American Type Culture Collection, Manassas, VA) were cultured in Advanced Dulbecco's minimal essential medium (DMEM; #6546) supplemented with 10% foetal bovine serum (#F1051), 200 mM L-glutamine (#G7513), 100 U mL^{-1} penicillin (#P4333), 100 µg mL^{-1} streptomycin (#P4333), and 80 µg mL^{-1} tylosin (#T3397) (DMEM Complete+AB) at 37 ^{o}C in a 5% CO_2 atmosphere. All cell culture reagents were from Sigma-Aldrich (Oakville, ON). Cells were grown in 75 cm^2 tissue culture flasks (canted neck 0.2 µm vented seal cap; #353136; BD Falcon, Mississauga, ON) in triplicate for the epigenetic assay and BioCoat Collagen I Cell Ware 6 well plates (#354400; Horsham, Pennsylvania, USA) for the gene expression assay.

2.1.1 Caco-2 Butyrate Supplementation Trials

An optimal NaB concentration of 5 mM for Caco-2 cell growth was determined by growing cells to day 5, and supplementing DMEM Complete+AB with 1, 5, 10, 30, and 100 mM NaB (#B5887; Sigma-Aldrich, Oakville, ON). Cells were grown for 2 days, before assessing confluence (~6.76 x 10^5 cells mL^{-1}) at day 7, with NaB supplemented according to Table 2.1. SCFA levels were detected using gas chromatography performed by Darrell Vedres (AAFC, Lethbridge, AB, Canada).

2.2 Bacteria and Growth Conditions

C. jejuni NCTC 11168 (K12E5)[292], *C. jejuni* 81-176[118], and *B. theta* were used throughout this study. Considerable genotypic and phenotypic variation exists with *C. jejuni*, and for this reason I chose to include two genotypically and phenotypically distinct clinical strains of the bacterium. *C. jejuni* 11168 was originally isolated from a human patient with diarrhea in 1977[293, 294], and it is

Table 2.1 Timeline for the addition of NaB and bacterial treatments.

7 day cell culture passage timeline	Activity
1	-Caco-2 cells subcultured into tissue culture flasks
2	
3	-Culture medium refreshed -NaB or PBS (± NaB) added
4	
5	-Culture medium refreshed
6	
7	-Culture medium removed -Caco-2 cells washed -Culture medium ± NaB added -Equilibration period (2 hours) -Confluence assessed -Bacteria added for 6 hours

the genome reference strain for the species[295]. A highly virulent variant of NCTC 11168 (K12E5) was recovered from a person infected by the strain[101]. *C. jejuni* 81-176 was originally isolated from the feces of a 9 year old girl suffering from *Campylobacter* enteritis[296], and is commonly used to study host-pathogen interactions in cell models[90, 122, 297]. The K12E5 strain will be referred to as strain "1", and the 81-1176 strain will be referred to as strain "2". *B. theta* (ATCC 29148-Δ*tdk*) is a common constituent of the intestinal microbiota of people[298], adult mice[299, 300], pig[301], and cow[301].

C. jejuni inoculum was prepared by growing the bacterium for 14-16 hours in Columbia broth (37 ^{o}C, 100 rpm, Difco, Detroit, MI) in a microaerobic atmosphere using a CampyGen gas pack (Oxoid, Ottawa, ON)[118]. *B. theta* was cultured for 14-16 hours in tryptone yeast extract glucose media (TYG; Appendix Table A2.1) at 37 ^{o}C in microaerobic atmosphere using a GasPak EZ Gas Generating Sachet (#260678; Thermo-Fisher Scientific, Ottawa, ON). Cells were prepared by removing medium and resuspending cells in DMEM Complete without antibiotics (DMEM Complete-AB. The density of bacteria in the inoculum was determined spectrophotomically with validation by spreading eight-fold dilutions onto Karmali agar (#CM0935; Oxoid, Ottawa, ON)[302] for *C. jejuni* or supplemented Bacto™ Brain Heart Infusion (BHIS; #237 500; BD Biosciences, Mississauga, ON) agar for *B. theta* (Appendix Table A2.2)[303]. Cultures were incubated at 37 ^{o}C in a microaerobic atmosphere, and the number of colonies was counted at the dilution yielding 30 to 300 colony forming units (CFUs) after 48 hrs of incubation.

2.2.1 Bacterial Persistence

B. theta and *C. jejuni* persistence was tested by growing Caco-2 cells in tissue culture flasks with DMEM Complete+AB in a 5% CO_2 atmosphere, then changing to DMEM Complete-AB before inoculating with the bacteria. *C. jejuni* was cultured on Karmali agar. *B. theta* was cultured on

supplemented BHIS agar. Every 60 min for 6 hours arbitrarily-selected screw cap microcentrifuge tubes were removed, vortexed (high for 10 sec), diluted in an 8-fold dilution series, and 100 µL of each dilution was spread in duplicate onto Karmali agar or BHIS agar for *C. jejuni* and *B. theta,* respectively. Cultures were incubated at 37 ^{o}C in a microaerobic atmosphere, and the number of colonies was counted at the dilution yielding 30 to 300 CFUs after 48 hrs of incubation.

2.3 Experimental Design

The experiment was arranged as a two (5 mM NaB and PBS alone) x two (*B. theta* and PBS alone) x three (*C. jejuni* K12E5 "1", *C. jejuni* 81-176 "2", and PBS alone) factorial (Table 2.2); three replicates were conducted on separate occasions.

2.3.1 Cell Culture

The cell cultures were maintained at 37^{o}C at 5% CO_2 in a humidified incubator[304]. For large scale samples, 5 mM NaB supplemented DMEM Complete+AB was replenished on day 5, and 7 as previously performed[74]. Samples were also grown in the absence of NaB as a control treatment. On day 7, when the cells reached confluence of 6.5 x 10^5 cells mL^{-1} (determined using a Countess Automated Cell Counter; #C10227; Invitrogen, Carlsbad, CA), the medium was aspirated, and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, #D5527; Sigma-Aldrich, Oakville, ON). Cells were then supplemented with new DMEM Complete-AB, and allowed to equilibrate at 37 ^{o}C for 2 hours before inoculation with a multiplicity of infection (MOI) of 100 CFU/enterocyte for *C. jejuni* K12E5 "strain 1", *C. jejuni* 81-176 "strain 2", and *B. theta*. After incubating for 6 hours, the medium was aspirated, and Caco-2 cells were washed with DPBS. For histone analysis, cells were trypsinized using Trypsin-EDTA (#25200-056; Thermo-Fisher Scientific, Ottawa, ON), collected in 15 mL Falcon tubes, centrifuged at 25 ^{o}C, 1,000 rpm for 5

Table 2.2 Complete factorial experimental design for the epigenetic and gene expression experiments with the + sign indicating media supplementation with NaB or inoculation with 100 CFU/enterocyte of *C. jejuni* K12E5, *C. jejuni* 81-176, or *B. theta*.

Variables:	**NaB**	***C. jejuni***		***B. theta***
	NaB 5 mM	***C. jejuni* K12E5 "strain 1"**	***C. jejuni* 81-176 "strain 2"**	***B. theta***
Treatment 0				
Treatment 1		+		
Treatment 2			+	
Treatment 3				+
Treatment 4		+		+
Treatment 5			+	+
Treatment 6	+			
Treatment 7	+	+		
Treatment 8	+		+	
Treatment 9	+			+
Treatment 10	+	+		+
Treatment 11	+		+	+

min, washed with DPBS, and flash frozen before being stored at -80 ^{o}C. For the gene expression assays, cells were harvested with RNALater (#AM7021; Life Technologies, Carlsbad, CA, USA) and stored at -80 ^{o}C until processed. CDNA samples were also stored at -20 ^{o}C until processed.

2.4 Epigenetics

2.4.1 Histone Extraction and Purification

Histones were extracted and precipitated as previously described with minor modifications[245]. Cells in a hypotonic lysis solution (10 mM Tris-Cl, pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1x solution of complete protease inhibitor cocktail (#1697 498; Roche, Mississauga, ON) and dounced 15 times before being acid extracted with 3 mL dilute sulphuric acid (0.2 N H_2SO_4)[245]. Histones were then purified using cellulose dialysis tubing with a 14 kDa molecular cut-off (#D9777; Sigma-Aldrich, Oakville, ON) as previously performed[245]. Dialyzed cells were then lyophilized and resuspended in ddH_2O for analysis.

2.4.2 Histone Acetylation Profile Analysis

Histones were visualized using 15% sodium dodecyl sulfate (SDS)-PAGE and compared to a Precision Plus Protein (#161-0373; Bio-Rad, Hercules, CA) and Calf thymus histone standard (#H9250; Sigma-Aldrich, Oakville, ON). Normalized samples were transferred to 2 µm cut off polyvinylidene fluoride (PVDF) (Sequi-Blot^{TM} PVDF Membrane Roll, #162-0184; Bio-Rad, Hercules, CA) activated in methanol for 1 min at 25 V for 2 hours at room temperature using 25 mM Tris/ 190 mM glycine (#161-0734; Bio-Rad, Hercules, CA): 20% methanol (#322415; Sigma-Aldrich, Oakville, ON) transfer buffer. PVDF was stored in Tris buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 1% Tween 20 (TBST; #170-6531; Bio-Rad, Hercules, CA); 5% skim milk overnight at 4 ^{o}C. The PDVF was rinsed in TBST, and then probed with either 1:7,250 rabbit IgG anti-H3 (#ab137760; Abcam, Toronto, ON) or 1:500 rabbit IgG anti-H3K9ac (#ab61231)

respectively in blocking buffer for 2 hours at room temperature. After three 5 min washes with TBST, PVDF was incubated with 1:10,000 donkey anti-rabbit IgG-FITC (#ab6798, Abcam, Toronto, ON) antibodies for 1 hour at room temperature, washed three times for 5 mins with TBST, and then rinsed briefly in TBS. The membrane was visualized on a Typhoon imager at 488 nm for the fluorescent secondary antibodies.

2.5 Host Immune Gene Expression

2.5.1 Caco-2 cDNA synthesis

Samples in RNALater (#AM7021; Life Technologies, Carlsbad, CA, USA) were thawed overnight at 4°C. Cells were pelleted by centrifugation (14,000 rpm for 15 min), RNALater was removed, and mRNA was extracted from cells using the RNeasy Mini Extraction Kit (#74104; Qiagen; Toronto, Ontario). The mRNA was quantified and qualified using an Agilent RNA 6000 Nano Bioanalyzer. Complementary DNA (cDNA) was then prepared using the QuantiTect® Reverse Transcription kit (#205311; Qiagen, Toronto, Ontario) after standardizing mRNA concentrations to 1,000 ng.

2.5.2 Immune Marker RT-qPCR

Primers for host targets were selected and developed against host AMPs defensins β1 (hBD-1), β3 (hBD-3), and α1 (hAD-1); TLRs 2, 5, 9; cytokines IL-12β, TNF, TGF-β; C-C- motif ligand (CCL) chemokines CCL2 (MCP-1), CCL25 (TECK), CCL28 (MEC); and the C-X-C- motif ligand (CXCL) chemokines CXCL5 (ENA-78), CXCL8 (IL-8), and CXCL10 (IP-10). The majority of the primers were developed for the current study using Primer3[305] in Geneious[306]. Primers were designed to gene reference sequences downloaded from NCBI to construct the gene array using targets identified by Drs. Richard R.E. Uwiera (University of Alberta, Edmonton, Alberta) and G. Douglas Inglis (AAFC, Lethbridge, Alberta). Each amplicon was designed to be between 140 -160 base pairs (bps), and to have a Tm of 58°C. Where possible, primers were designed across flanking introns.

All primers were blasted in NCBI to ensure specificity to the gene of interest. Primer sets were evaluated using pooled cDNA from control and *C. jejuni* treated Caco-2 cells. Pooled cDNA was prepared in a 5-fold dilution series, 5 dilutions, plus a no template control (NTC). PCR was completed using QuantiTect SYBR Green (#204243; Qiagen; Toronto, Ontario), standard conditions. Primers with amplification efficiencies between 95-105% were considered for the study. A dissociation curve was also run to ensure that only a single product was produced. As a final check, a control sample of RNA was included with each qPCR run with samples, to ensure that genomic DNA was not being amplified.

The EpMotion 5020 (Eppendorf, Mississauga, ON) was used for creating master mixes (forward primer, reverse primer and SYBR green) as well as for dispensing cDNA into 384 well plates. Immune markers (Table 2.3) were quantified using real-time quantitative polymerase chain reaction (RT-qPCR), using triplicates for each of the three replicates. The cycling conditions were an initial denaturation for 15 min at 95^{o}C, 40 cycles of denaturation for 15 sec at 95^{o}C, and annealing/ extension for 30 sec at 58^{o}C. For all samples, melt curve analysis was conducted to confirm amplification specificity. PCR and fluorescence detection were carried out in the 7900 HT Fast Real-Time PCR System (Thermo-Fischer Scientific, Ottawa, Ontario).

Expression of five housekeeper genes including actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucuronidase β (GusB), peptidylprolyl isomerase B (PpiB), and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was quantified by qPCR (Table 2.3). Expression data of defensins, TLRs, cytokines, and chemokines was standardized to the housekeeper genes (n = 3) that met stability criteria (i.e. geNorm values between 0.5 to 1.0) using the Biogazelle qbase software (Biogazelle, Zulte, Belgium). The resultant adjusted values (i.e. to account for differential extraction efficiency) are used in the analyses (i.e. relative

Table 2.3 Oligonucleotide primers and PCR product sizes for immune markers. Genes successfully optimized for RT-qPCR reaction conditions have a 'Y' in the 'Run' column.

Immune Marker Gene name (name)	Oligonucleotide Sequences		Source	Reference Sequence	Gene ID	Run
	5' primer	3' primer				
Defensins						
DEFβ1 (hBD-1)	CTGCTGTTTACTCTCTGCTTACTTTT	CCTCCACTGCTGACGCA	[137]	NM_005218.3	1672	Y
DEFβ103A (hBD-3)	TTATTGCAGAGTCAGAGGCGG	CCACACTTTACAACACTCTCGT	This Study	NM_018661.3	414325	Y
DEFβ4a (hBD-2)	AGACTCAGCTCCTGGTGAAGC	GCTCCACTCTTAAGGCAGGTAA	[307]			
DEFα1 (hAD-1)	TCCTTGCTGCCATTCTCCTG	GCTTTGGAGCCAAGCTTTCG	This Study	NM_001042500	1667	Y
DEFα2 (hAD-2)	TCCTTGCTGCCATTCTCCTG	GCTTTGGAGCCAAGCTTTCG	This Study	NM_001042500		
DEFα3 (hAD-3)	AGAGCTGATGAGGTTGCTGC	TGCAATGCACGCTGGTATTC	This Study	NM_005217.3		
DEFα4 (hAD-4)	GCAAGAGGTGATGAGGCTCC	CTGTTCGCCGGCAGAATACTA	This Study	NM_001925.1		
DEFα5 (hAD-5)	GCTGATGAGGCTACAACCCA	ACTCACGGGTAGCACAACG	This Study	NM_021010.1		
DEFα6 (hAD-6)	GGGGCAAATGACCAGGACTT	TGACAGTGCAGGTCCCATAG	This Study	NM_001926.3		
Toll-like Receptors						
TLR2	AGCACTGGACAATGCCACAT	GCCCTGAGGGAATGGAGTTT	This Study	NM_003264.3	7097	Y
TLR4	TCCTGCGTGAGACCAGAAAG	TCCGTGATAAAACGGCAGCA	This Study	NM_003266.3		
TLR5	GCTTCCTCTTGCTGTTTCT	AGTTCTGGGCTAAAGGGTGA	This Study	NM_003268.5	7100	Y
TLR9	CAAGGTGTACCCGCTACTGG	TCCCCTCTCAGACAGCCTAC	This Study	NM_017442.	54106	Y

				3		
Cytokines						
TGF-α	CCGCTGAGTGACCCGCC	ACAGCGTGCACCAACGTACC	This Study	NM_001099691.2		
TGFβ1 (TGF-β)	GCTGCTGTGGCTACTGGTGC	CATAGATTTCGTTGTGGGTTTC	[136]	NM_000660.5	7040	Y
IL-2	TGCATTGCACTAAGTCTTGCAC	GCATCCTGGTGAGTTTGGGA	This Study	NM_000586.3		
IL-4	ACATTGTCACTGCAAATCGACACC	TGTCTGTTACGGTCAACTCGGTGC	This Study	NM 000589.3		
IL-5	CTCTTGGAGCTGCCTACGTG	TTTCCACAGTACCCCCTTGC	This Study	NM_000879.2		
IL-6	CTGACCCAACCACAAATGCC	ATCTGAGGTGCCCATGCTAC	This Study	NM_000600.3		
IL-7	CTCGCAAGTTGAGGCAATTTCT	CTTTGTTGGTTGGGCTTCACC	This Study	NM_000880.3		
IL-10	AGGCAACCTGCCTAACATGC	GGCAACCCAGGTAACCCTTA	This Study	NM_000572.2		
IL-12β	TCCTCCCTTGAAGAACCGGA	TGACAACGGTTTGGAGGGAC	This Study	NM_002187.2	3593	Y
IL-15	CATTTTGGGCTGTTTCAGTGC	GGGGTGAACATCACTTTCCG	This Study	NM_000585.4		
IL-17						
CSF2 (GMCSF)	CCATGATGGCCAGCCACTAC	CTGGCTCCCAGCAGTCAAAG	This Study	NM_000758.3		
TNF	TCTCGAACCCCGAGTGACAA	TATCTCTCAGCTCCACGCCA	[308]	NM_000594.3	7124	Y
CCL motif chemokines						
CCL2 (MCP-1)	GAACCGAGAGGCTGAGACTA	GGGGCATTGATTGCATCTGG	This Study	NM_002982.3	6374	Y
CCL5 (RANTES)	GGTACCATGAAGGTCTCCGC	TACTCCTTGATGTGGGCACG	This Study	NM_002985.2		

CCL25 (TECK)	CTCCCCAAGAGACACAGGAAG	TACAGCATGAGGGCCTGCTT	This Study	NM_001201359.1	6370	Y
CCL28 (MEC)	TGTGTCAGCCCGCACAACCA	TCGTGTTTCCCCTGATGTGCCCT	This Study	NM_001301875.1	56477	Y
CXCL motif chemokines						
CXCL5 (ENA-78)	CTCTTGACCACTATGAGCCTCC	CGCAACGCAGCTCTCTCAA	This Study	NM_002994.3	6374	Y
CXCL8 (IL-8)	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC	Primer Bank Database	NM_000584	3576	Y
CXCL10 (IP-10)	CCTTTCCCATCTTCCAAGGGT	GGAGGATGGCAGTGGAAGTC	This Study	NM_001565.3	3627	Y
Housekeepers						
ACTB	GCAGGAGTATGACGAGTCCG	TCACCTTCACCGTTCCAGTT	This Study	NM_001101.3	60	Y
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	*138*	NM_001256799.2	2597	Y
GusB	ATCAACAACACACTCACCCC	TGGGATACTTGGAGGTGTCA	This Study	NR_120531.1	2990	Y
PpiB	CTGTCCGGGCTGCTTTC	GGTCAAAATACACCTTGACGG	This Study	AY962310.1	5479	Y
HPRT1	TCAGGCGAACCTCTCGGCTT	TCACTAATCACGACGCCAGGGCT	This Study	M26434.1	3251	

Note 1: Primer bank database: http://pga.mgh.harvard.edu/cgi-bin/primerbank. Dr. Richard R.E. Uwiera (University of Alberta, Edmonton, Alberta) identified these gene targets. Members of Dr. G. Douglas Inglis's laboratory (Jenny Gusse, Dr. Lisa D. Kalischuk, Kirsty Brown, and Dr. 'TD' Thangadurai Ramu; AAFC, Lethbridge, Alberta) developed the novel primers for the array.

Note 2: Genes not underlined in Table 2.3 are targets for which primers were designed and attempts to optimize amplification were performed by lab technicians Jenny Gusse, Kirsty Brown, and post-doctoral fellow Dr. Thangadurai "TD" Ramu with the primer sets failing to meet the RT-qPCR reaction parameters. Underlined genes represent the targets that were successfully amplified under the reaction parameters, and were investigated further.

expression to other treatments). Normalized data was expressed as relative expression[309]. The mean of the three observations per replicate was calculated and used in analyses.

2.5.3 Gene Expression Statistical Analysis

Data are expressed below as means ± standard error measurements (SEM) for three or more independent biological replicates unless indicated otherwise.

2.5.4 Statistical Analysis

For each gene target, the relative gene expression data were $\log_{10}$ transformed to normalize variance, and the transformed data were analyzed using the mixed procedure of SAS (SAS® software; SAS Institute Inc., Cary, NC, USA). The experiment was analyzed for main effects and interaction effects for the three experimental variables (i.e. C. j, B. t, and NaB). A P value of ≤0.058 was defined as statistically significant, and a P value of >0.058 and ≤0.100 was considered to be biologically significant (see Table 3.1). Where appropriate, individual treatments within or across factors were compared using a protected least squares (LS) means test; an α value of 0.058 was applied. Values for individual treatments were averaged across factors only where appropriate (i.e. in the absence of a significant interaction). For example, when a significant interaction was observed only for the C. j and NaB factors, values were averaged over the B. t factor for graphical presentation (e.g. Figure 3.4).

Chapter 3 Results

3.1 Caco-2 Cell Culture

Due to the wide range of NaB concentrations previously reported in host gene expression experiments, a variety of NaB concentrations (0-100 mM) were tested to verify the optimal NaB concentration for Caco-2 cell culturing. Gas chromatography revealed that NaB concentrations remained fairly constant over time in the 0 and 1 mM NaB cultures (Figure 3.1A). At 5 mM NaB, cultures display significant decreases in NaB concentrations (Figure 3.1A) and increased growth rates were observed, suggesting uptake and utilization during growth. At 10 mM NaB, Caco-2 cell growth was observed to decrease significantly after 2 days, and corresponded with similar levels of NaB concentration (Figure 3.1B). There were deleterious effects on cell density at concentrations over 10 mM. At 30 mM NaB cells no longer adhered to the tissue culture flask. At 100 mM cells were not viable resulting in a lack of uptake shown by non-fluctuating NaB concentrations (Figure 3.1B). Based upon these observations, I decided to use 5 mM NaB for future Caco-2 cultures. 5 mM is below the threshold for NaB induced apoptosis in colonic cancer cells, and is consistent with previous studies investigating the epigenetic activity of NaB[310].

Following the optimization of Caco-2 NaB cell culture conditions, the viability of *B. theta* and *C. jejuni* in Caco-2 culture media in the presence and absence of NaB, and under Caco-2 atmospheric conditions (37 ^{o}C in a 5% CO_2 microaerobic atmosphere) over time was determined using serial dilutions of extracted cells at 0, 2, 4, and 6 hours post inoculation and plated on selective media (Figure 3.2; n = 2). Both *B. theta* and *C. jejuni* were determined to have between 30 and 300 CFU mL^{-1} at a dilution factor of 1 x 10^{-6}, which is consistent with previously determined values for *C. jejuni*[101].

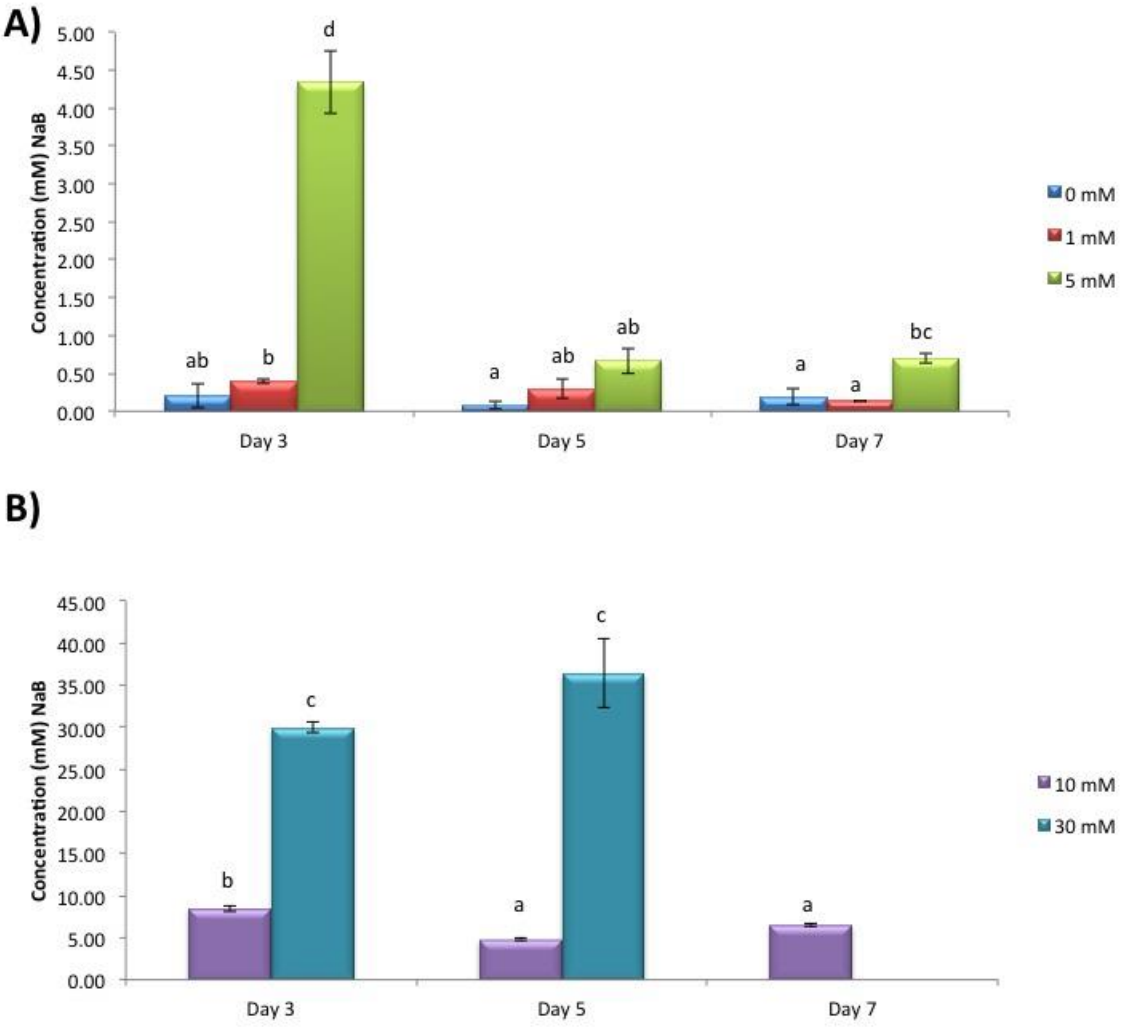

Figure 3.1 Caco-2 cell line NaB supplementation optimization. NaB concentrations in Caco-2 culture media were quantified on day 3, 5 and 7 by gas chromatography. The average NaB concentrations in panel A show the 0, 1, and 5 mM NaB samples, and in panel B show the 10 and 30 mM samples. The cells in the 30 mM treatment were no longer attached to the tissue culture flasks after day 5 and were not included in this analysis. Vertical lines associated with histogram bars are standard deviation. Histogram bars not accompanied by the same letter differ ($\alpha = 0.058$).

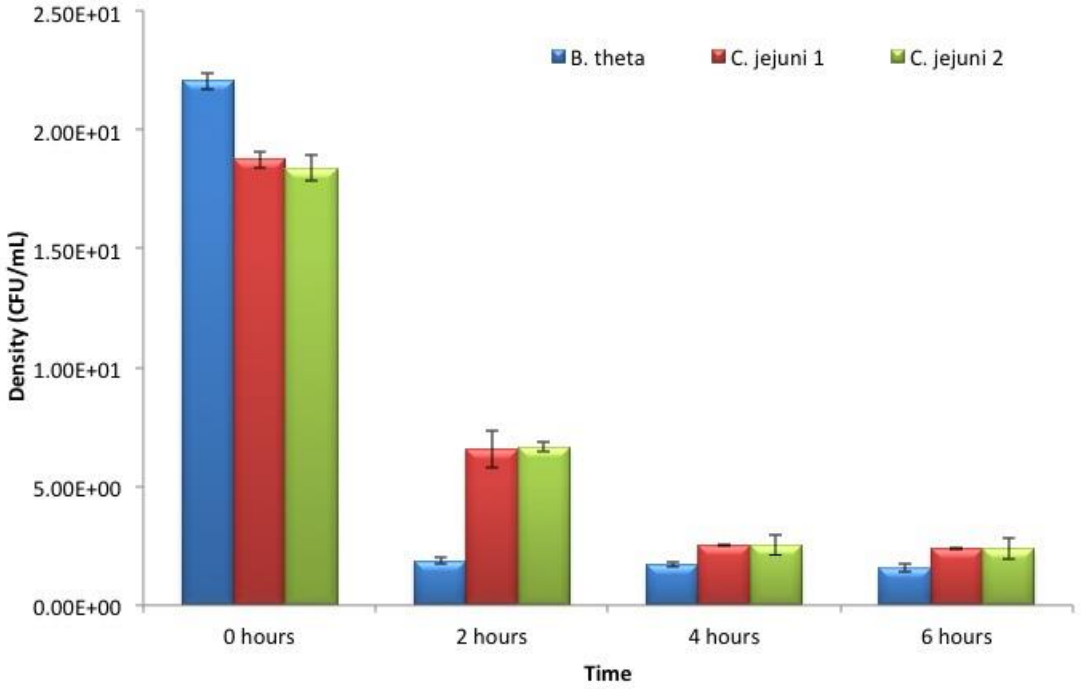

Figure 3.2 Bacterial survival histogram for anaerobic enteric microbes grown in cell culture conditions. Average (n = 2) *B. theta* and *C. jejuni* (K12E5 "1" and 81-176 "2") density (CFU mL^{-1}) over a 6-hour time course following inoculation into confluent Caco-2 cells growth in DMEM Complete-AB without NaB, in tissue culture flasks. Vertical lines associated with histogram bars are standard deviation.

3.3 Histone Acetylation Profiles

Histones were extracted from Caco-2 cells grown under conditions described in the complete factorial (Table 2.2). The histone concentrations were diluted to consistent levels (Figure 3.3A). H3 was probed using anti-H3 antibodies to verify that the histone levels were consistent (Figure 3.3B). The histones were also probed with anti-acetylated H3K9 antibodies (Figure 3.3B) to determine if there were changes to global histone H3K9 acetylation levels. Pair-wise comparisons of acetylation levels in the absence (treatments 0-5) and presence (treatments 6-11) of NaB indicate that NaB appears to increase acetylation levels under most conditions. *B. theta*, in treatment 9, reduces the acetylation due to NaB alone (treatment 6). *B. theta* also reduces acetylation due to NaB in the presence of *C. jejuni* strain 1 (treatment 10). Notably, *B. theta* has the opposite effect in the presence of NaB and *C. jejuni* strain 2 (treatment 11) as this condition produces the highest observed H3K9ac levels.

3.4 Host Immune Responses

To further understand potential downstream effects of altered histone tail acetylation in Caco-2 cells, RT-qPCR was performed using cDNA generated from extracted RNA to quantify the expression of selected genes in host immune responses. Primers were designed for defensins, TLRs, cytokines, and chemokines RT-qPCR (Table 2.3). Evidence for induction of these genes provide insight into the Caco-2 cells' ability to detect, respond and defend 6 hours post inoculation with *B. theta* and *C. jejuni,* in NaB supplemented and lacking media. Changes from the standardized level of expression with a P value < 0.058 were considered statistically significant, while results with a P value < 0.1 were considered biologically significant. Main effects were analyzed using a protected means separation test (P < 0.058). P-values associated

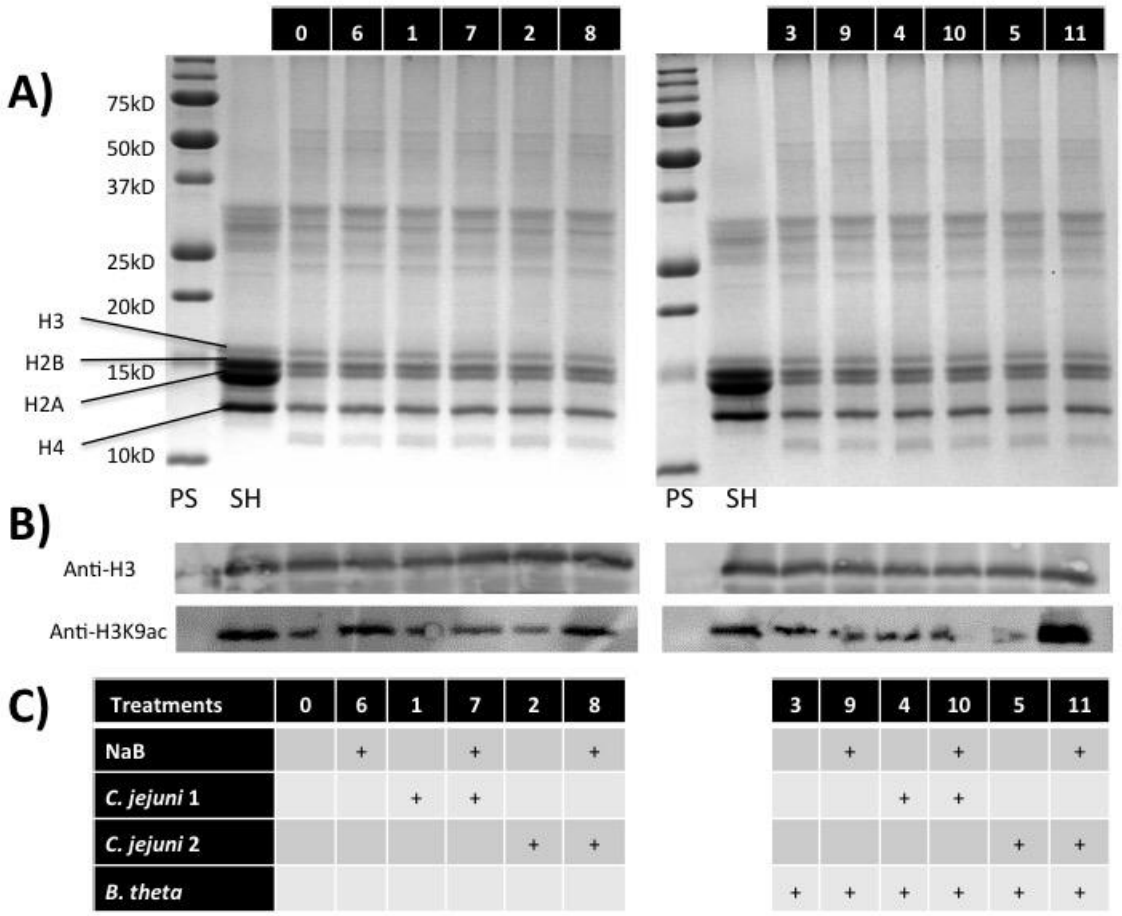

Figure 3.3 Analysis of Caco-2 histone H3K9 acetylation levels. (A) 15% SDS-gels of acid extracted histones from Caco-2 cells treated with NaB and *C. jejuni* strains 1 and 2 without (left) and with *B. theta* (right) (Table 2.2). Histone levels were diluted to consistent concentrations using histone H3 levels as a marker. Electrophoresed histones are compared to a molecular weight protein standard (PS) and histone standard (SH). Core histone mobility of H3, H2B, H2A, and H4 is labeled on the left. **(B)** Western blot of core histones in (A) that have been probed with anti-H3 antibodies (top) and anti-H3K9ac antibodies (bottom). **(C)** Tables indicate the sample conditions from the complete factorial. "+" indicates presence of the condition.

with the variance in defensin, TLR, cytokine, and chemokine expression are shown in Table 3.1.

3.4.1 Defensins

Defensins are involved in the prevention of microbial attachment to the intestinal epithelium. They can be constitutively expressed or inducible. Three defensin genes were investigated as the Caco-2 cells were challenged with NaB, *C. jejuni* and *B. theta* in the factorial design. HAD-1 was the only defensin expressed in the small intestine tested, and expression levels were unchanged in all treatments. NaB strongly affects transcript levels of the constitutively expressed hBD-1. NaB, *C. jejuni* and *B. theta* all interact to affect the inducible hBD-3.

3.4.1.1 hBD-1

The complete factorial design (Table 2.2) revealed an interaction between *C. jejuni* and NaB (Table 3.1). In this work an 'interaction' is defined as when two or three variables (e.g. *C. jejuni*, *B. theta* or NaB) affect transcription levels differently together than any of the individual variables independently. For example, *C. jejuni* did not have a main effect increasing or decreasing hBD-1 expression, but the two strains did induce hBD-1 differently, in the absence of NaB. However, NaB interacts with *C. jejuni* to induce similar hBD-1 expression profiles (Figure 3.4B), and both *C. jejuni* strains induced a similar level of expression to the PBS + NaB treatment (treatment 6) when NaB was included. The presence of *C. jejuni* did not significantly increase or decrease the NaB effect. NaB had a stronger effect than *C. jejuni*, while *B. theta* had no effect on hBD-1 expression.

3.4.1.2 hBD-3

Unlike hBD-1, the inducible hBD-3 has altered expression levels in response to interactions between all three variables (Table 3.1); however, individually the three variables affect hBD-3 to

Table 3.1 P-values for main effects and interaction effects for defensins, TLRs, cytokines, and chemokines. A P value of ≤ 0.058 was defined as statistically significant, and a P value > 0.058 and ≤ 0.100 was considered to be biologically significant. Main variable effects for NaB, *B. theta* and *C. jejuni* (collectively as a species instead of as the two *C. jejuni* strains) and interaction effects where the target gene was upregulated are indicated in red (dark red = statistically significant; light red = biologically significant), and downregulated are indicated in green (dark green = statistically significant; light green = biologically significant).

		Main and Interaction Effects (P Values)						
	Marker	**C. j**	**B. t**	**C. j/B. t**	**NaB**	**C. j/NaB**	**B. t/NaB**	**C. j/B. t /NaB**
	Defensins							
Figure 3.4	**hBD-1**	0.745	0.962	0.479	< 0.001	0.058	0.427	0.140
Figure 3.5	**hBD-3**	0.088	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002
	TLRs							
Figure 3.6	**TLR2**	0.380	0.523	0.872	0.064	0.401	0.690	0.831
Figure 3.7	**TLR5**	0.466	0.272	0.536	< 0.001	0.048	0.410	0.093
Figure 3.8	**TLR9**	0.808	0.056	0.407	< 0.001	0.330	0.139	0.293
	Cytokines/Chemokines							
Figure 3.9	**TNF**	0.105	< 0.001	0.286	< 0.001	0.468	0.440	0.271
Figure 3.10	**ENA-78**	0.275	< 0.001	0.097	0.012	0.113	0.870	0.556
Figure 3.11	**TECK**	< 0.001	0.060	0.833	< 0.001	0.055	0.019	0.773
Figure 3.12	**IL-8**	< 0.001	< 0.001	0.590	0.047	< 0.001	0.007	0.137
Figure 3.13	**MCP-1**	0.029	0.002	0.414	0.019	0.280	0.111	0.507
Figure 3.14	**IP-10**	< 0.001	< 0.001	< 0.001	0.238	0.051	0.001	0.014
Figure 3.15	**TGF-β**	0.687	0.583	0.817	0.025	0.267	0.936	0.563
Figure 3.16	**IL-12β**	0.677	0.176	0.176	0.839	0.529	0.135	0.237

Note 1: As the *C. jejuni* factor possesses three levels in the 2 x 2 x 3 factorial (PBS, strain 1, or strain 2), it was necessary to apply a mean separation test to statistically compare the three treatments for this factor (i.e. in the event that a significant main effect and/or interaction effect are present)
Note 2: Means and SEM for individual treatments (e.g. averaged over factors) are presented for each gene within subsequent figures only where warranted (e.g. a significant main effect but a non-significant interaction effect was observed).

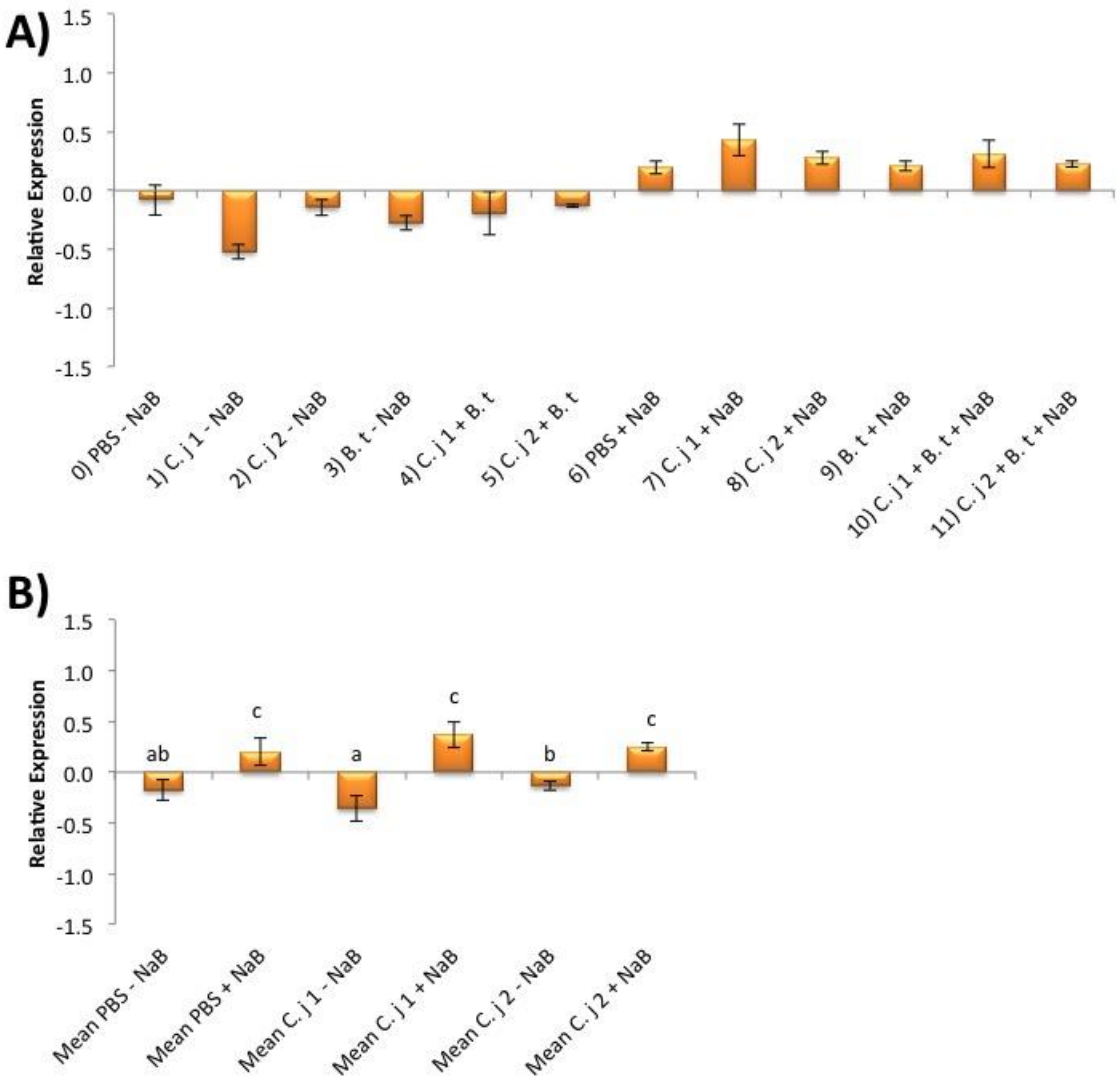

Figure 3.4 Relative expression levels of hBD-1. A) Caco-2 cell transcription of hBD-1 mRNA relative to the baseline exposed to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB and both *C. jejun*i strains interact to influence hBD-1 expression (Table 3.1), but *C. jejun*i as a species does not independently affect hBD-1 expression. Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

a lesser degree. There are two significant induction changes in the absence of NaB. *B. theta* reduces hBD-3 expression (treatment 3), and *B. theta* co-inoculated with *C. jejuni* strain 1 (treatment 4) decreased hBD-3 expression. Neither *C. jejuni* strain (treatment 1 and 2) had an effect on its own, and likewise, NaB (treatment 6) did not affect expression by itself (Figure 3.5). Treatments 8, 10 and 11 exemplify the interaction between the three variables (Figure 3.5). NaB has the strongest influence on induction, with altered expression in all NaB treatments except with *C. jejuni* strain 1 (treatment 7) and *B. theta* (Figure 3.5; treatment 9). HBD-3 responds differently to the two *C. jejuni* strains; however, the presence of both *B. theta* and NaB enables the Caco-2 cells to induce hBD-3 equally in response to both *C. jejuni* strains in a three-way interaction.

3.4.2 TLRs

TLRs are involved in detecting PAMPs in the enteric epithelium. Detection initiates signalling cascades and the activation of transcription factors, such as AP-1 and NF-κB. TLR signalling results in a wide variety of cellular responses, for example the production of inflammatory cytokines that direct the adaptive immune response. They require careful regulation of their expression because a lack of TLRs would fail to trigger host responses, whereas an excess of TLR signalling leads to an excess of inflammation[311]. Three Caco-2 TLRs were investigated in the factorial design. Expression levels of TLR2, responsible for detecting mostly Gram-positive bacteria, were unchanged except in the presence of NaB. The bacterial flagellin detecting TLR5 responded similarly to the constitutively expressed hBD-1. TLR9 (detects unmethylated CpG islands) is upregulated in the presence of NaB or *B. theta* alone independently of one another.

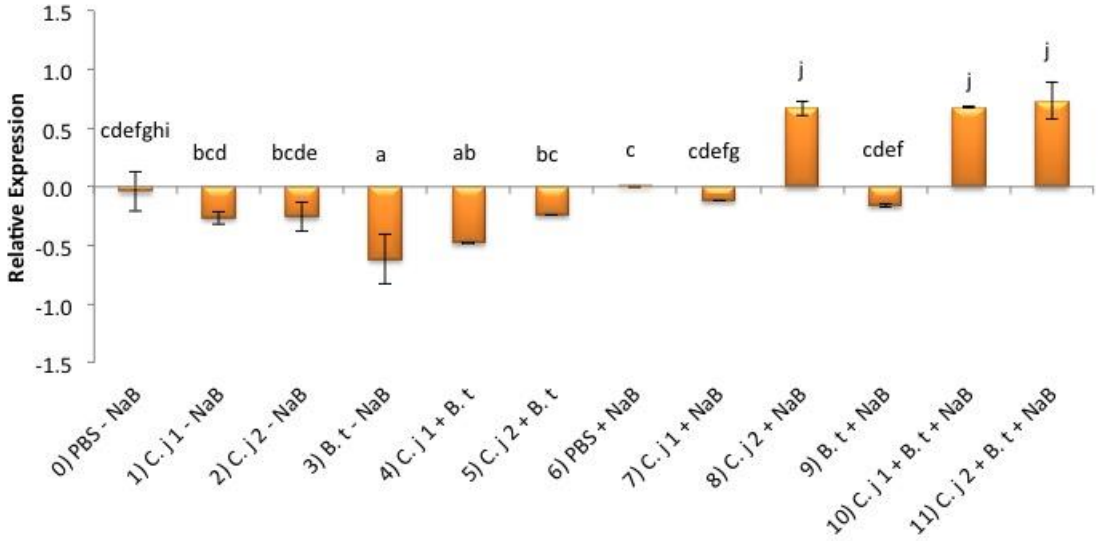

Figure 3.5 Relative expression levels of hBD-3. Caco-2 cell expression of hBD-3 mRNA relative to the baseline in response to factorial treatments (Table 2.2) showing the three-way interaction between *B. theta*, *C. jejuni* and NaB (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

3.4.2.1 TLR2

There were no differences with an α value of 0.058 for any of the 12 treatments (Figure 3.6A) for TLR2. The complete factorial design (Table 2.2) revealed a trend for NaB (Figure 3.6) on TLR2 transcription, although it was weaker than with the other TLRs. TLR2 expression was decreased when NaB was present. This is the only investigated gene for which NaB had a negative effect.

3.4.2.2 TLR5

Similar to hBD-1, *C. jejuni* and NaB interact to influence TLR5 expression (Table 3.1). NaB exerts a larger effect than *C. jejuni*. In the PBS treatments without bacterial stimuli (treatments 0 and 6), NaB significantly increases TLR5 expression (Figure 3.7B). This trend is repeated in the presence of both *C. jejuni* strain 1 and strain 2 (Figure 3.7B). In the absence of NaB, *C. jejuni* strains do not induce TLR5 differently. This is in contrast to hBD-1, where in the absence of NaB, the two strains did induce different expression levels: however, the presence of NaB has a stronger effect than the *C. jejuni* strain effect (Figure 3.7B).

TLR5 and hBD-1 expression patterns could be similar due to the feedback mechanism between β-defensins and TLRs. β-defensins can induce TLR signalling and recruitment/activation of leukocytes[312]. TLR5 detects Gram-negative bacterial flagellin, and hBD-1 has documented activity against fungi and anaerobic, Gram-positive commensal *Bifidobacterium* and *Lactobacillus* species[314]. With both TLR5[182] and hBD-1[313] reported to play key roles in maintaining the DGM population, similar PAMPs could influence their expression.

3.4.2.3 TLR9

Statistical analysis of TLR9 transcript expression demonstrates NaB and *B. theta* have main variable effects (Table 3.1). TLR9 displays a general trend for increased expression levels in the

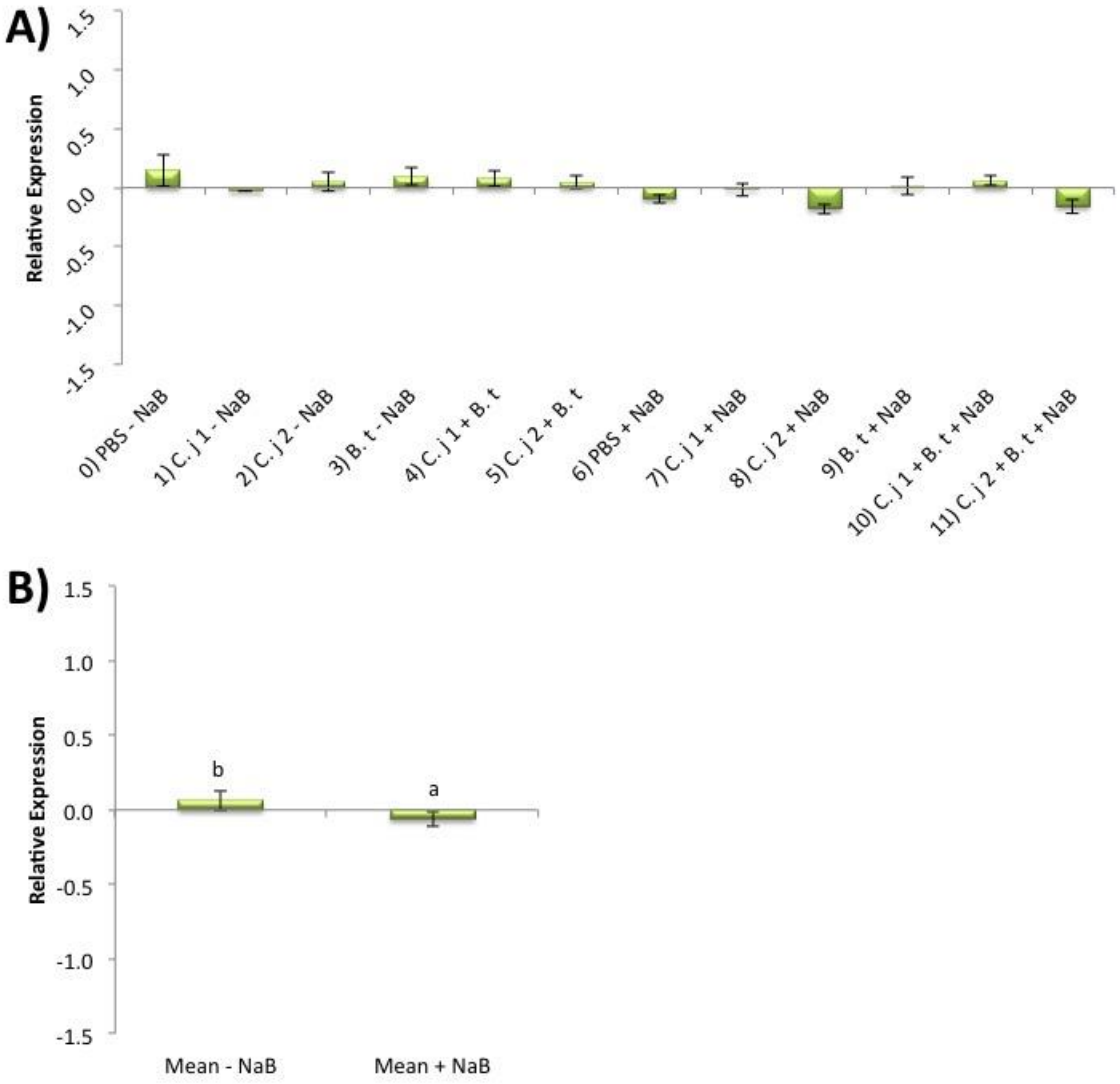

Figure 3.6 Relative expression levels of TLR2. A) Caco-2 cell transcription of TLR2 mRNA relative to the baseline exposed to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB induced TLR2 expression averaged across all conditions (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.1$).

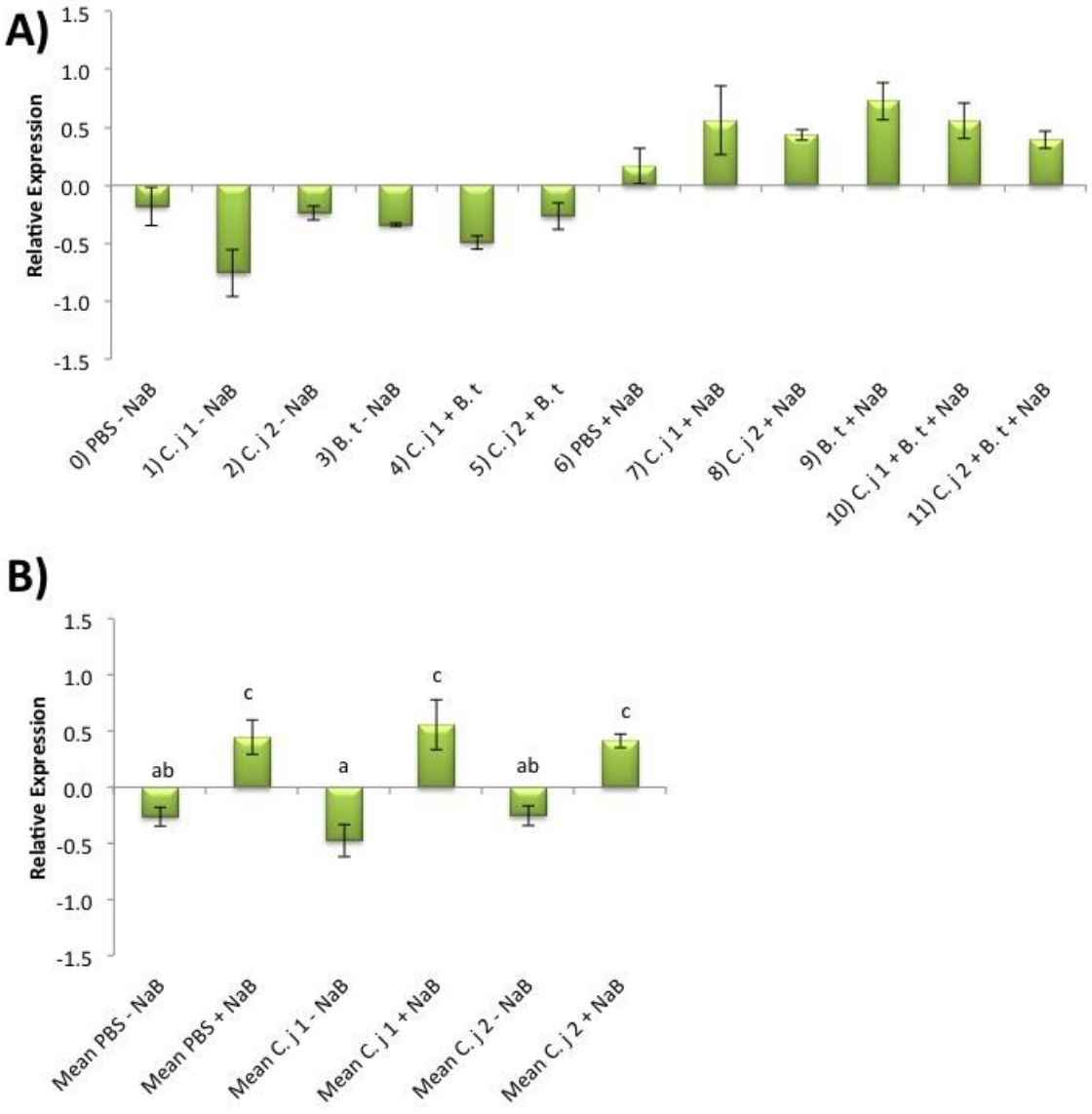

Figure 3.7 Relative expression levels of TLR5. A) Caco-2 cell transcription of TLR5 mRNA relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB interacts with both strains of *C. jejuni* (Table 3.1), on average across *B. theta* treatments. Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

presence of NaB (Mean + NaB; treatments 6-11) when compared to the mean of the treatments in the absence of NaB (Mean – NaB; treatments 0-5) in Figure 3.8B. *B. theta* also has a treatment effect on TLR9 (Figure 3.8C) and displays increased expression levels in the presence of *B. theta* (Mean + B. t; treatments 3, 4, 5, 9, 10, and 11) when compared to expression in the absence of *B. theta* (Mean – B. t; treatments 0, 1, 2, 6, 7, and 8). Together, these results indicate that TLR9 expression is sensitive to both NaB and *B. theta*, but not *C. jejuni*.

3.4.3 Cytokines and Chemokines

Cytokines and chemokines are involved in the communication and coordination of immune responses. The presence, absence, and quantity of the various cytokines/ chemokines, in an area, determine the inflammation profile at that site. Expression of these genes that regulate inflammation can be constitutive or inducible depending on the presence of triggers. Given the potential for damage to the host, the expression of many cytokines/ chemokines is limited, and requires multiple signals to become upregulated. Two genes that regulate inflammation, TNF and ENA-78 were both upregulated by NaB and *B. theta*. The homeostatic TECK and inflammatory IL-8 both were more responsive to *C. jejuni* and *B. theta* in the presence of NaB. Inflammatory cytokine, MCP-1 was increased in the presence of *C. jejuni*, *B. theta* and NaB, whereas another inflammatory cytokine, IP-10 was primarily upregulated in the presence of bacteria with NaB implicated in increasing the response to *B. theta* and *C. jejuni* strain 2. The regulatory TGF-β is upregulated by NaB. IL-12β expression, the main inducer of a Th1 response, is not affected by any of the main treatments.

3.4.3.1 TNF and ENA-78

Both NaB and *B. theta* exhibit main treatment effects on TNF and ENA-78 relative expression

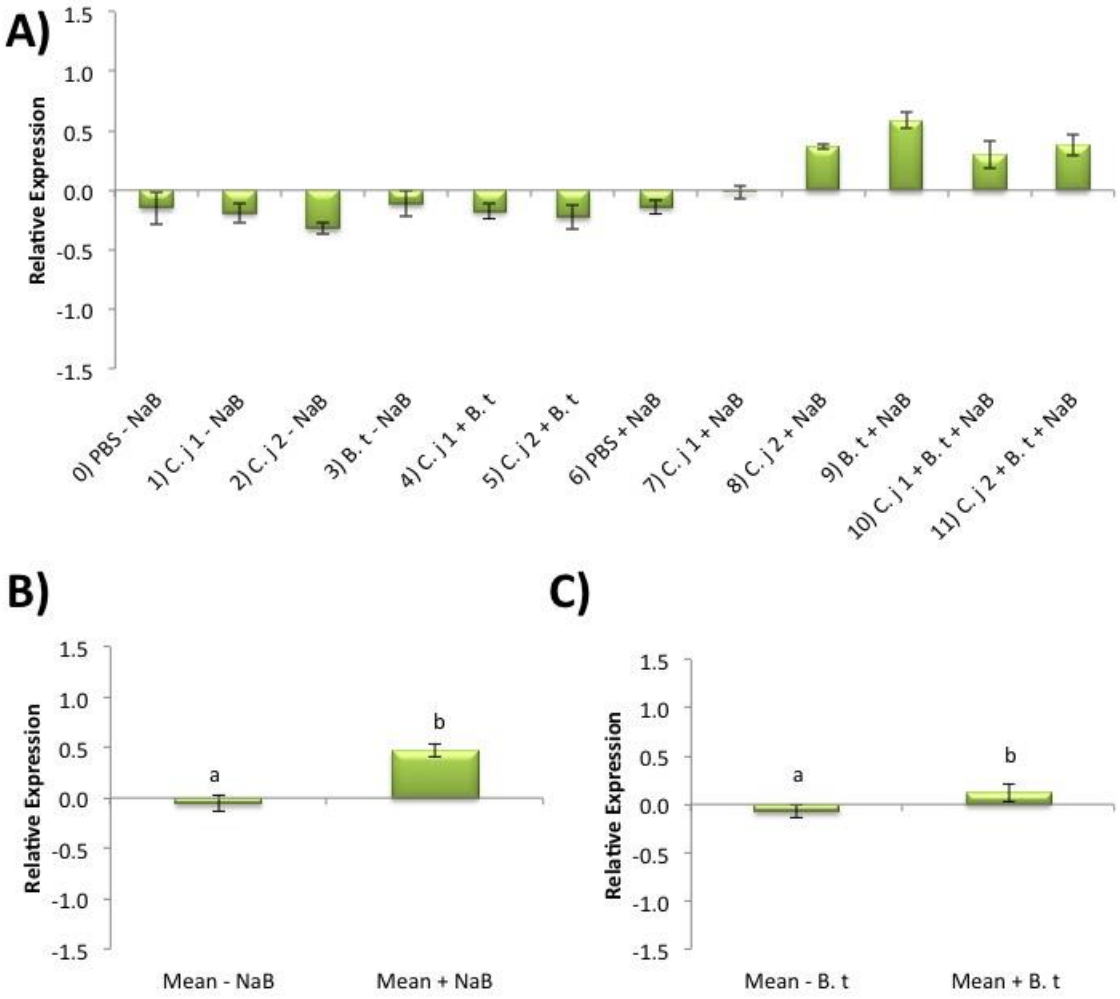

Figure 3.8 Relative expression levels of TLR9. A) Caco-2 cell TLR9 mRNA transcription relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB treatment effect averaged across *B. theta* and *C. jejuni*. **C)** *B. theta* treatment effect averaged across NaB and *C. jejuni* treatments. Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

(Table 3.1) similar to TLR9. Both NaB and *B. theta* had stronger treatment effects for TNF than for TLR9, while only *B. theta* strongly affected ENA-78. In the absence of NaB (Mean – NaB; treatments 0-5), TNF expression is increased in the presence of *B. theta* (Figure 3.9C), and was the only cytokine to display a treatment effect in the presence of *B. theta* (Mean + B. t; treatments 3, 4, 5, 9, 10, and 11).

Similar to TNF, NaB and *B. theta* exhibited main effects on relative ENA-78 expression. *B. theta* influenced all three chemokines, in contrast to only influencing TNF of the investigated cytokines. In the absence of NaB (Mean – NaB; treatments 0-5), ENA-78 relative expression was less than in the presence of NaB (treatments 6-11; Figure 3.10B). ENA-78 and MCP-1 are the only chemokines to display a treatment effect for *B. theta* independently of NaB, which resulted in increased expression (Figure 3.10C) when *B. theta* was present (Mean + B. t; treatments 3, 4, 5, 9, 10, and 11). These results show that there are main effects by both NaB and *B. theta* (Table 3.1).

3.4.3.2 TECK and IL-8

The complete factorial design (Table 2.2) of this experiment revealed that interactions between NaB and both *C. jejuni* and *B. theta* (Table 3.1) alter both TECK and IL-8. Both *C. jejuni* strains increase TECK transcription, in the presence of NaB, with strain 2 inducing more of a change averaged across treatments 8 and 11 (Figure 3.11B), whereas in the presence of NaB, strain 1 (treatments 7 and 10) increased TECK relative expression to the same level as NaB did averaged across the *C. jejuni* lacking treatments (treatments 6 and 9). These results reveal an interaction between NaB and *C. jejuni* to induce transcription of TECK. Similarly, NaB induces expression in the treatments with *B. theta* (Mean B. t + NaB; treatments 9-11) in Figure 3.11C, revealing an

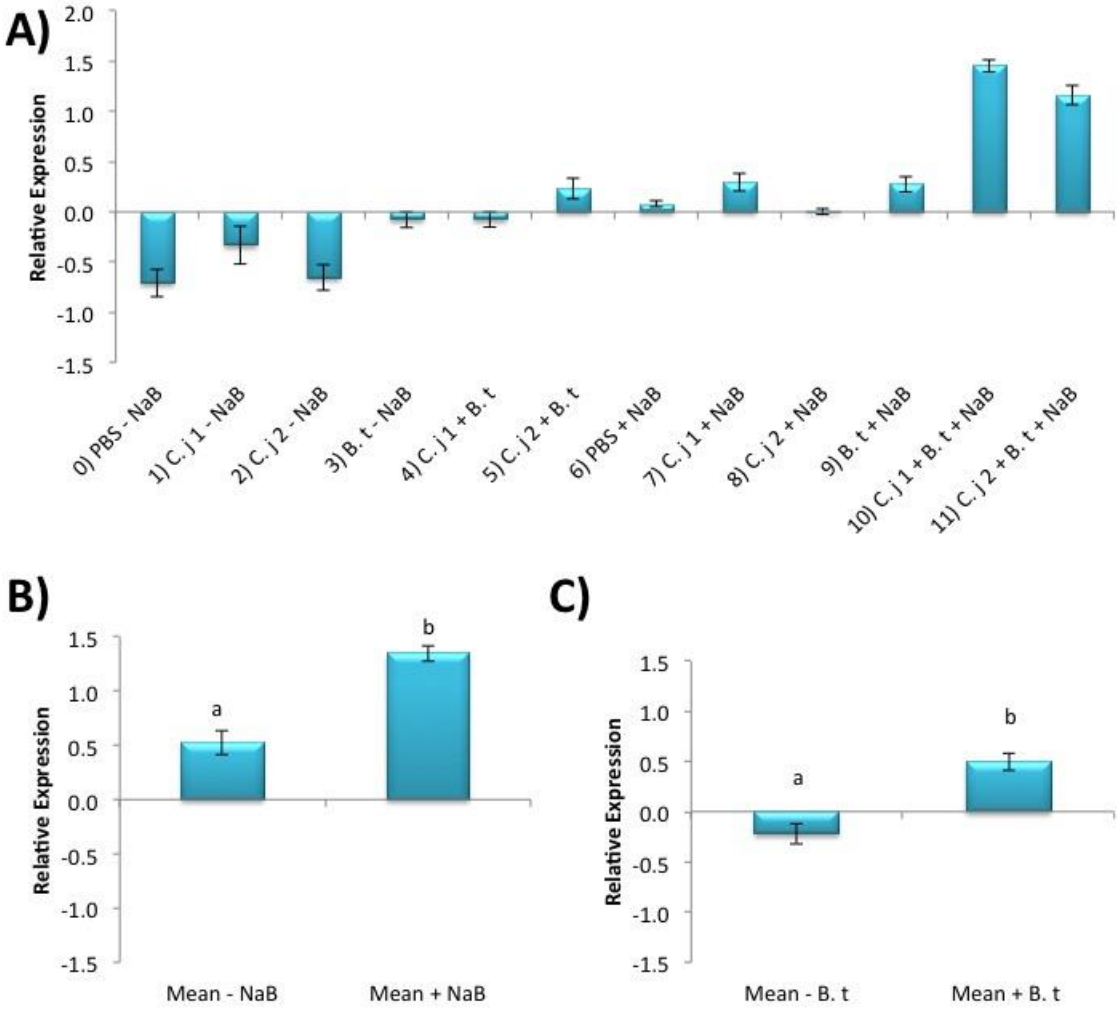

Figure 3.9 Relative expression levels of TNF. A) Caco-2 cell expression of TNF mRNA relative to baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB treatment effect (Table 3.1) averaged across the *B. theta* and *C. jejuni* treatments influencing Caco-2 TNF expression. **C)** Averaged treatments with and without *B. theta* showing the *B. theta* treatment effect (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

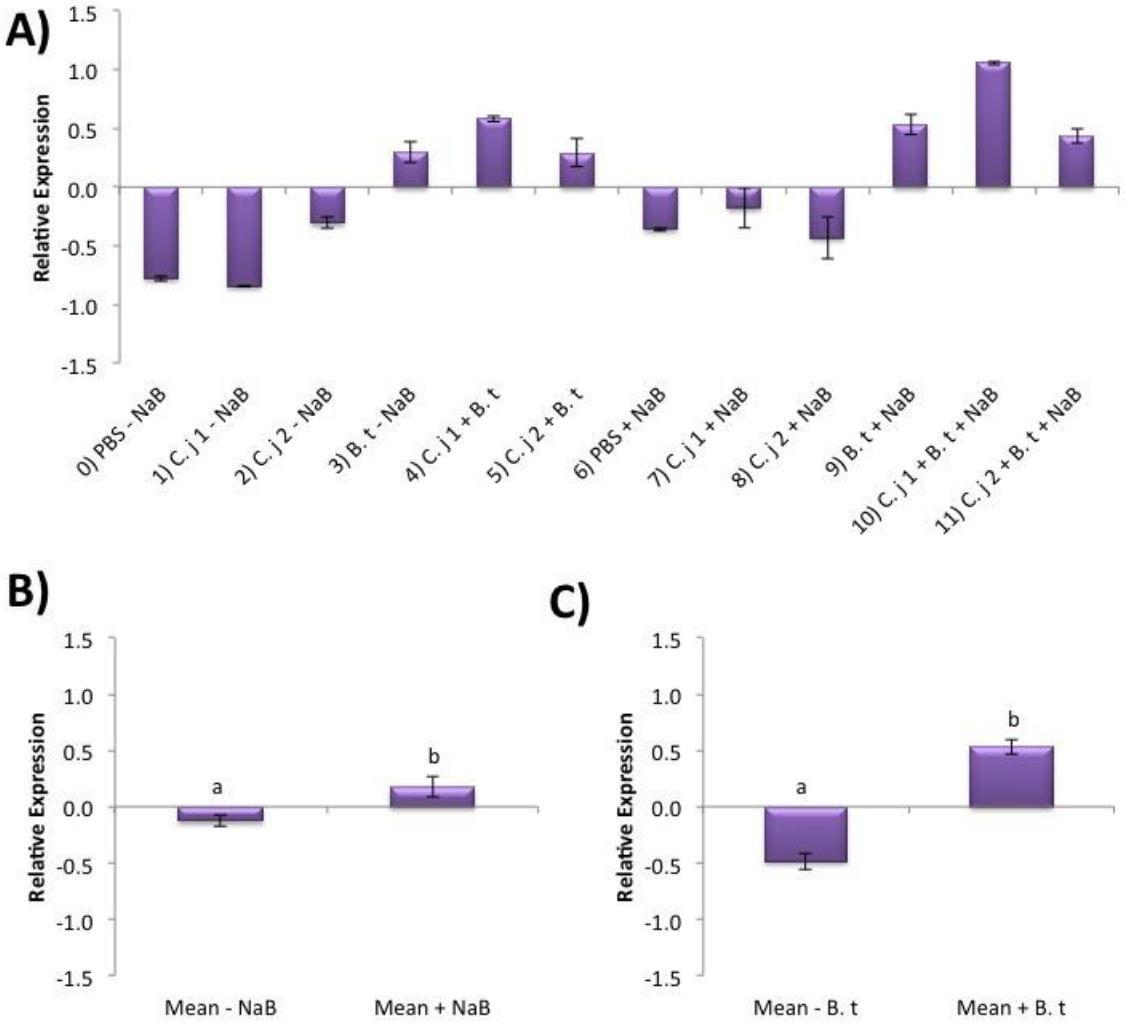

Figure 3.10 Relative expression levels of ENA-78. A) Caco-2 cell transcription of ENA-78 relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** NaB treatment effect averaged across *C. jejuni* and *B. theta* treatments (Table 3.1). **C)** *B. theta* treatment effect averaged across NaB and *C. jejuni* treatments (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

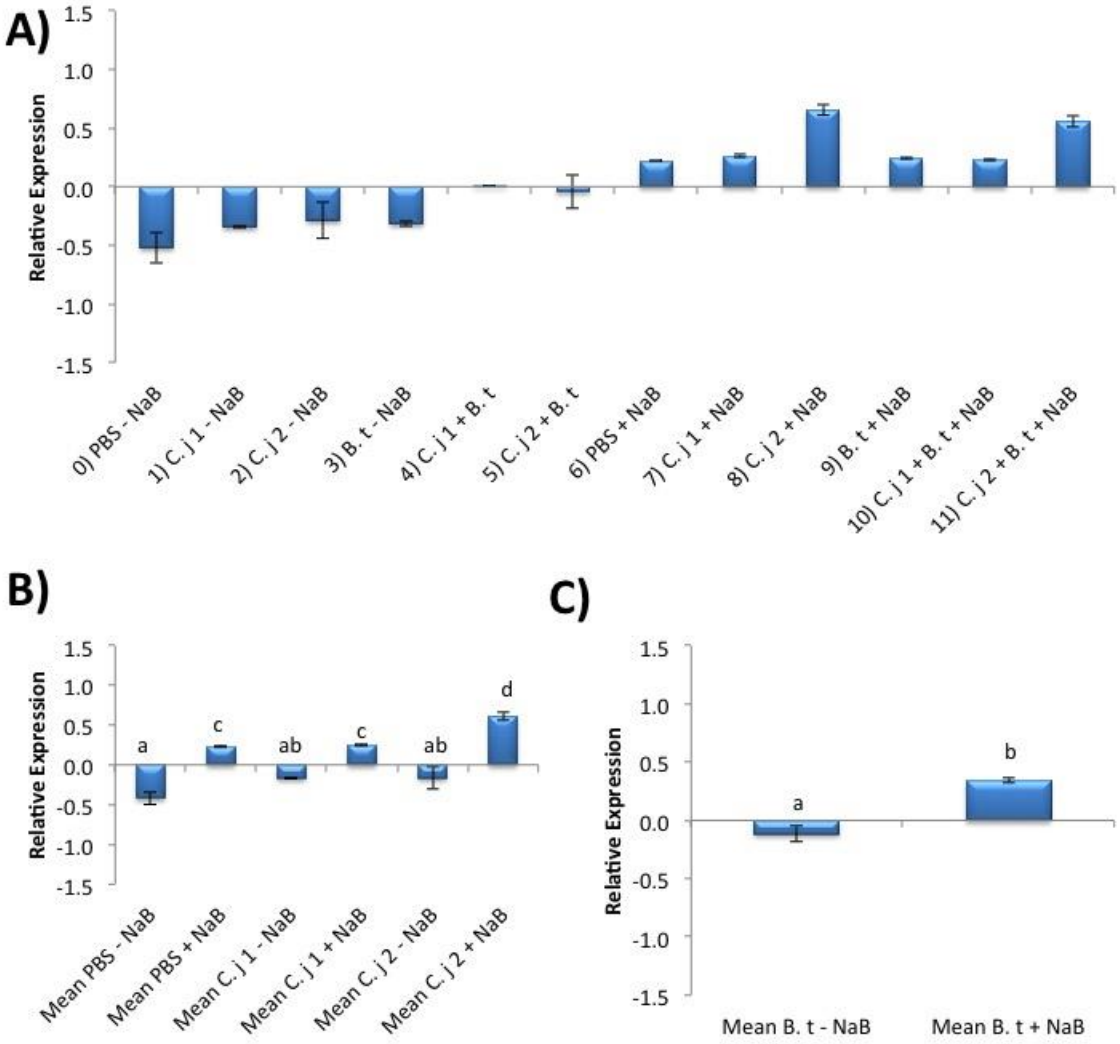

Figure 3.11 Relative expression levels of TECK. A) Caco-2 cell expression of TECK mRNA relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** Coculture of both *C. jejuni* strains with and without NaB showing the interaction between *C. jejuni* and NaB (Table 3.1). **C)** *B. theta* treatment interaction with NaB averaged across *C. jejuni* treatments (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars not that are accompanied by the same letter differ ($\alpha = 0.058$).

interaction between NaB and *B. theta*. In contrast, Caco-2 cells inoculated with *B. theta* in the absence of NaB (Mean B. t – NaB; treatments 3-5) showed lower relative expression levels (Figure 3.11C). For the PBS control, *C. jejuni* strain 1, *C. jejuni* strain 2, and *B. theta*, the addition of NaB increased TECK expression.

C. jejuni strain 2 and *B. theta* influence IL-8 expression similar to what was observed for TECK expression. NaB also influences IL-8 and TECK expression in similar manners, although NaB alone (PBS control) decreases IL-8 expression, which is in contrast to TECK. Notably, *C. jejuni* strain 1, in the presence and absence of NaB, gives rise to significantly different expression levels. Consequently, there is an interaction between NaB and *C. jejuni* that is strain dependent (Figure 3.12C). These results show that NaB can increase Caco-2 sensitivity to different Gram-negative bacteria in a strain dependent manner.

3.4.3.3 MCP-1, IP-10 and IL-12β

All single variables display main effects for MCP-1 expression (Figure 3.13), while none of the multi-variable treatments yield interaction effects (Table 3.1). MCP-1 transcript levels are increased when *C. jejuni* is present to similar magnitudes (Figure 3.13A) for both strains (strain 1: treatment 1, 4, 7, and 10; strain 2: treatments 2, 5, 8, and 11). Inoculation with *B. theta* (Mean + B. t.; treatments 3, 4, 5, 9, 10, and 11) also increased MCP-1 transcription (Figure 3.13B) in comparison to the treatments without *B. theta* (Mean – B. t; treatments 0, 1, 2, 6, 7, and 8). In addition, the presence of NaB (treatments 6-11) increased expression of MCP-1 (Figure 3.13D). These results show *C. jejuni*, *B. theta* and NaB independently influence MCP-1 expression.

All three variables also showed main effects for IP-10 relative expression, but in contrast to MCP-1, all three variables display interaction effects. IP-10 expression displayed variable results

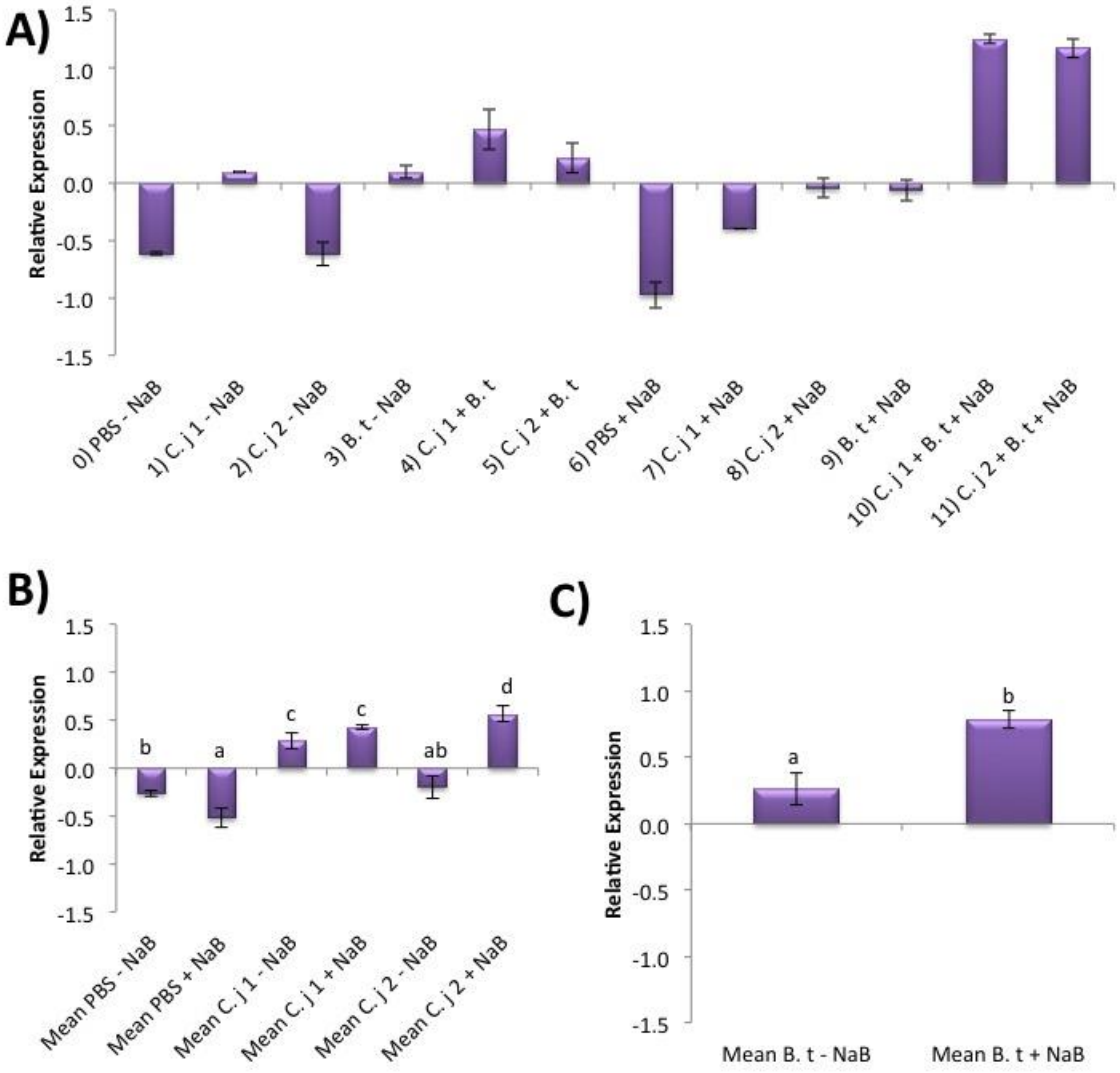

Figure 3.12 Relative expression levels of IL-8. A) Caco-2 cell transcription levels of IL-8 relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** Interaction between NaB and both strains of *C. jejuni* averaged across *B. theta* treatments (Table 3.1). **C)** Interaction between *B. theta* and NaB across *C. jejuni* treatments (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ ($\alpha = 0.058$).

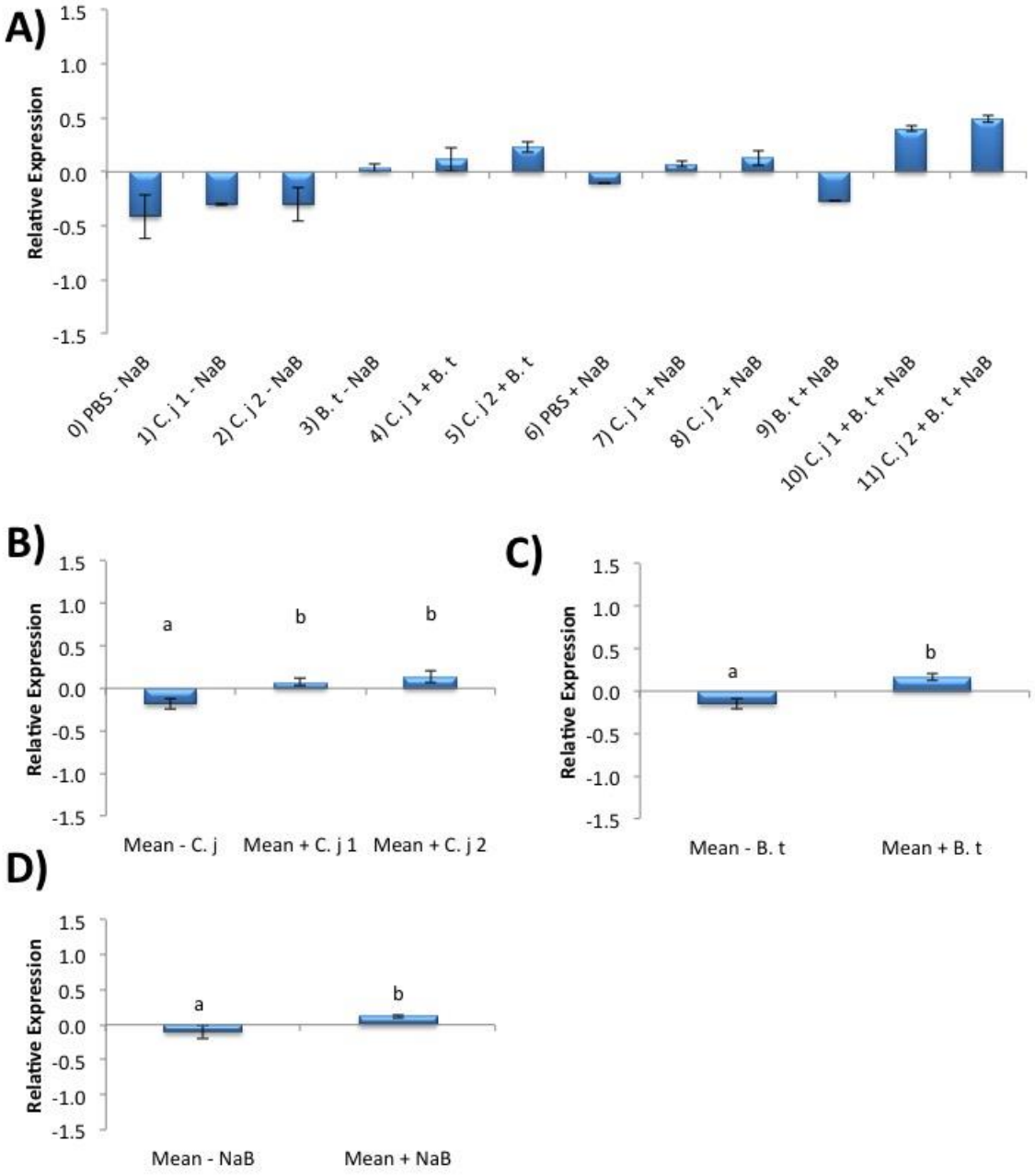

Figure 3.13 Relative expression levels of MCP-1. A) Caco-2 cell expression of MCP-1 mRNA relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** Treatments averaged across *B. theta* and NaB treatments showing the *C. jejuni* treatment effect (Table 3.1), **C)** *B. theta* treatment effect averaged across NaB and *C. jejuni* treatments (Table 3.1). **D)** NaB treatment effect averaged across *B. theta* and *C. jejuni* treatments. Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ (α = 0.058).

for all the conditions tested (Figure 3.14). In the absence of bacterial stimuli, NaB (Figure 3.14; treatment 6) did not elicit induction of IP-10 above that of the negative control (Figure 3.14; treatment 0) and did not display a main treatment effect (Table 3.1), similar to IL-12β (Figure 3.15). All three bacterial treatments (*C. jejuni* strain 1, strain 2 and *B. theta*) increased expression of IP-10 to varying levels. Co-inoculating *B. theta* with either *C. jejuni* strain did not affect IP-10 levels (treatments 4 and 5 in comparison to treatments 1 and 2 respectively). NaB did induce an expression change for *C. jejuni* strain 10 (treatment 11) relative to treatments 2 and 5 (Figure 3.14).

IL-12β was the only gene investigated that does not display a main effect when treated with *C. jejuni*, *B. theta* or NaB (Table 3.1). This is notable, as IL-12β is known to differentiate T cells into the Th1 subset (Figure 1.4), and *C. jejuni* has previously been shown to initiate a Th1 response by inducing IL-12β expression[130]. Similarly, *B. theta* has only been shown to weakly increase IL-12β in human monocyte-derived DCs[314], and NaB has been reported to have anti-inflammatory activity by inhibiting IL-12β in human monocytes[127]. While the origins of these differences are not clear, IL-12β may require other factors to initiate induction beyond constitutive levels in Caco-2 cells.

3.4.3.4 TGF-β

TGF-β is mainly implicated in maintaining immune tolerance through the regulation of lymphocyte proliferation, differentiation and survival with several other functions in most cells[201]. TGF-β is important in both the initiation and resolution of inflammatory responses by influencing chemotaxis, activation, and survival of lymphocytes, NK cells, DCs, macrophages, mast cells, and granulocytes[202]. Whether TGF-β is acting in initiation or resolution of

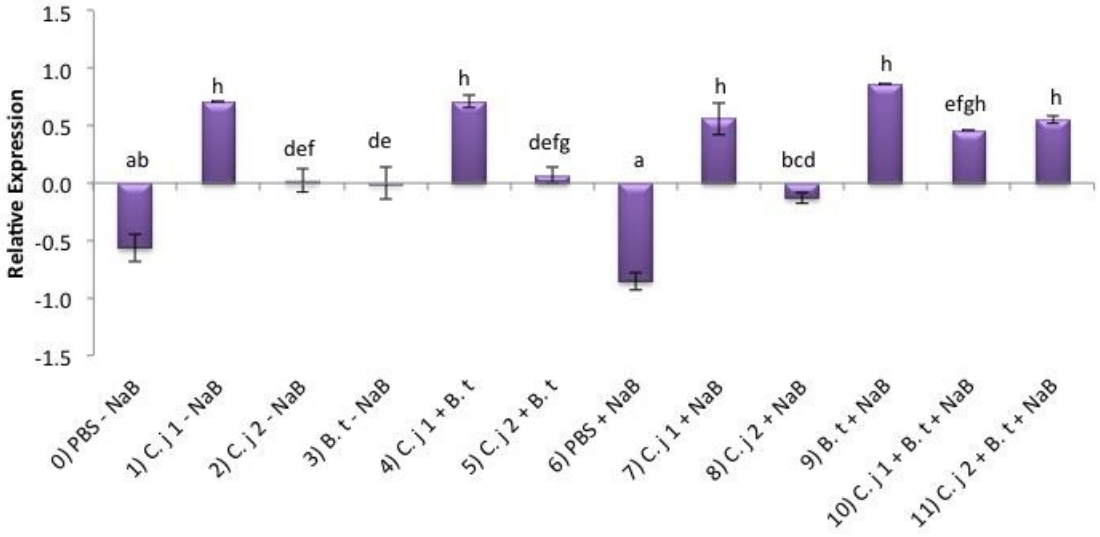

Figure 3.14 Relative expression levels of IP-10. Caco-2 cell expression of IP-10 relative to the baseline in response to factorial treatments (Table 2.2) showing the three-way interaction between *B. theta*, *C. jejuni* and NaB (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars not accompanied by the same letter differ ($\alpha = 0.058$).

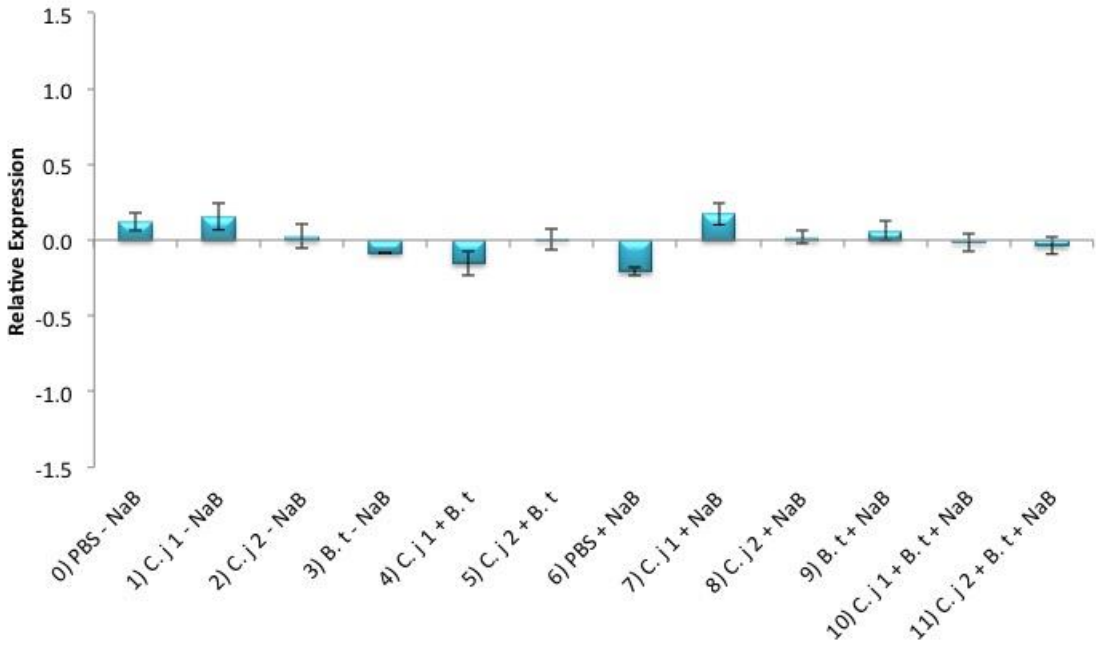

Figure 3.15 Relative expression levels of IL-12β. Expression of Caco-2 IL-12β mRNA relative to the baseline exposed to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, C. jejuni (PBS, strain 1, strain 2) +/- and B. theta +/-. Vertical lines associated with histogram bars are SEM.

inflammation can be location dependent.

Sonicated *C. jejuni* induced TGF-β expression in INT-407 cells[315], whereas *B. theta* has not been shown to influence TGF-β expression. 0.1 and 1 mM NaB is believed to increase transcription of TGF-β in HK-2 cells for up to 30 minutes[71]. NaB was the only variable with an effect on the expression of the generally regulatory TGF-β (Table 3.1). When NaB was present (treatments 6-11), TGF-β transcription increased (Figure 3.16B), although the effect was weak. Given the role of TGF-β in either Th17 or Treg T helper cell subsets, many other factors are likely implicated in controlling TGF-β transcription. The timelines for various immune responses are not well characterized given the stimuli in this experiment. TGF-β is one of the only immune markers that I can actually make a prediction about given its role in the resolution of inflammation. My results suggest that TGF-β may not be heavily induced within 6 hours of bacterial inoculation without NaB, in Caco-2 cells.

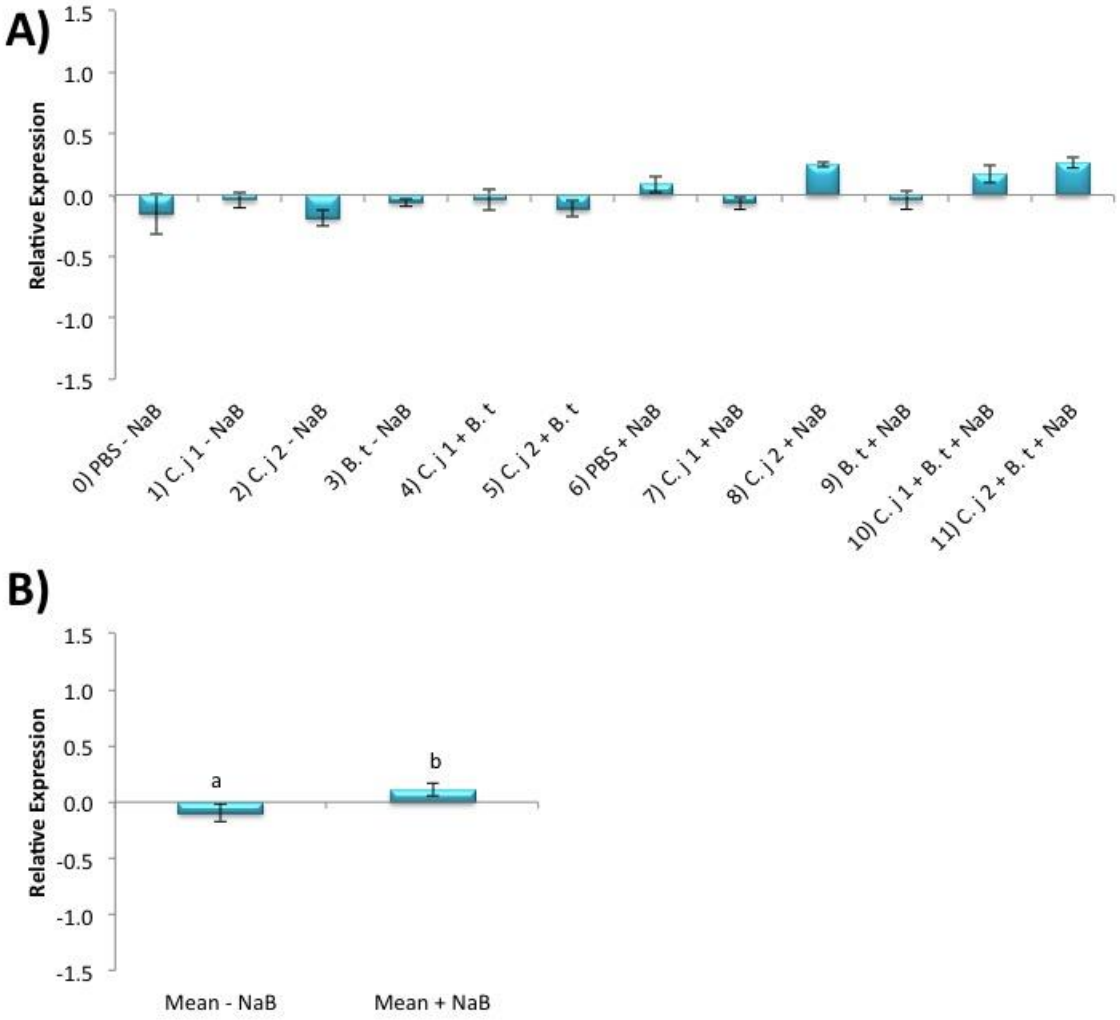

Figure 3.16 Relative expression levels of TGF-β. A) Caco-2 cell expression of TGF-β mRNA relative to the baseline in response to factorial treatments (Table 2.2) in the presence/ absence of NaB +/-, *C. jejuni* (PBS, strain 1, strain 2) +/- and *B. theta* +/-. **B)** Treatments containing NaB averaged across *B. theta* and *C. jejuni* treatments showing the NaB treatment effect (Table 3.1). Vertical lines associated with histogram bars are SEM. Histogram bars that are not accompanied by the same letter differ (α = 0.058).

Chapter 4 Discussion

4.1 Butyrate, *B. theta* and *C. jejuni* Affect Caco-2 Cell Line Transcription

The DGM generally mediates health effects through its collective composition and metabolic properties; however, reproducible experimental systems to investigate this relationship are currently lacking. Therefore, I first set out to establish a model system to explore the NaB-colonocyte-bacteria interaction. Caco-2 cells were selected for this system because they are commonly used for colonic cell culture in the field.

4.1.1 NaB-Induced H3K9ac and Modulation by Bacteria

The H3K9 motif in Caco-2 was chosen as a potential *in vitro* biomarker for correlating enteric NaB levels with changes to host gene expression *in vivo* because elevated H3K9ac levels are present in actively transcribed genes[316]. H3K9 acetylation profiles were monitored in histone extracts diluted to consistent H3 protein levels (Figure 3.3) to verify that 5 mM NaB had an effect on global Caco-2 H3K9ac levels. In particular, NaB has been implicated in immune gene transcription[317]. NaB supplementation has been shown to both increase and decrease transcription of factors involved in the host immune response[318, 319]. Additionally, bacteria have been linked to changes in histone modifications[289] as well as altered host immune responses[320]. In this work, NaB and *C. jejun*i were capable of changing histone H3K9 acetylation levels, previously proposed to directly influence the expression of immune gene transcription[321, 322]. While *B. theta* was far less effective in changing global histone H3K9 acetylation levels, the three experimental variables did affect Caco-2 immune gene expression (Table 3.1).

Global H3K9ac levels decrease in the presence of *C. jejuni* for treatments 7 and 8 (Figure 3.3). This decrease indicates that the presence of a bacterial species can modify the NaB effect on the Caco-2 cell histone acetylation profiles. Intriguingly, the *C. jejuni* strains modulated H3K9

acetylation differently in the presence of NaB. *C. jejuni* strain 1 (treatment 7) decreases acetylation levels to a greater extent than strain 2 (treatment 8), in the presence of NaB. The mechanism behind *C. jejuni*'s capacity to alter NaB induced H3K9ac levels is currently unknown, and warrants further investigation at other concentrations of NaB. Bacterial factors such as capsular composition, outer membrane proteins, or secreted factors could influence histone acetylation. Treatment 11 with *C. jejuni* strain 2, NaB and *B. theta* yielded the highest acetylation levels, even above NaB alone (treatment 6), whereas strain 1 in treatment 10 did not have a strong effect. Based upon these results, H3K9 acetylation may serve as a biomarker for monitoring the effects of low concentrations of NaB on colonic histone acetylation.

4.2 Caco-2 Cell Immunomodulation

NaB has been previously shown to alter the gene expression profile of cell lines, in response to inflammatory stimuli[127, 268, 323-325]. In this study, the effects of NaB supplementation, with *B. theta* and two strains of *C. jejuni*, on the expression of Caco-2 genes involved in the enteric mucosal immune response were investigated using an in-house immune response RT-qPCR array (Table 2.3). The current study helps to provide a more complete picture of how NaB influences immune responses to beneficial or harmful bacteria, and expands on previous studies that focused on fewer genes with only one or two treatments. Using this approach, I have identified that NaB can influence the induction of both defensins and TLRs in Caco-2 cells, *B. theta* did not influence defensin production, but cytokine and chemokine expression (both dependently and independently of NaB) were affected (Table 3.1). *C. jejuni* can negatively influence host defensin, TLR and cytokine/chemokine expression, and together, *B. theta* and NaB interact to influence several Caco-2 responses to *C. jejuni* (Table 3.1).

Most of the effects attributed to NaB in the literature are due to changes in HDACi activity leading to elevated levels of histone acetylation and affecting the activity of many transcription factors, including NF-κB[326, 327]. In this study, HDACi activity alone leads to elevated levels of host response gene expression, but it cannot explain the NaB induced decreases in gene expression. Further experiments will be required to support the previous studies to determine how NaB affects immune gene expression by directly or indirectly influencing NF-κB activation[126, 319]. In addition, the results of this study suggest that investigation of H3K9ac levels *in vivo* and chromatin immunoprecipitation PCR with anti-H3K9ac may be a valuable approach for determining the relationship between H3K9ac and specific gene expression in Caco-2 cells before proceeding into animal studies. Although histone modifications and chromatin remodelling have been reported during bacterial infections[287], neither *C. jejuni* nor *B. theta* have been shown to influence Caco-2 histone tail acetylation in previous work.

Both β-defensins (hBD-1 and hBD-3), all three TLRs (TLR2, TLR5 and TLR9), and two regulatory cytokines (IL-12β and TGF-β) had expression levels similar to the derived baseline expression (Figure 4.1), suggesting that they were unstimulated by NaB, *B. theta* or *C. jejuni*. HBD-1[154], TLR5[175] and TGF-β[328] are expressed constitutively, along with the p35 chain heterodimeric subunit of IL-12β[329]. TLR2 expression has been reported to be at very low levels[175], while literature is lacking for IEC expression levels of TLR9. My results are consistent with the understanding that these immune markers are constitutively expressed, and it also suggests that they are required to maintain physiological inflammation. Further, these immune markers can be up- or downregulated in response to pro- or anti-inflammatory challenges.

The pro-inflammatory TNF and the tested chemokines (MCP-1, TECK, ENA-78, IL-8, and IP-10) all expressed at levels lower than the baseline in absence of NaB, *B. theta* or *C. jejuni*. These findings suggest that it is beneficial to limit inflammation signals in the absence of stimuli, and are consistent with previous reports that TNF[328], MCP-1[70], TECK[221], ENA-78[330], and IP-10[70] are all inducible. For example, TNF is a potent pyrogen, and the immune system would likely avoid creating concentration gradients for leukocyte responses unless there was a trigger. The lack of detectable hAD-1 mRNA in Caco-2 cells indicates that this defensin may not be expressed from colonocytes *in vivo*. Alternatively, Caco-2 cells are known to present different phenotypes under different culture conditions, and can transform into small intestine-like cells after eight days. Therefore, at this point, hAD-1 may be more likely to be detected in Caco-2 cells.

4.2.1 NaB Influences Caco-2 Immune Processes

The observations that both defensin and two of the three TLR genes have increased expression levels in response to NaB (Table 3.1) validate my first hypothesis that NaB would induce defensin and TLR expression. The higher number of genes induced by NaB supplementation has also been reported in previous microarray studies employing IECs[331-333]. The reported inhibition of HDAC activity could in part explain these results. HDACi leads to increases of global histone acetylation levels, and is generally associated with elevated levels of gene expression[334]. The data presented here (Figure 3.3) provides support that histone acetylation at H3K9 would be a promising target for chromatin immunoprecipitation PCR assays to confirm if this modification is regulating host immunomodulatory genes. NaB can regulate the transcription of immune markers through HDACi activity, but it has been suggested to also regulate the expression of some genes by other mechanisms such as NF-κB[335] and Gpr109a[282]. The effects of NaB on

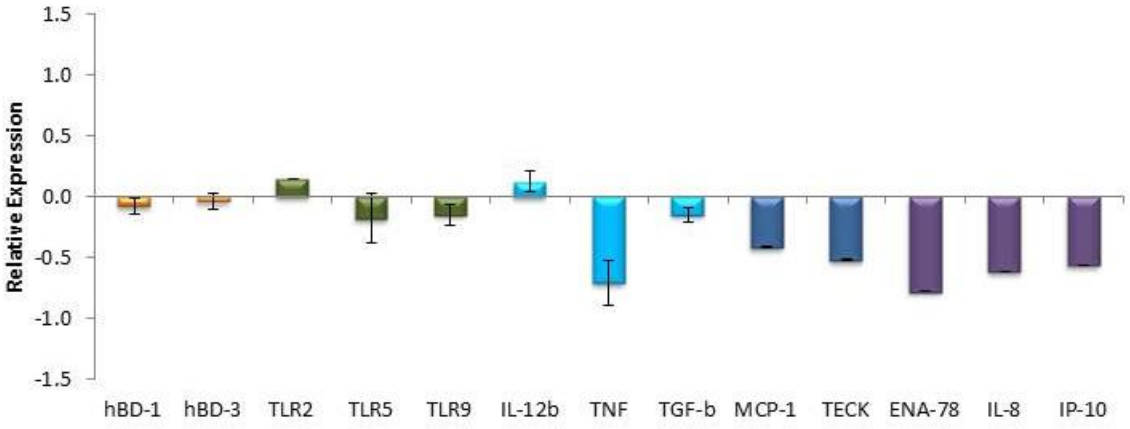

Figure 4.1 Unstimulated expression of Caco-2 immune markers. Caco-2 immune gene marker expression in the unstimulated treatment 0, relative to baseline expression. Defensins hBD-1 and hBD-3, TLRs 2, 5 and 9, and cytokines IL-12β and TGF-β were close to the baseline. TNF and chemokines MCP-1, TECK, ENA-78, IL-8, and IP-10 were lower than baseline. Vertical lines associated with histogram bars are SEM.

defensin expression have largely been cell line or animal model dependent. The constitutively expressed hBD-1[153] has been shown to be upregulated by 4 mM phenylbutyrate in the U937 monocytic cell line[85] over a 24 hour period, and bovine epithelial cells showed an increase in expression when treated with 10 mM NaB for 24 hours[84]. In contrast, 2 mM NaB in the HT-29 IEC line has been shown to increase expression of the inducible hBD-3[336]. The results presented in this study do not directly correlate with the HT-29 cell line results, though NaB did increase expression of both defensins with a bacterial co-stimulant. The present study therefore supports a growing body of evidence for host AMP production being dependent on DGM composition and SCFA production. It has been established that NaB and *B. theta* both contribute to mucus formation[28], and with mucus (mucin) having been implicated in defensin production, there may be a feedback system between mucus[337], acetate producing bacteria (e.g. *B. theta*) and NaB producing bacteria (e.g. *F. prausnitzii*) leading to defensin responses to pathogens.

Host PRRs are crucial in maintaining physiological inflammation and the health of the host. NaB downregulated TLR2 in contrast to the effect NaB had on TLR5 and TLR9. Previously, 5 mM NaB was observed to down-regulate TLR4 expression in HT-29 cells[338]; however, this is the only instance where NaB downregulates a TLR (i.e. TLR2) in the literature. This is also the first time that NaB treatment has been shown to increase expression of both TLR5 and 9. Previously, NaB has been shown to block TLR2-agonists, acting in an anti-inflammatory fashion in peripheral blood mononuclear cells from patients with IBD[339]. A similar action may result from reducing expression of TLR2 itself. When homeostasis is perturbed in diseases such as IBD, TLR2 can become deregulated[340]. NaB could serve as a means of returning TLR2 expression back to homeostatic levels to prevent prolonged inflammation, acting as an effective modulator of the

initial steps of acute inflammation. NaB could have increased TLR5 expression through an H3K9ac mediated recruitment of undefined transcription factors as it is not NF-κB regulated.

4.2.2 *B. theta* Influences Caco-2 Immune Gene Expression

Although *B. theta* did not display any hallmark anti-inflammatory properties (e.g. promoting Caco-2 expression of defensins and down-regulating TLRs and pro-inflammatory cytokines) as hypothesized, NaB did increase some cytokine responses in an interaction with *B. theta*, which validates the potential for *B. theta* to be considered immunomodulatory. To date, the majority of research associated with Bacteroides modulation of the enteric immune system has focused on *B. fragilis. B. fragilis* has been identified as an opportunistic pathogen and greater threat to human health than *B. theta*[80]. In order to begin elucidating the role of *B. theta* in host gene expression and subsequent immune responses, I investigated the induction of immune genes in Caco-2 cells in the presence of *B. theta* (Table 2.2).

A notable finding is that *B. theta* decreases hBD-3 expression. Given the importance of defensins to the enteric mucosal immune system, *B. theta* decreasing hBD-3 expression appears to contradict my second hypothesis that *B. theta* would promote defensin production. While the importance of this decrease in expression is not entirely clear, it could contribute to increasing the persistence of the bacterium in the mucosa since hBD-3 has activity against strictly anaerobic bacteria such as Bacteroides. This activity has been shown to be oxygen dependent[167], so down-regulation of hBD-3 may help maintain an anaerobic environment. The gene expression data was not likely influenced by *B. theta* changing H3K9ac levels. *B. theta* has the smallest effect on H3K9ac levels (Figure 3.3), which correlates with it also having the fewest expression responses (Table 3.1).

B. theta interacted with NaB to influence expression of hBD-3, IL-8, TECK, and IP-10, which have chemoattractive properties. In the case of hBD-3 and IP-10, there was an additional interaction with *C. jejuni*, *B. theta* and NaB. The interactions with *B. theta* and *C. jejuni* could be an artefact of increased bacterial load. Alternatively, it could be that *B. theta* increases the sensitivity to *C. jejuni* and through expression of hBD-3 and IP-10, helps direct chemoattraction so that the host can defend itself more efficiently. *B. theta* has been previously linked with increasing IFN-γ in CD4+ T cells[341], but not IP-10. Increased expression of IFN-γ and IP-10 would facilitate a more robust response to Th1-type inflammation[342].

TLR5 influences the DGM by inducing antibodies against flagella[182] that slow flagellated bacteria. *B. theta* has been shown to restrict signalling induced by flagellin[24], the main TLR5 agonist, but little is known with respect to TLR5 expression. *B. theta* did not affect TLR5 expression levels (Figure 3.7). In contrast, *B. theta* did affect TLR9 expression. Further experiments are required to elucidate whether this was through an NF-κB dependent mechanism, which is important for detecting pathogens and maintaining commensal homeostasis.

Bacteroides spp. have been reported to enhance the susceptibility of hosts to *C. jejuni* infection[343]. Some harmful bacteria can benefit from *Bacteroides* spp. altering the mucus structure of the intestine when nutrients are limiting[75]. My co-culture treatments with *B. theta* and *C. jejuni* may provide further insight into this relationship. A number of cytokine and chemokine transcripts were lower in response to *C. jejuni* alone than in co-culture with *B. theta*: TNF, MCP-1, TECK, ENA-78, and IL-8. Alternatively, *B. theta* could be increasing *C. jejuni*'s pathogenicity[343].

4.2.2.1 *B. theta* Induces Cytokine Expression

Commensal bacterial members of the DGM are known to initiate pro-inflammatory responses, contributing to host health and well-being by stimulating the immune system[81, 344]. In contrast, other commensal members inhibit or suppress IEC inflammatory responses by modulating immunity through PRRs, inhibiting NF-κB, or increasing the secretion of anti-inflammatory cytokines[345-347]. In this regard, *B. theta* could be a member of the commensal DGM population that stimulates the immune system to maintain a balance between pro- and anti-inflammatory responses by increasing the expression of TNF, MCP-1 and ENA-78 independently, or in combination with NaB increasing TECK and IL-8 expression.

4.2.3 *C. jejuni* Triggers a Pro-inflammatory -like Response in Caco-2 cells whilst Negatively Affecting AMP and TLR Production

Although the third hypothesis that *C. jejuni* would decrease host defensin and TLR expression was not validated, *C. jejuni* did induce pro-inflammatory chemokine expression. Previous studies have reported that hBD-1 expression was not affected by *C. jejuni* NCTC 11168 or 81-176 in infected Caco-2 or HT-29 cells *in vitro*[151]. This result was also shown using pro-inflammatory (IL-1α, IFN-γ and TNF-α) and LPS from *E. coli* O111:B4[149]. My results are consistent with these findings, as the presence of *C. jejuni* does not affect hBD-1 expression in the absence of NaB (Figure 3.4B). However, in the absence of NaB the two strains did induce hBD-1 transcription differently from one another (Figure 3.4B). In contrast, the presence of NaB increases hBD-1 expression in the presence of *C. jejuni* in a strain independent manner (Figure 3.4B). Determining the mechanism by which the two strains induce expression differently in the

absence of NaB will require future investigation into the genetic and phenotypic differences between the K12E5 (strain 1) isolate and 81-167 (strain 2).

Neither *C. jejuni* nor *B. theta* affected TLR2 expression. This finding is expected, as TLR2 is commonly associated with detecting Gram-positive bacteria[348]. Previously however, *C. jejuni* IA 3902 increased TLR2 expression in Guinea pig subplacental trophoblasts[311]. Again, the differences in cell types and/ or bacterial strains may account for the contrasting expression patterns. Neither TLR5 nor TLR9 displayed strong reactions in the presence of *C. jejuni*. While there is an interaction between NaB and *C. jejuni* in the case of TLR5, *C. jejuni* does not enhance or repress the NaB induced expression profiles. These findings suggest that *C. jejuni* has little effect on the expression of TLR5 or TLR9, in contrast to NaB. This is consistent with findings that both TLR5 and TLR9 are unable to detect *C. jejuni* DNA[185] and the heavily glycosylated flagella of *C. jejuni*[349].

4.2.4 NaB and *B. theta* Influence Caco-2 Responses to *C. jejuni*

A main objective of this study was to determine the degree to which NaB and *B. theta* could modulate immune responses triggered by *C. jejuni*. HBD-3 and IP-10 provide support for the fourth hypothesis that together *B. theta* and NaB interact to modulate responses induced by *C. jejuni*. In the case of hBD-3, pathogens have been shown to decrease defensin expression, such as *Shigella flexneri* in HT-29 cells[350] or *Vibrio cholerae* in IECs[351] in order to provide a colonization advantage. So instead of further decreasing hBD-3 expression NaB and *B. theta* were expected to increase hBD-3 expression in the presence of *C. jejuni*. Previous studies have shown that both *C. jejuni* NCTC 11168 and 81-176 increased hBD-3 expression with induction highest 10 hours post infection in Caco-2 and HT-29 cells[151]. In the current study HBD-3 was not affected by either

strain of *C. jejuni*, *B. theta* or NaB independently, but in the presence of *B. theta* and NaB, hBD-3 expression was upregulated. This suggests either multiple levels of regulation or multiple regulatory inputs are involved in hBD-3 expression. Further, if *B. theta* and NaB decrease the hBD-3 response time of the Caco-2 cells to *C. jejuni*, this could be an example of immune stimulation.

B. theta and NaB influence the pro-inflammatory cytokine TNF and chemokines (MCP-1, TECK, ENA-78, IL-8, and IP-10). Of the genes investigated, the largest increases in expression levels result from the addition of NaB (hBD-1, TLR5 and TECK) or both NaB and *B. the*ta (hBD-3, TLR9, TNF, MCP-1, ENA-78, IL-8, and IP-10). These results (e.g. hBD-3, TNF, MCP-1, TECK, ENA-78, IL-8, and IP-10) suggest that Caco-2 cells have a greater potential to respond and attract immune cells to the damaged area when *B. theta* and NaB are present. The addition of *B. theta* increases the expression of cytokines and chemokines in response to *C. jejuni*. For example, *C. jejuni* alone may be capable of avoiding MCP-1 induction, but *B. theta* and NaB help to counter this activity. A priming activity that elevates host defences and sensory potential in addition to more robust and concise inflammatory responses to a pathogen such as *C. jejuni* does suggest some benefits to Caco-2 cells in having *B. theta* present with NaB. Although the results of this study are not yet definitive, it suggests that *B. theta* is a candidate immunomodulating member of the human DGM.

4.3 Gene Expression Summary

There were a number of novel immune target primer sets designed for this experiment that yielded novel insight into the roles of NaB, *B. thet*a and *C. jejun*i on Caco-2 immune responses. The RT-qPCR results supported the immunoblotting in that NaB and *C. jejuni* exerted larger

influences on Caco-2 histone tail acetylation and gene expression. This is the first reported indication that NaB can decrease TLR2 expression and increase both TLR5 and TLR9. The results of this study suggest that NaB and *B. theta* may have additional effects in the presence of harmful stimuli.

Chapter 5 Conclusions and Future Directions

5.1 Conclusions

The work in this study focused on characterizing and understanding how NaB, a DF fermentation by-product, might influence host responses to *C. jejuni* and *B. theta*. The Caco-2 cell line was introduced as a model system for investigating the host responses to a commensal bacterium, *B. theta*, and two strains of a known prevalent foodborne pathogen, *C. jejuni*. Immunoblotting for acetylated H3K9 and RT-qPCR was used to measure modulations in epigenetic structure and gene expression, respectively. This is the first report of NaB and enteric bacteria modulating H3K9ac levels in Caco-2 cells, which may provide a useful tool for investigating similar responses *in vivo*.

Based on the data presented herein, I demonstrate that NaB, *B. theta* and *C. jejuni* are capable of influencing Caco-2 cell immune gene expression. More specifically, I have described the roles of NaB, *B. theta* and *C. jejuni* in host AMP and PRR responses under these conditions. I propose the immune markers (Table 2.3) chosen for this study represent valuable targets for further investigation in intestinal cell lines and animal models. Further, I have shown an interaction between NaB and *B. theta* in response to *C. jejuni*. Consequently, *B. theta* may be considered an immunomodulating commensal bacterium, and the target of further investigation. *B. theta* is a prominent member of the human DGM is less commonly associated with intestinal infections than *B. fragilis*, and is capable of influencing Caco-2 immune responses. Further mechanistic and *in vivo* studies are required to clarify results concerning the viability of the bacteria, the concentration of NaB utilized, cell line used as a model system, and the assays employed.

5.2 Future Directions

The histone H3K9 acetylation increase agrees with what has been published about the effect of NaB on general histone acetylation[258, 271]. Similarly, the effect that *C. jejuni* had on histone acetylation is consistent with reports of pathogens affecting chromatin remodelling and altering histones during infection[287]. Further, *B. theta* had less detectable effects on acetylation and had weaker effects on gene expression in comparison with *C. jejuni* and NaB. Given combined treatments of NaB and these bacteria generate differences in histone tail acetylation, H3K9 may be useful for further describing interactions between DGM and hosts. In order to provide a more complete definition of H3K9 as a suitable biomarker for regulation of factors that regulate host immune responses, investigation into H3K9 methylation with respect to host immune factors is warranted. A more comprehensive analysis with anti-H3K9ac using chromatin immunoprecipitation coupled to the inflammatory gene array introduced here for Caco-2 cells could provide a stronger correlation between histone acetylation and gene expression. Indeed, by profiling the histone modifications and nuclear protein binding events at the gene promoter regions, more direct correlations can be drawn between the histone modifications and each gene's known inflammatory role. A chromatin immunoprecipitation PCR array would also provide insight into the epigenetic molecular mechanisms and biological pathways behind the immune responses induced by *C. jejuni*, *B. theta* and NaB. This approach could be applied to an animal (e.g. murine) model before human application.

In addition to histone acetylation, this work examined the relative transcription levels of selected genes implicated in the host immune response. The Western blots present in this study are the first to show that *C. jejuni* and NaB both influence Caco-2 global acetylation patterns.

Further, this is the first time that *B. theta* has been investigated in the context of histone tail acetylation. There were a number of novel immune target primer sets designed for this experiment that yielded novel insight into the roles of NaB, *B. thet*a and *C. jejun*i on Caco-2 immune responses. The RT-qPCR results supported the immunoblotting in that NaB and *C. jejuni* exerted larger influences on Caco-2 histone tail acetylation and gene expression. This is the first reported indication that NaB can decrease TLR2 expression and increase both TLR5 and TLR9. To better understand how NaB, *B. theta* and *C. jejuni* affect H3K9 acetylation and immune responses in a complex organism, future *in vivo* models are required to investigate if NaB has an effect on the production of defensins, TLRs and cytokines. The results of this study suggest that NaB and *B. theta* may have additional effects in the presence of harmful stimuli; *in vivo* colitis models should also be pursued to investigate ameliorative capabilities of NaB and *B. theta* in disease states such as campylobacteriosis. Expanding this study to examine additional treatments such as including a NaB producing bacterium (e.g. *F. prausnitzii*) instead of artificially supplementing with NaB could yield further immunomodulatory potential, and could clarify some of the effects that NaB was shown to have in this study. Expanding this study to include *B. fragilis* would also resolve questions surrounding the immunomodulatory potential of *B. theta* in direct comparison to a *bone fide* pathogen from Bacteroides.

It was expected that some of the genes would show *C. jejuni* strain specific responses given the wide variety of strains employed throughout *C. jejuni* research[352]. However, given the high genetic diversity between human *C. jejuni* isolates, future comprehensive tests into the virulence potential of various strains could prove useful. An additional dead/heat killed control and different atmospheres for the bacterial inoculation phases of the study to favour *B. theta* or

C. jejuni could determine if the effects shown in this study were the result of bacterial activity since the current experiment was carried out in a cell culture atmosphere. Ultimately this will help define the cell responses to these conditions. Once this is clarified, further testing into the ability or inability of the PRRs (e.g. TLR2, TL5 and TLR9) to detect the different *C. jejuni* strains could provide insight into why the two strains in this study induced different responses.

Mechanistic studies such as internalization/permeability assays would aid in determining if NaB and *B. theta* are decreasing *C. jejuni* internalization, or if *B. theta* accompanies *C. jejuni* across the epithelial layer. Utilizing genomic information from both *C. jejuni* strains and mutagenesis tools to disrupt or delete genes of various proteins associated with virulence in conjunction with internalization/ permeability assays would provide insight into pathogenicity and bacterial translocation into the blood stream.

Further efforts to repeat this experiment with alternative cell lines or primary cultures would provide insight into the immunomodulatory roles of NaB, *B. theta* and *C. jejuni*. The HT-29 or LS174T cell lines could provide cell line dependent induction patterns. The Caco-2 cell line is one of the most widely used cell lines, in part because it serves as a small intestinal cell model after 8 days or a large intestinal cell model before 8 days, but the HT-29 cell line can differentiate and produce a number of cell types such as GCs. Different cell types present alternative interaction surfaces. The LS174T cell line is well characterized for its mucin production capabilities that influence defensin activation. Given the relationship between defensin activity and mucin secretion, a cell line that produces more mucin than the Caco-2 line may reveal additional information about *B. theta*'s role in host AMP secretion. Further, primary cultures are the first cell culture from the host before being immortalized into a cell line. Cell

culture results are inherently biased because they are cancerous lines. Repeating this experiment with primary cultures may provide a closer representation of physiological responses to stimuli before proceeding into animal studies.

Any future efforts concerning this research should seek to provide a more thorough understanding of how colonocytes participate in the innate and adaptive immune responses, how *B. theta* participates in changing host immune responses, and whether NaB with or without *B. theta* can assist the host in defending against enteric pathogens to increase colonization resistance against enteric pathogens. Given that there are dozens of genes that regulate host immunity, a number of cell types[4], upwards of 500 species of bacteria, fungi and archaea[353] and an unknown number of active immunomodulins in the GIT, expanding this research to include more of each tested component will help to confirm and resolve my findings.

Stock images from somersault18:24 (www.somersault1824.com used for Figures: 1.1 and 1.5 (modified from http://www.actrec.gov.in/histome/lead_in.php).

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Appendices

Appendix 1

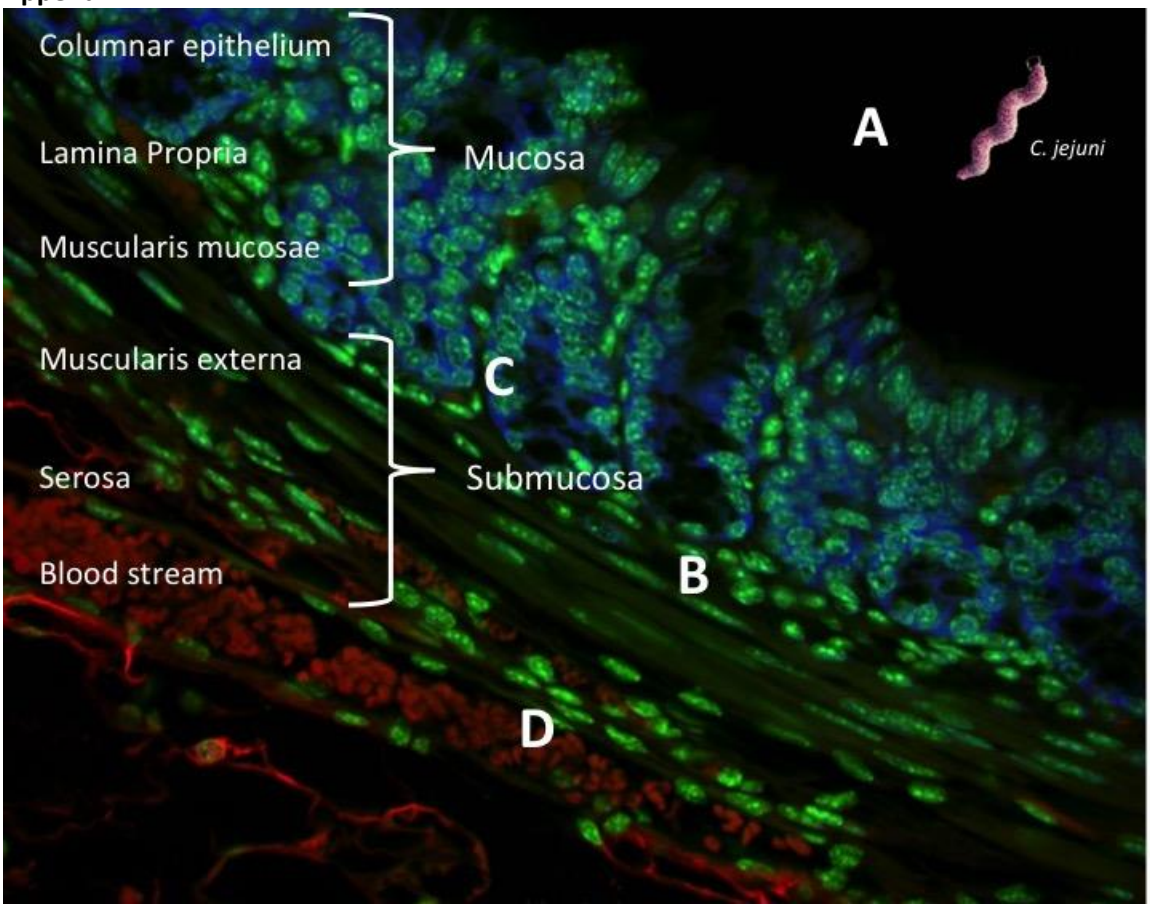


Figure A1.1 Transepithelial route of *C. jejuni* infection. 40x oil immersion confocal micrograph showing the path translocating *C. jejuni* take to enter the bloodstream **(D)**, from the intestinal lumen **(A)** through the mucosa **(B)** and smooth muscle **(C)** possibly via capillaries. DAPI nuclear stain is shown as green, with red blood cells shown in red, and epithelial cells in blue (*C. jejuni* not drawn to scale).

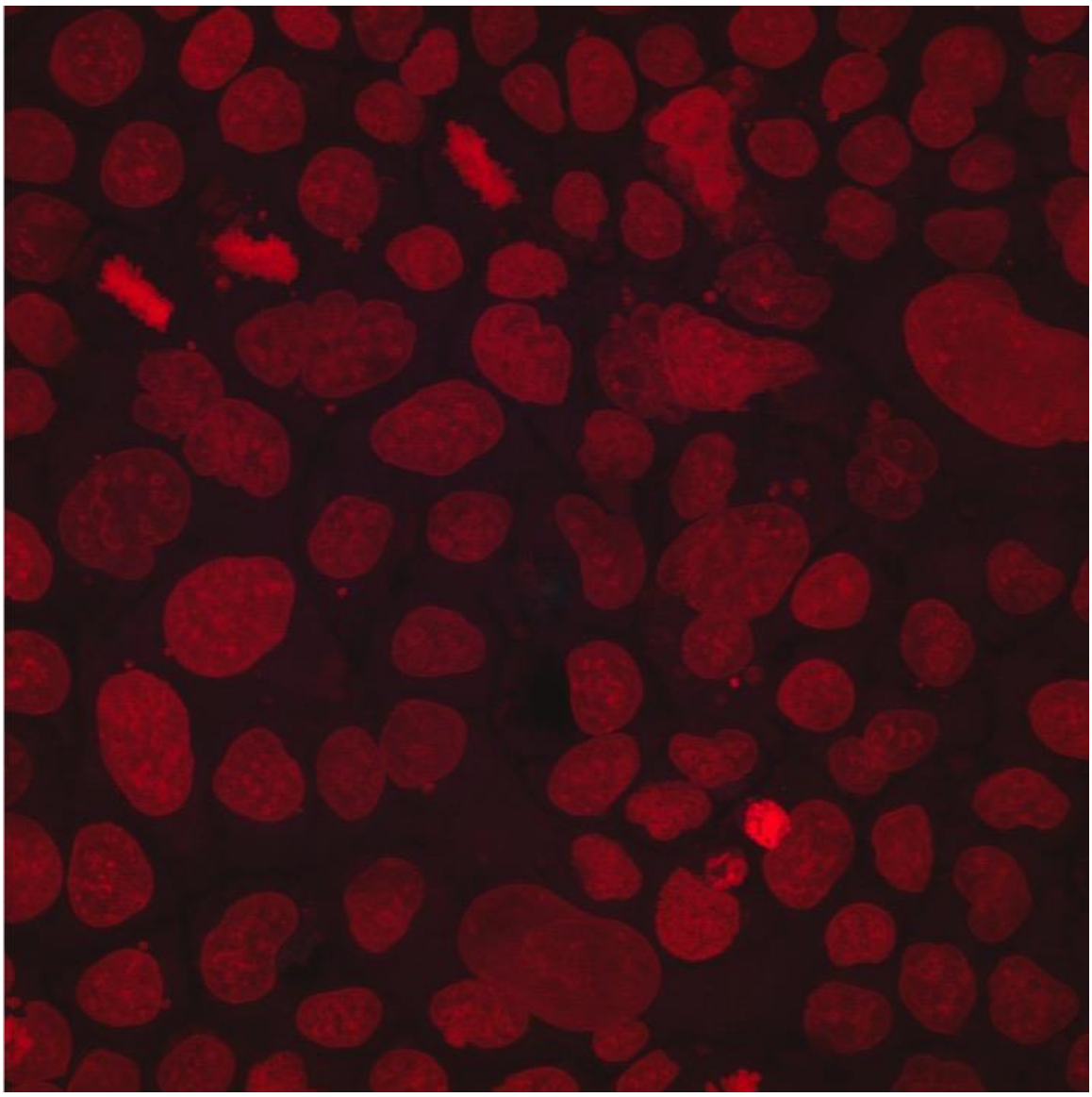


Figure A1.2 Caco-2 cell monolayer. Formaldehyde fixed Caco-2 cell monolayer, visualized with the DRAQ5 nuclear stain displaying cobblestone morphology.

Table A1.1 Literature results for *C. jejuni* inoculation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene * = NF-κB regulated	Literature N/E = no effect, N/A = not applicable	Cell line/animal	Anticipated in Caco-2 cells	My result Stat sig 0.058
C. jejuni	hBD-1 Constitutively expressed	1) 11168: No effect (N/E)[151] 2) 81-176: N/E[151] 3) Unaffected by pro-inflammatory or bacterial molecules[149]	1) Caco-2 and HT-29 2) Caco-2 and HT-29 3) Caco-2 and HT-29	N/E or decrease	K12E5 decrease 81-176 N/E
	hBD-3	1) 11168: Up[151] Highest 10hrs post infection 2) 81-176: Up[151] Highest 10hrs post infection 3) *Shigella flexneri* decreases hBD-3[350]	1) Caco-2 and HT-29 2) Caco-2 and HT-29 3) HT-29	Increase/decrease, temporal factor	K12E5 N/E 81-176 N/E
	TLR2*	1) *C. jejuni* IA 3902: Up[311]	1) Guinea pig subplacental trophoblasts	Increase	K12E5 N/E 81-176 N/E
	TLR5	N/A for K12E5 N/A for 81-176		N/E	K12E5 decrease 81-176 N/E
	TLR9*	11168: N/A 1) 81-176: Surface TLR9 expression **down** (Western blot, IHC, flow cytometry)[354]	1) Colitis mouse model, T84 cells	Increase (but Caco-2 cells may not express TLR9 apically)	K12E5 N/E 81-176 N/E
	IL-12β*	1) 11168: Minor increase[355] 81-176: N/A	1) Dendritic cells from bone marrow	Increase? Small effect	K12E5 N/E 81-176 N/E
	TNF*	1) 11168: Up[356] 2) 81-176: Up[129]	1) C57BL/6 mice 2) Dendritic cells	Increase	K12E5 N/E 81-176 N/E But, *C. j* did have interactions
	TGF-β	1) Isolated *C. jejuni*: Sonicated *C. jejuni* induced TGF-b[315] 81-176 and 11168: N/A	1) INT-407 cells	Decrease?	K12E5 N/E 81-176 N/E

	MCP-1*	11168: N/A 1) 81-176: Up[70]	1) INT-407 cells	Increase	K12E5 N/E 81-176 N/E
	TECK	1) N/A for 11168 2) N/A for 81-176		No effect	K12E5 N/E 81-176 N/E
	MEC*	11168: N/A		No effect	K12E5 N/E
	ENA-78*	81-176: Increase from 6-12 hrs post infection[229]	T84	Increase but time dependent and cell type	K12E5 N/E 81-176 N/E
	IL-8*	1) 11168: Up[151] 2) 81-176: Up[151]	1) Caco-2 and HT-29 2) Caco-2 and HT-29	Increase	K12E5 increase 81-176 N/E[322]
	IP-10*	1) 11168: N/A 2) Up [321]	1) 2) Dendritic cells	Increase	K12E5 Increase >81-176 81-176 Increase <K12E5

Table A1.2 Literature results for *B. theta* inoculation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	Cell line/animal	Anticipated in Caco-2 cells	My result
B. thetaiotaomicron	hBD-1	N/A (Unaffected by pro-inflammatory or bacterial molecules)[149] (*Salmonella Dublin*, Salmonella *Typhi*, enteroinvasive *E. coli*.	Caco-2 and HT-29	No effect	N/E
	hBD-3	N/A		No effect	Decrease
	TLR2*	N/A		No effect	N/E
	TLR5	*B. theta* restricts signalling induced by flagellin, a TLR5 agonist, and flagellated pathogens [24] N/A about induction		Increase	N/E
	TLR9*	N/A		Increase	N/E
	IL-12β*	Weak increase[314]	Human peripheral blood mononuclear cells (PBMC) Monocyte-derived DCs	Increase	N/E
	TNF*	Decreases *S. enteridis* induced TNF expression (thesis in Caco-2)[357]	Caco-2	Decrease	N/E
	TGF-β	1) Barely detectable[358] 2) *B. fragilis* enterotoxin induces IL-8 and TGF-β[359]	1) DCs 2) HT-29, T84, Caco-2 and IEC-6	Low expression	N/E
	MCP-1*	?		Increase	Increase
	TECK	N/A		Increase	N/E
	MEC*	N/A		Increase	N/E
	ENA-78*	N/A		Increase	Increase
	IL-8*	1) No response in ileal biopsies[78] 2) Decreased response in Crohn's ileal biopsies	1) biopsies from ileal tissue	Increase	Increase

		tissue[78] -short increase due to limit of NF-κB activity by *B. theta*[24] 3) Colonized germ free mouse w *B. theta*, decreased IL-8[360]	2) biopsies from Crohn's patients 3) Germ free mice		
	IP-10*	N/A But, *B. theta* and other anaerobic bacteria increase IFN- γ in CD4+ T cells[341]		Increase	Increase

Table A1.3 Literature results for NaB supplementation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	Cell line/animal	Anticipated in Caco-2 cells	My result
5 mM NaB	hBD-1	1) Phenylbutyrate induced (4 mM)[85] through CAMP gene expression (U937 downregulated) 2) Down (4 mM)[85] 3) Up[84]	1) Lung epithelial cell line VA10, HT-29, A498, and U937 cells 2) Monocytic cell line U937 3) Bovine epithelial cells	Increase	N/E alone, but overall NaB effect
	hBD-3	1) Phenylbutyrate (4 mM) N/E[85] 2) NaB (2 mM) increases expression, with antibiotics decreasing expression[336]	1) Lung epithelial cell line VA10, HT-29, A498, and U937 cells 2) HT-29	Increase	N/E alone, but overall NaB effect
	TLR2*	1) Blocks TLR2-agonists (anti-inflammatory in patients w IBD)[339] (0.06-1 mM) 2) N/E[361] (4, 8, 12, 24 mM)	1) Peripheral blood mononuclear cells (PBMC) of IBD patients/healthy controls 2) Caco-2	N/E	N/E alone
	TLR5	1) N/A for TLR5 2) 5 mM NaB downregulates TLR4 expression[338]	1) 2) TLR4: HT-29	Increase	N/E alone, but overall NaB effect
	TLR9*	N/A		N/E	N/E
	IL-12β*	1) Inhibits IL-12β	1) Human monocytes	N/E	N/E
	TNF*	1) Blocks TLR2-agonists (anti-inflammatory in patients w IBD)[339] (0.06-1 mM)	1) Peripheral blood mononuclear cells (PBMC) of IBD patients/healthy controls	Decrease	Increase
	TGF-β	Up[71] (0.1, 1 mM for up to 30 min)	HK-2 cells	Increase	N/E, but overall NaB effect
	MCP-1*	1) (2.5-20 mM) reversibly decreases MCP-1	1) Caco-2	Decrease	N/E

		secretion, but cells were grown for 2 weeks, not 1[86]			
	TECK	N/A		Increase	Increase
	MEC*	Increases MEC (2 mM)[87]	HCA-7, HT-29, HCT-8, Caco-2	Decrease	N/E
	ENA-78*	Decreased (5 – 25 mM)[362]	Human normal colon myofibroblasts (CCD-18Co)	Decrease	N/E
	IL-8*	1) Transiently down-regulates Pam3CSK4-triggered IL-8 production (3 or 5 mM)[277] by inducing expression of A20, a negative regulator of the NF-κB pathway 2) Increase (5 mM)[86]	1) Caco-2 and SW480 2) Caco-2	Context dependent	Decreased IL-8 alone[363] (Increased with bacteria)
	IP-10*	(0.1 – 10.0 mM) ≥ 5 mM NaB blocks IP-10 release[323]	Human intestinal myofibroblasts	Decrease	N/E

Table A1.4 Literature results for the combination of *C. jejuni* and *B. theta* inoculation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	My result
C. jejuni /B. theta	hBD-1	N/A	K12E5: N/E 81-176: N/E
	hBD-3	N/A	K12E5: N/E 81-176: Decrease
	TLR2*	N/A	K12E5: N/E 81-176: N/E
	TLR5	N/A	K12E5: N/E (not different from *B. t* alone or *C. j* K12E5 alone) 81-176: N/E
	TLR9*	N/A	K12E5: N/E 81-176: N/E
	IL-12β*	N/A	K12E5: N/E 81-176: N/E
	TNF*	N/A	K12E5: N/E 81-176: Increase (not different from *B. t* or 81-176 alone)
	TGF-β	N/A	K12E5: N/E 81-176: N/E
	MCP-1*	N/A	K12E5: Increase from negative 81-176: Increase from negative, increase from *C. j* alone, not different from *B. t* alone
	TECK	N/A	K12E5: Increase 81-176: Increase from negative, increase from *B. t* alone but not *C. j*
	MEC*	N/A	K12E5: N/E 81-176: Not investigated
	ENA-78*	N/A	K12E5: Increase (not different from *B. t* alone) 81-176: Increase, but not different from *C. j* or *B. t* alone

	IL-8*	N/A	K12E5: Increase (not different from *B. t* alone) 81-176: Increase from negative and from *C. j* alone, but not *B. t*
	IP-10*	N/A	K12E5: Increase (different from *B. t* alone) 81-176: Increase from negative, but not from *C. j* or *B. t* alone.

Table A1.5 Literature results for *C. jejuni* inoculation in combination with NaB supplementation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	Cell line/ animal	Anticipated in Caco-2 cells	My result
C. jejuni /5 mM NaB	hBD-1	N/A			K12E5: Increase 81-176: Increase
	hBD-3	N/A			K12E5: N/E 81-176: Increase
	TLR2*	N/A			K12E5: N/E 81-176: Decrease
	TLR5	1) N/A 2) Down[127] (anti-inflammatory) (0.5 or 1 mM) in the presence of *S. aureus*.	1) 2) PBMC	Increase	K12E5: Increase 81-176: Increase
	TLR9*	N/A			K12E5: Increase 81-176: N/E from negative, but increase from *C. j* 81-176 alone
	IL-12β*	N/A 1) Down with heat killed *S. aureus* cells[127]	1) Human monocytes		K12E5: N/E 81-176: N/E
	TNF*	N/A			K12E5: Increase 81-176: Increase
	TGF-β	N/A for campy -infection with V. cholera decreased TGF-β 1.5-fold in INT407 cells[364], but upregulated in T84 and Caco-2		Increase	K12E5: N/E 81-176: N/E
	MCP-1*	N/A 2) LPS stimulated with NaB had no effect on MCP-1 expression over NaB alone[86]	1) 2) Caco-2	Decrease or increase	K12E5: Increase 81-176: Increase
	TECK	N/A			K12E5: Increase

					81-176: Increase
	MEC*	N/A			K12E5: N/E
	ENA-78*	N/A			K12E5: N/E 81-176: N/E
	IL-8*	1) NaB inhibits pathogen (synthetic triacylated lipoprotein Pam3CSK4) triggered IL-8 expression[277] 2) NaB enhances IL-8 secretion in response to IL-1b and LPS[74] 3) LPS stimulated with NaB increases IL-8 expression more than NaB alone[86]	1) Caco-2 2) 3) Caco-2	Decrease	K12E5: N/E 81-176: Increase
	IP-10*	N/A			K12E5: Increase 81-176: N/E

Table A1.6 Literature results for *B. theta* inoculation in combination with NaB supplementation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	My result
B. theta/ 5mM NaB	hBD-1	N/A	Not different from negative, but increase from *B. t* alone
	hBD-3	N/A	Not different from negative, but increased from *B. t* alone
	TLR2*	N/A	N/E
	TLR5	N/A	Increase from negative and *B. t* and NaB alone
	TLR9*	N/A	Increased from negative, *B. t* alone, NaB alone
	IL-12β*	N/A	N/E (trend, NaB alone decreased relative to negative)
	TNF*	N/A	Increase from negative, but not from *B. t* or NaB alone
	TGF-β	N/A	N/E
	MCP-1*	N/A	N/E
	TECK	N/A	Increased from negative and from *B. t* alone. Same as NaB alone
	MEC*	N/A	N/E
	ENA-78*	N/A	Increased from negative and from NaB alone, but the same as *B. t* alone
	IL-8*	N/A	Not different from negative or *B. t* alone. Different from NaB alone (*B. t* effect)
	IP-10*	N/A	Increased from *B. t* and NaB alone.

Table A1.7 Literature results for *B. theta* and *C. jejuni* inoculation in combination with NaB supplementation of cell culture or animal models contrasted with results from this study.

Stimulus	Gene	Literature N/E = no effect, N/A = not applicable	My result
C. jejuni /B. theta/5 mM NaB	hBD-1	N/A	K12E5: Increased from Neg, *B. t* and *C. j* alone. Not different from NaB alone, or NaB/*C. j* or NaB/*B. t* 81-176: Increased from Neg, *B. t* and *C. j* alone. Not different from NaB alone, or NaB/C. j or NaB/*B. t*
	hBD-3	N/A	K12E5: Increased from Neg, NaB, *B. t* and *C. j* alone, NaB/*C. j* K12E5 and NaB/*B. t* 81-176: Increased from Neg, *B. t*, NaB, *C. j* alone, and NaB/*B. t* but the same as NaB/C. j 81-176
	TLR2*	N/A	K12E5: N/E 81-176: Same as NaB/*C. j* 81-176, decreased from negative
	TLR5	N/A	K12E5: Increased from Neg, *B. t*, NaB, and *C. j* alone. Not different from NaB/*C. j* or NaB/*B. t* 81-176: Increased from *B. t*/*C. j* 81-176, but the same as all others.
	TLR9*	N/A	K12E5: N/E (Due to large error) 81-176: Increased from negative, *C. j* 81-176, *B. t*, NaB alone, *B. t*/*C. j* 81-176, and NaB/*C. j* 81-176. The same as NaB/*B. t*
	IL-12β*	N/A	K12E5: N/E 81-176: N/E
	TNF*	N/A	K12E5: Increased from everything 81-176: Increased from negative, *C. j* 81-176. Not different from *B. t*, NaB, or NaB/*C. j* 81-176, NaB/ *B. t*
	TGF-β	N/A	K12E5: N/E 81-176: N/E
	MCP-1*	N/A	K12E5: Increased from Neg, *C. j* K12E5. Not different from *B. t* or NaB alone, or *B. t*/*C. j* k12E5 or NaB/*C. j* k12E5. Increased from *B. t*/NaB

			81-176: Increased from negative, *C. j* 81-176, and NaB/ *B. t*. Not different than *B. t*, NaB, or *B. t*/ *C. j* 81-176
	TECK	N/A	K12E5: Increased from negative, *C. j* alone, *B. t* alone. Not different from NaB, NaB/*C. j* K12E5, *B. t*/*C. j* K12E5 or NaB/*B. t* 81-176: Increased from negative, *C. j* 81-176, *B. t*, *B. t*/*C. j* 81-176. The same as NaB/*C. j* 81-176, NaB/ *B. t* (NaB effect)
	MEC*	N/A	K12E5: N/E
	ENA-78*	N/A	K12E5: Increased from everything 81-176: Increased from negative, *C. j* 81-176 and NaB alone, and NaB/*C. j* 81-176. Not different than *B. t* alone, *B. t*/*C. j* 81-176 or NaB/*B. t*
	IL-8*	N/A	K12E5: Increased from negative, *C. j* K12E5, *B. t*, NaB, NaB/*B. t*, and Nab/*C. j* K12E5. Not different from *B. t*/*C. j* K12E5 81-176: Increased from negative, *C. j* 81-176, *B. t*, and NaB alone, NaB/*C. j* 81-176, and NaB/*B. t*. The same as *B. t*/ *C. j* 81-176.
	IP-10*	N/A	K12E5: Increased from negative, *B. t* and NaB alone. Same as *C. j* K12E5 alone, *B. t*/ *C. j* K12E5, Na/*C. j* K12E5, and NaB/*B. t* 81-176: Increased from negative, *B. t*, *C. j* 81-176 and NaB alone, *B. t*/*C. j* 81-176, NaB/*C. j.* The same as NaB/*B. t*

Appendix 2 Comparative Graphs Between Baseline and Induced Expression Levels

Note: These graphs are a sacrifice between statistically accurate representations and visually understandable representations. This comes at the cost of not being able to compare between different genes on the same graph. Instead, these graphs are meant to be compared vertically, e.g. hBD-1 for the negative control in comparison qualitatively to hBD-1 when exposed to *C. jejuni* K12E5.

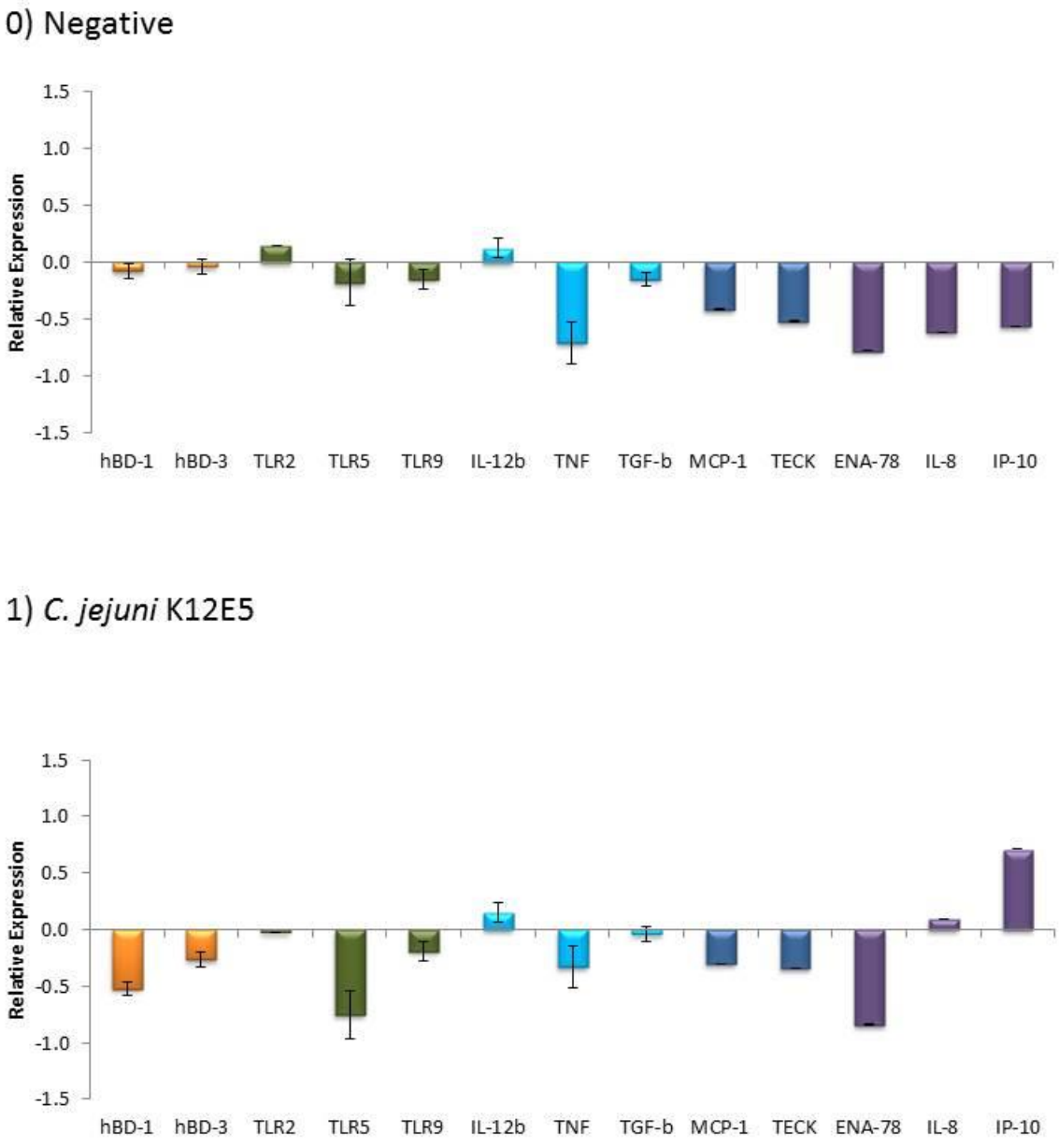
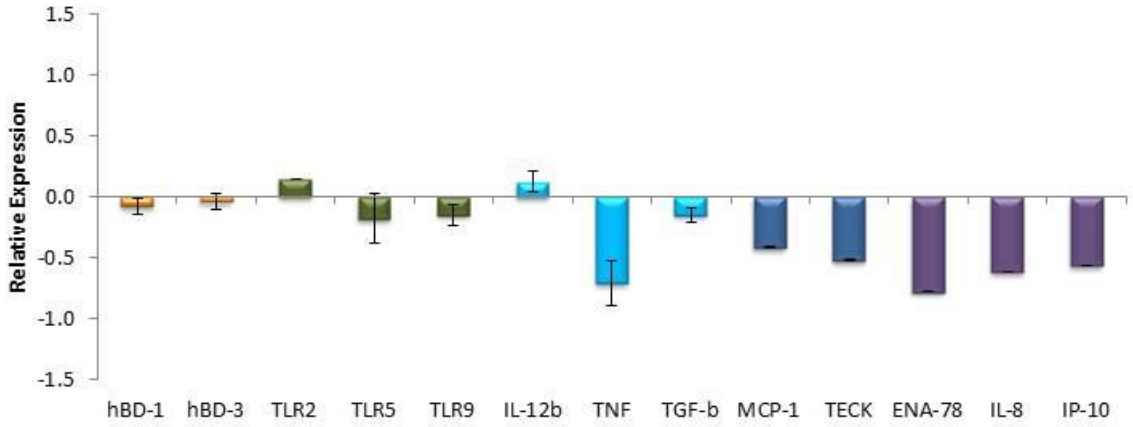


Figure A2.1 Comparison between baseline expression and *C. jejuni* K12E5 induced expression. Vertical lines associated with histogram bars are SEM.

0) Negative



2) *C. jejuni* 81-176

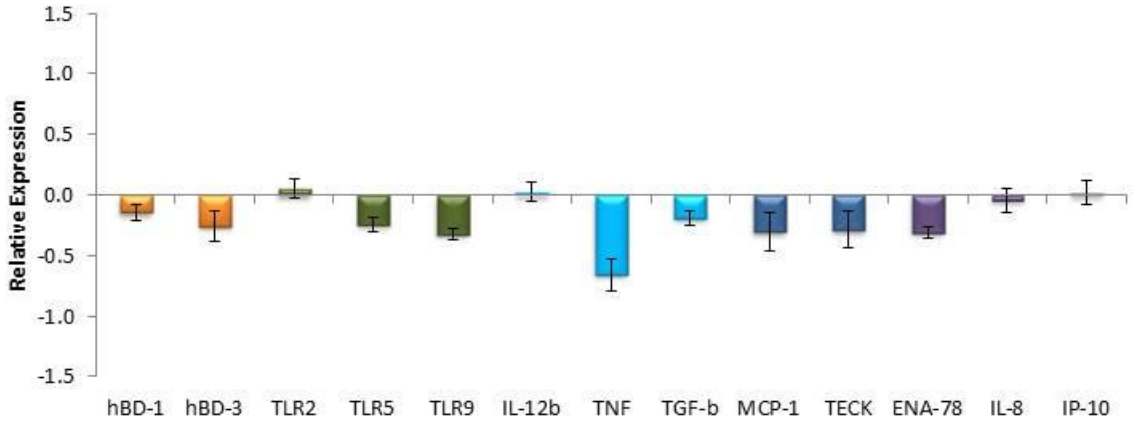


Figure A2.2 Comparison between baseline expression and *C. jejuni* 81-176 induced expression. Vertical lines associated with histogram bars are SEM.

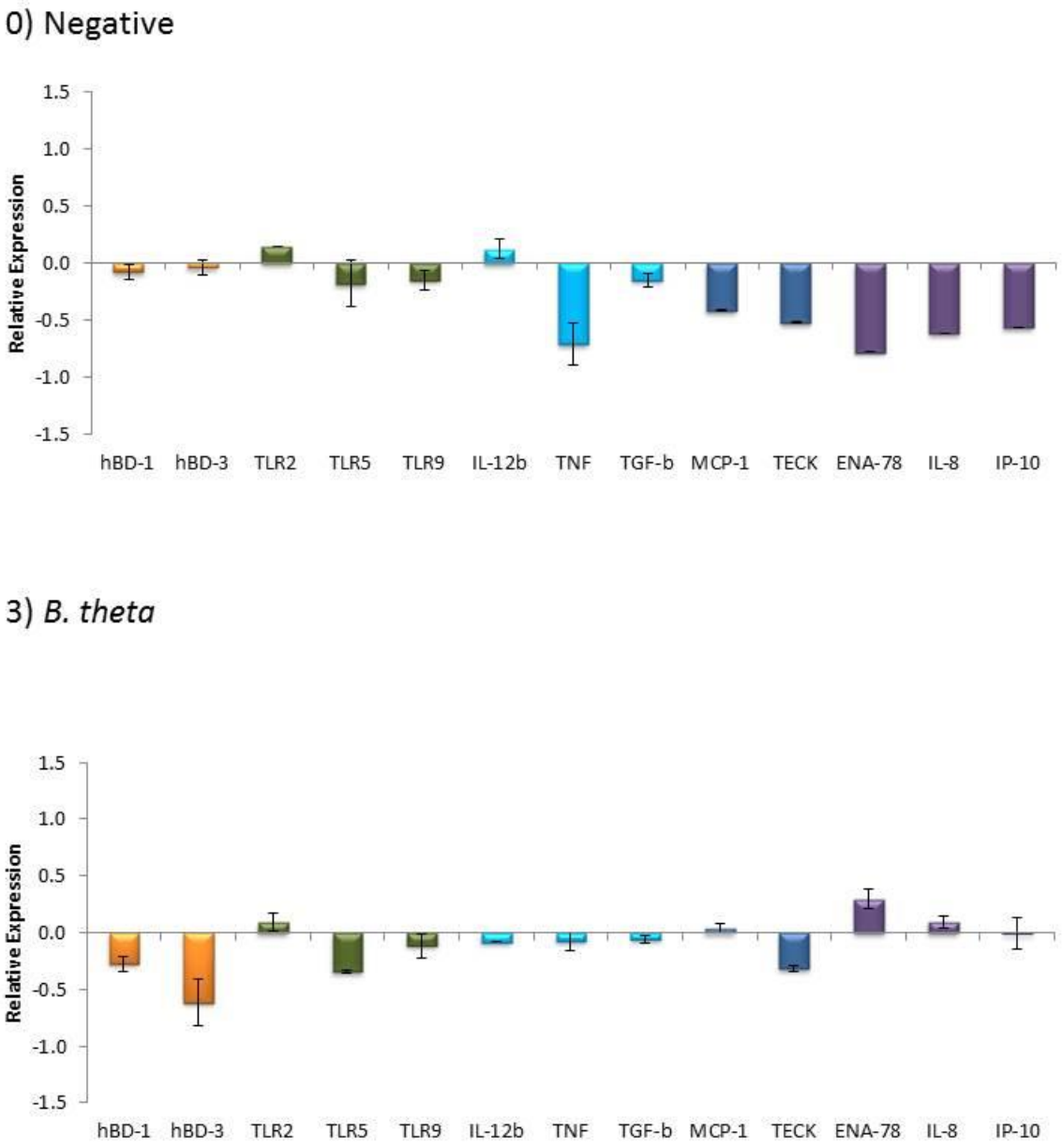


Figure A2.3 Comparison between baseline expression and *B. theta* induced expression. Vertical lines associated with histogram bars are SEM.

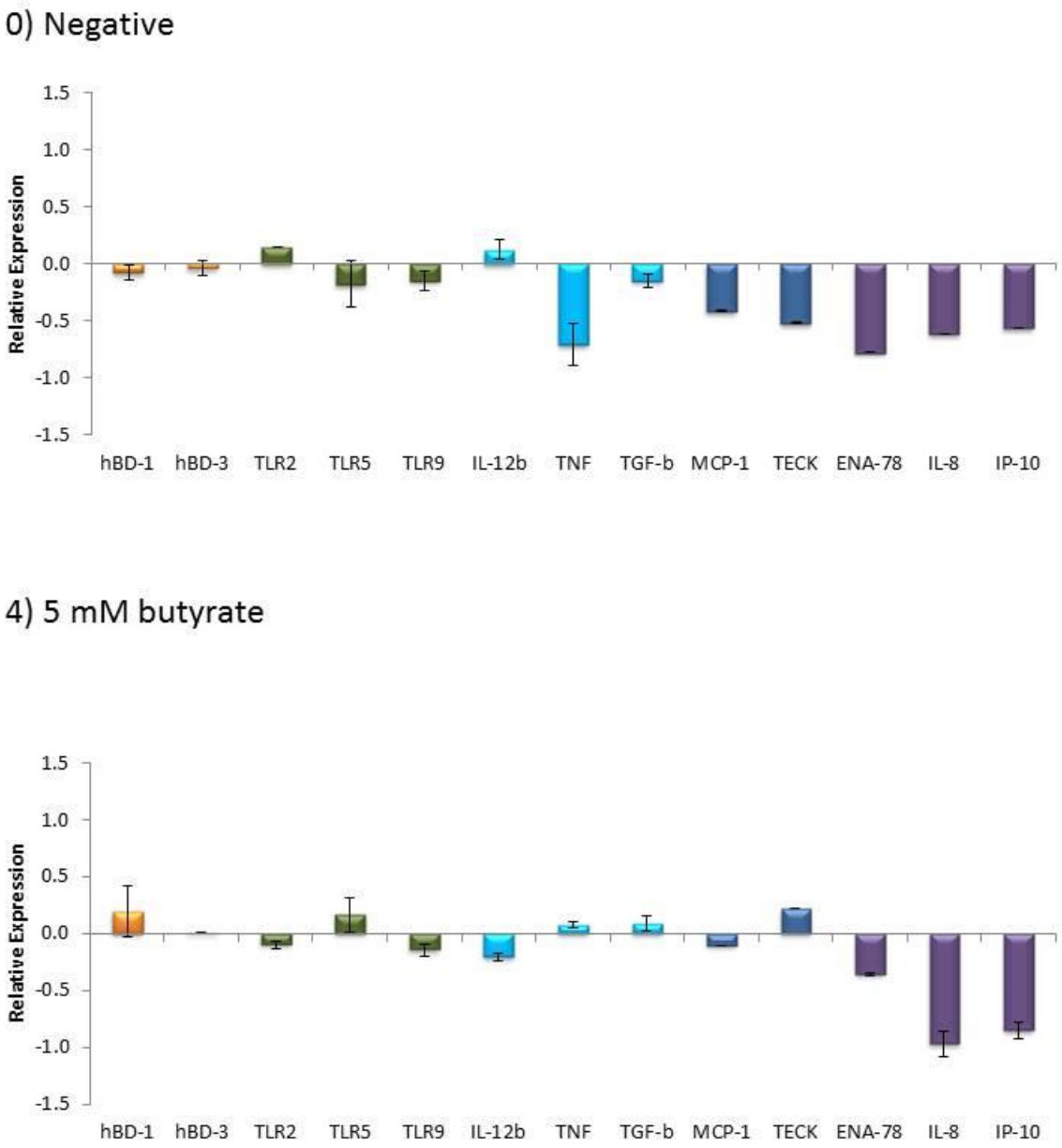


Figure A2.4 Comparison between baseline expression and 5 mM NaB induced expression. Vertical lines associated with histogram bars are SEM.

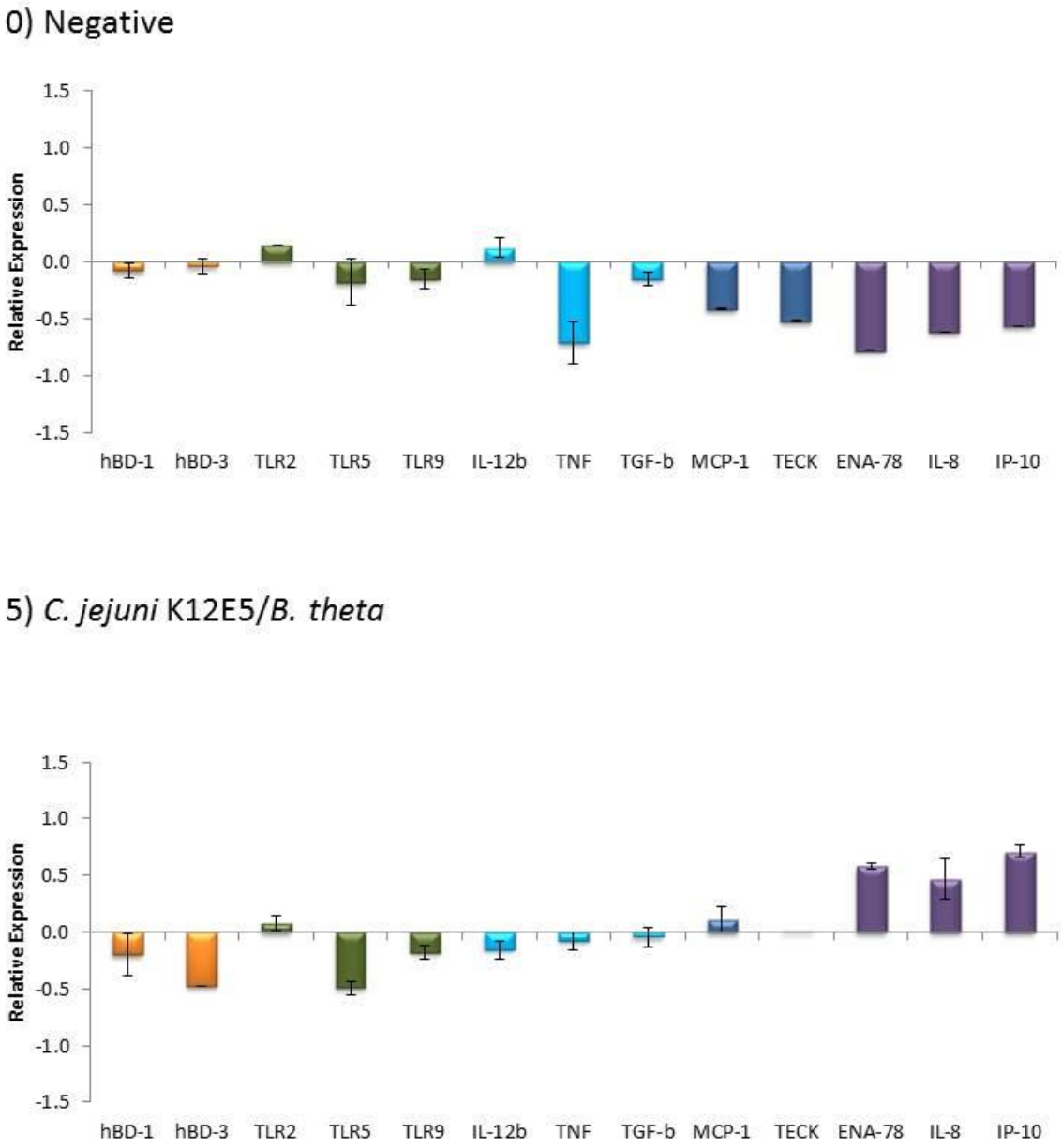


Figure A2.5 Comparison between baseline expression and *C. jejuni* K12E5 and *B. theta* induced expression. Vertical lines associated with histogram bars are SEM.

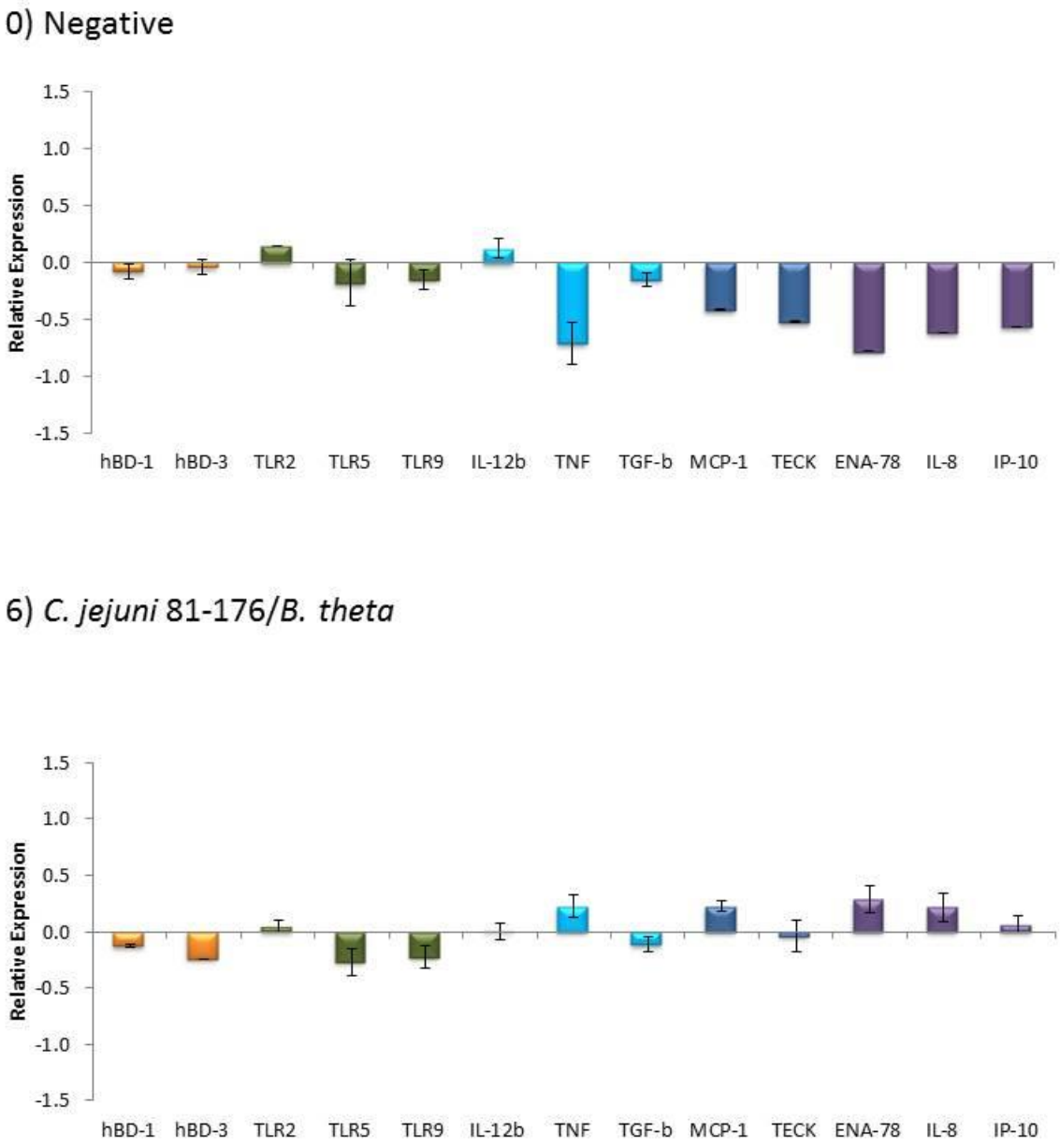
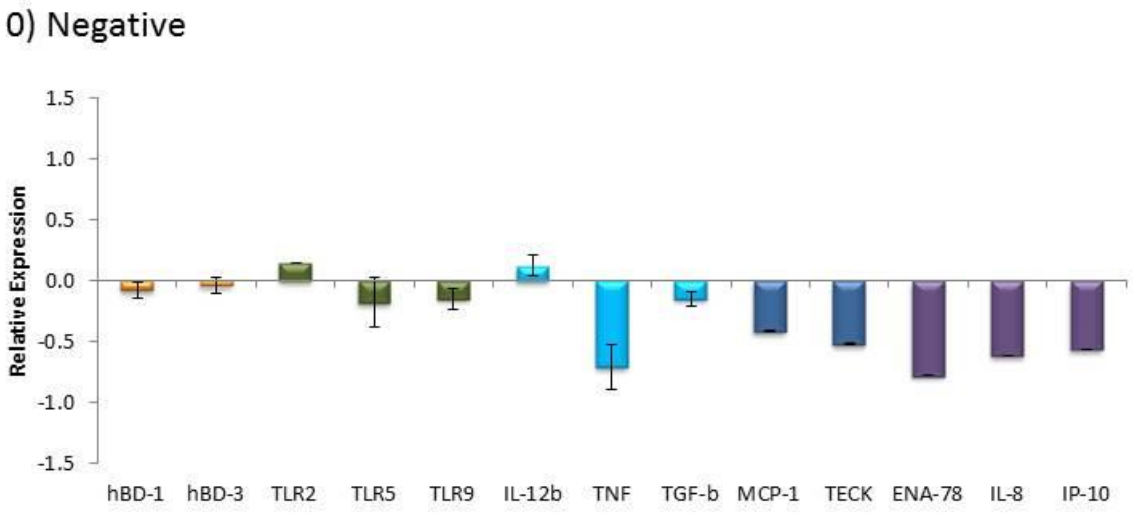


Figure A2.6 Comparison between baseline expression and *C. jejuni* 81-176 and *B. theta* induced expression. Vertical lines associated with histogram bars are SEM.



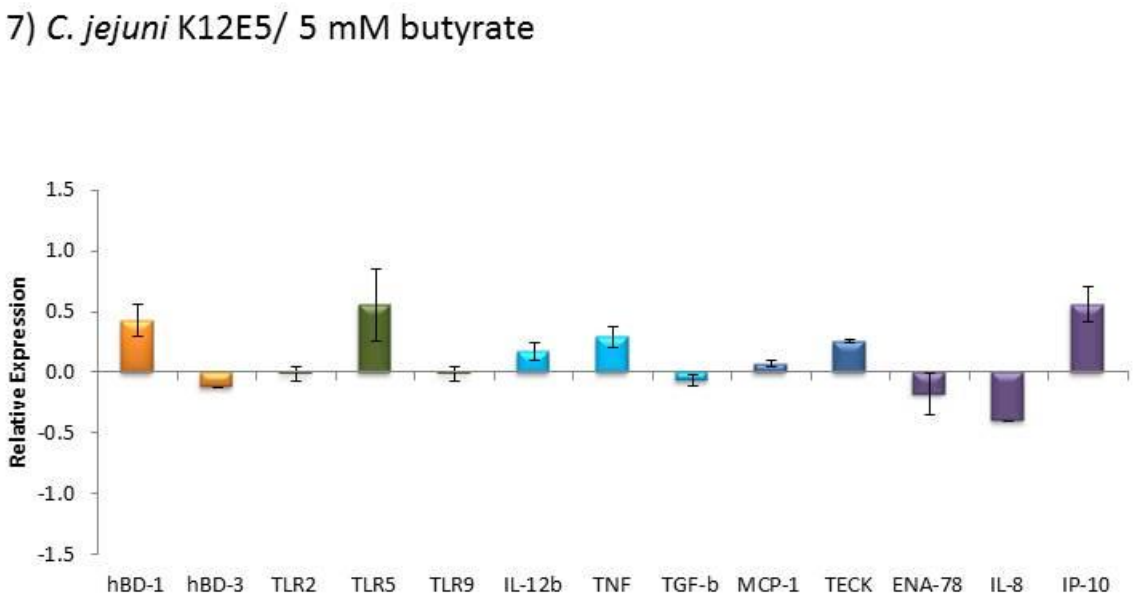
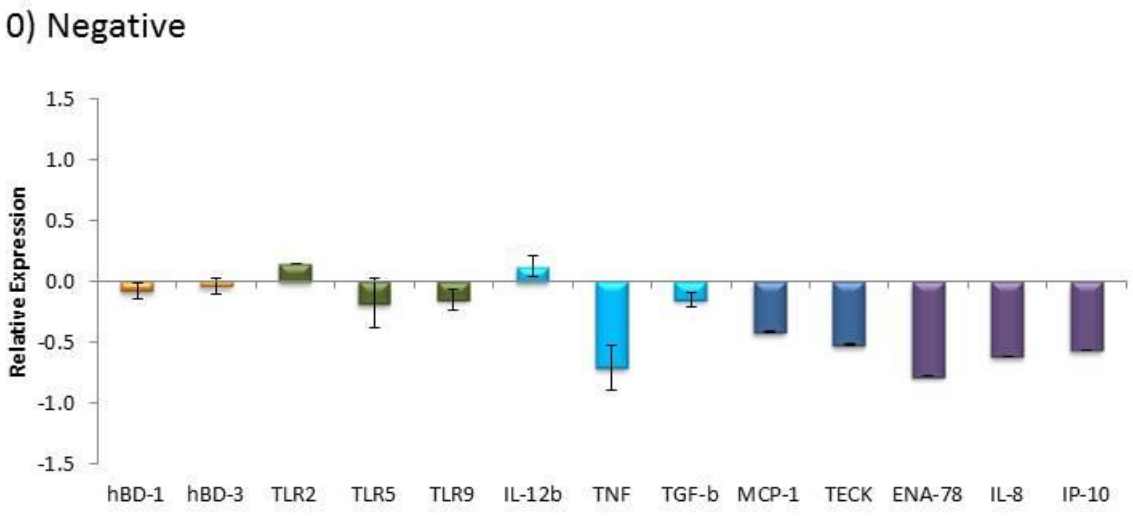


Figure A2.7 Comparison between baseline expression and *C. jejuni* K12E5 and NaB induced expression. Vertical lines associated with histogram bars are SEM.



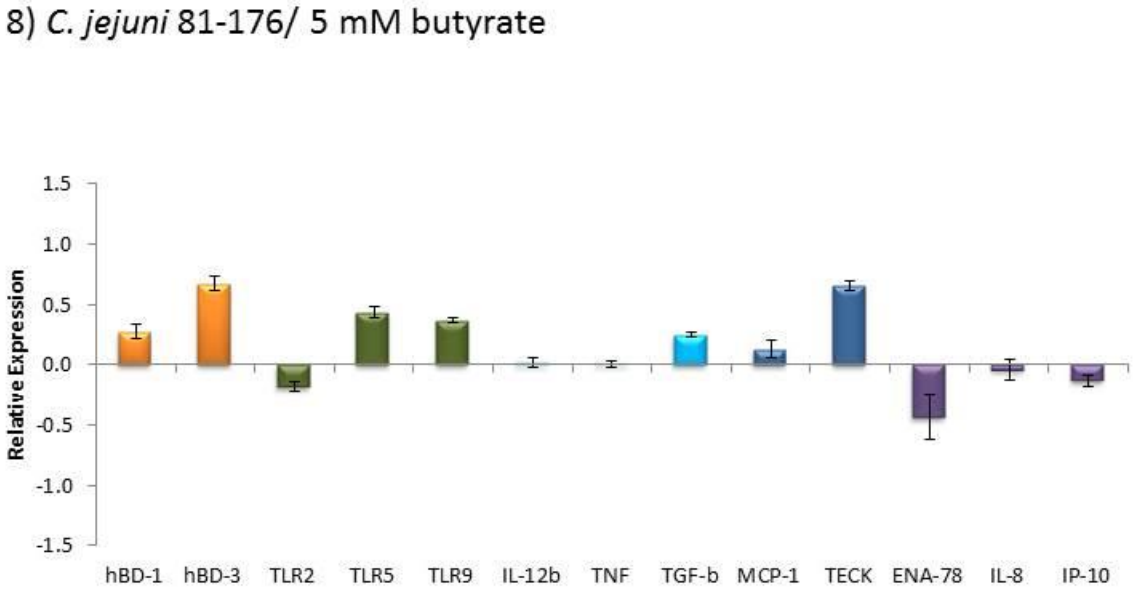


Figure A2.8 Comparison between baseline expression and *C. jejuni* 81-176 and NaB induced expression. Vertical lines associated with histogram bars are SEM.

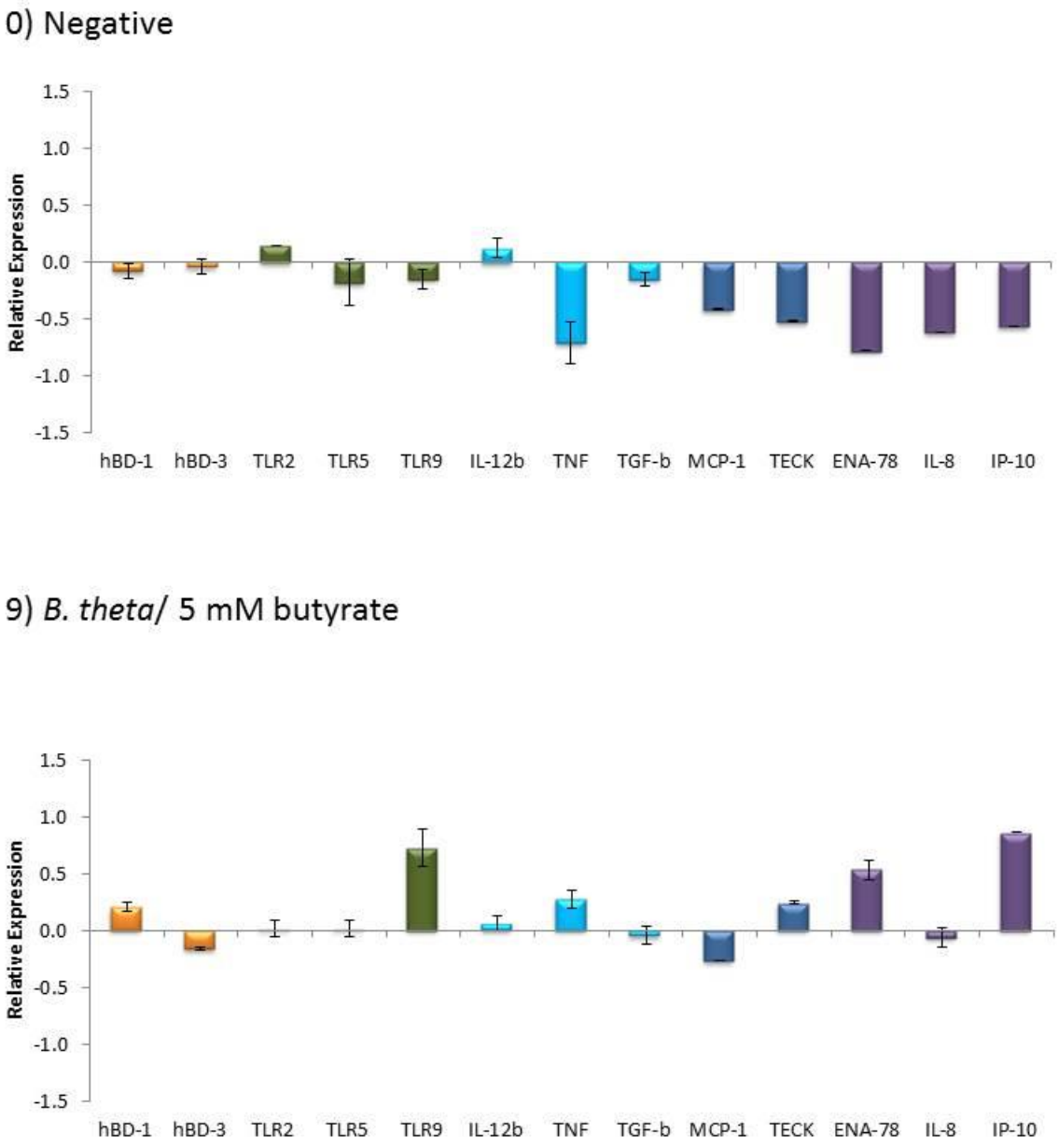
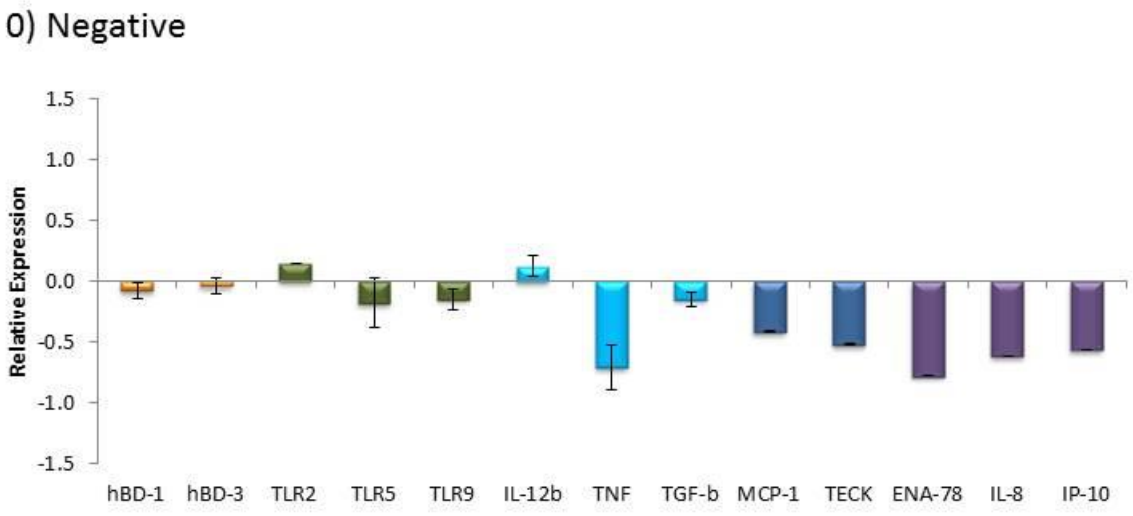


Figure A2.9 Comparison between baseline expression and *B. theta* and NaB induced expression. Vertical lines associated with histogram bars are SEM.



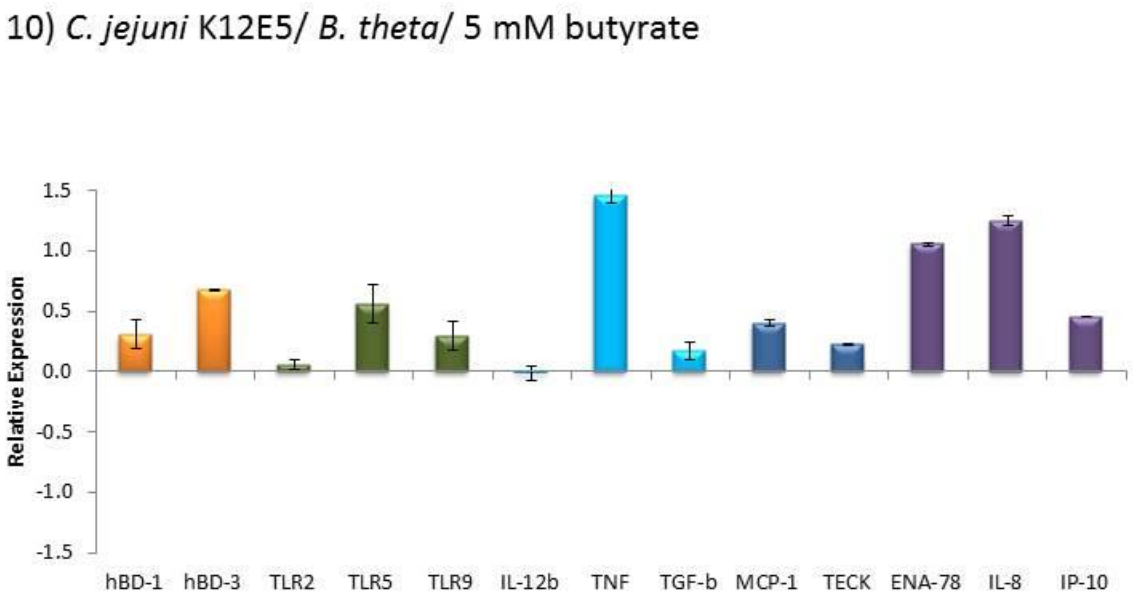
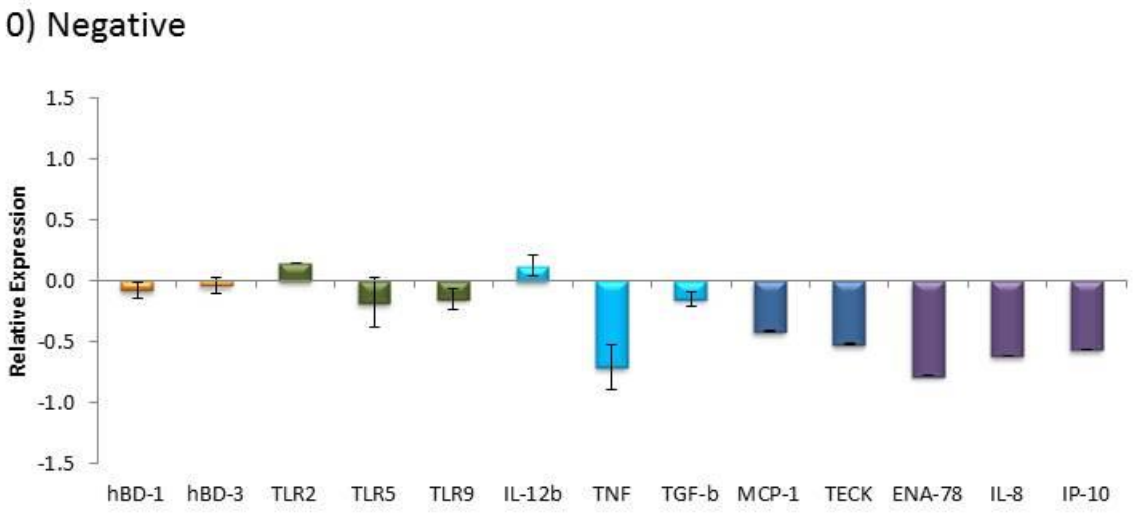


Figure A2.10 Comparison between baseline expression and *C. jejuni* K12E5, *B. theta* and NaB induced expression. Vertical lines associated with histogram bars are SEM.



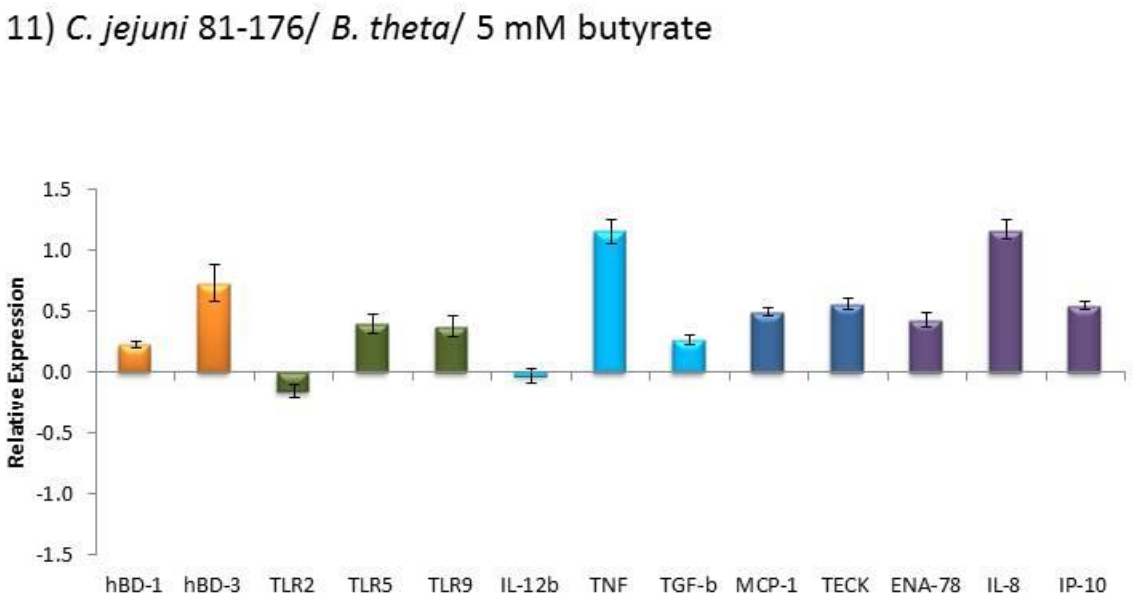


Figure A2.11 Comparison between baseline expression and *C. jejuni* 81-176, *B. theta* and NaB induced expression. Vertical lines associated with histogram bars are SEM.

Appendix 3 Media

Table A3.1 Tryptone yeast extract glucose (TYG) medium for *B. theta*

	100 mL
Tryptone Peptone	1 g
Bacto Yeast Extract	0.5 g
Glucose	0.2 g
Cysteine (Free base)	0.5 g
1M KPO_4 pH 7.2	10 mL
Resazurin (0.25 mg mL^{-1})	0.4 mL
Vitamin K solution (1 mg mL^{-1})	0.1 mL
TYG Salts	4 mL
0.8% $CaCl_2$	0.1 mL
$FeSO_4$ (0.4 mg mL^{-1})	0.1 mL
Histidine (0.2 M pH 8.0)-hematin	0.1 mL

Media without histidine-hematin was autoclaved for 20min. TYG salt solution includes $MgSO_4$-$7H_2O$, $NaHCO_3$, and NaCl. Note: Hemin (0.1% in 0.005 M NaOH), Resazurin (0.1% in dH_2O)

Table A3.2 Supplemented brain heart infusion (BHIS) medium for *B. theta*

	100 mL
Brain Heart Infusion Broth Powder (Difco)	3.7 g
Cysteine (Free base)	0.1 g
Hemin solution (0.1% in 0.005 M NaOH)	1 mL
Resazurin (0.25 mg mL^{-1})	0.4 mL
Histidine (0.2 M pH 8.0)-hematin	0.1 mL
$NaHCO_3$ solution (10%)	2 mL
Agar	1.5 g

Media was autoclaved for 20min before adding $NaHCO_3$ solution. Note: Hemin (0.1% in 0.005 M NaOH), Resazurin (0.1% in dH_2O).

Appendix 4 Confocal Microscopy

A4.1 Immunocytochemistry

Caco-2 cells were grown to confluence in CultureWell™ Chambered Coverglasses (#C37005; Sigma-Aldrich, Oakville, ON), and then washed with DPBS. At room temperature, cells were incubated with the 3.5% formaldehyde for 10 mins, followed by three DPBS washes. Fixed cells were permeabilized with 0.2 % Tween 20 (#170-6531; Bio-Rad, Hercules, CA) for 20 mins, and then washed three times with DPBS. Nuclei were stained with 1:500 DRAQ5 (ab108410) (Abcam, Toronto, ON): gelatin (#170-6537, Bio-Rad, Hercules, CA), and then washed three times with DPBS. Cells were then imaged on an Olympus Fluoview FV1000 Confocal Laser Scanning microscope.

A4.2 Fluorescence *in situ* hybridization

A4.2.1 Materials and Methods

A4.2.1.1 Mice

Twelve 3-week old female C57BL/6J mice obtained from Charles River Laboratories (Wilmington, MA) were group housed[365]. Following a 14 day adaptation period, randomly selected mice were divided into four treatment groups comprised of three mice per treatment. *C. rodentium* was used as the acute incitant of Th1 / Th17 mediated inflammation, wherein mice were orally gavaged with a suspension of *C. rodentium* cells (100 µL; $3x10^9$ CFU mL^{-1}) on two consecutive days, while being fed a control diet (Dyet #103455GI, Dyets Inc., Bethlehem, PA) for the entirety of the experiment. Conditions specified by the Canadian Council of Animal Care were met (**http://ccac.ca/en_/standards/guidelines**), and the Lethbridge Research Centre Animal Care Committee approved the project before commencement of the main study (Animal Use Protocol Review #1322).

A4.2.1.2 Treatments

Treatment A mice (n = 3) were orally gavaged with phosphate buffered saline (PBS) (26.7 mM KCl, 14.7 mM KH_2PO_4, 1379 mM NaCl, 80.6 M Na_2HPO_4-$7H_2O$, pH 6.91), with alternating day PBS enemas being administered (control treatment). Treatment B mice (n = 3) were gavaged with PBS and administered alternating day NaB enemas (100mM). Treatment C mice (n = 3) were gavaged with *C. rodentium* and administered alternating day PBS enemas. Treatment D mice (n = 3) were gavaged with *C. rodentium* and administered alternating day NaB enemas (100mM).

A4.2.1.3 Experimental Design

The experiment was designed as a complete factorial experiment arranged as a completely randomized design, with two levels of immunological stress and two levels of NaB supplementation (Table A3.1). Samples for tissue analysis were collected 14 days post *C. rodentium* infection. This time point was used based on the histological inflammation observed from a previous pilot study examining peak *C. rodentium* infection under the same conditions. Experiment carried out by Janelle Jiminez, Tara Shelton, Kathaleen House, and Jenny Gusse (AAFC, Lethbridge).

Table A4.1 Summary of mice treatments administered to female C57BL/6J mice.

	NaB - (PBS)	NaB + (100 mM NaB)
Cr-	Treatment **A**: Cr-NaB-	Treatment **B**: Cr-NaB+
Cr+	*Treatment **C***: Cr+ NaB -	*Treatment **D***: Cr+ NaB +

*Cr-: PBS gavaged, Cr+: *C. rodentium* gavaged; Supplementation with PBS (0 mM NaB) or 100 mM NaB by enema.

A4.2.1.4 *Citrobacter rodentium* Inoculation

GFP-labelled *C. rodentium* (received from Dr. Bruce Vallance, University of British Columbia) was used to inoculate mice in Treatments C and D. The bacterium was grown aerobically on Lysogeny Broth agar (LA) with 15 µg mL^{-1} chloramphenicol (Sigma-Aldrich, Oakville, ON) at 37 ^{o}C for 24 hours. Biomass was removed from the surface of LA and transferred into sterile Lysogeny Broth (LB) supplemented with 15µg mL^{-1} chloramphenicol. Cultures were maintained for 2 hours at 37 ^{o}C at 100 rpm, until the OD_{600} reading measured above 0.1. Cultures were then centrifuged at 12.3 *g* for 15 mins. The supernatant was removed, and pelleted cells were re-suspended in 3 mL PBS. Cell density was enumerated by diluting the cell suspension in a 10-fold dilution series, 100 µL was spread on LA, cultures then maintained at 37 ^{o}C for 24 hours, and CFU were counted at the dilution yielding 30 to 300 CFU per culture. Cell densities ranged from 2.3 to 5.6 x 10^{9} CFU mL^{-1}. Sterile syringes were filled with 110 µL of the cell suspension and stored at room temperature in a biological safety cabinet until used (maximum of 45 mins).

A4.2.1.5 NaB Supplementation

NaB solutions were diluted with 1X PBS solution to attain the final concentration of 100 mM; each solution was adjusted with 10 M sodium hydroxide (NaOH) to a pH between 7.2 and 7.4. The NaB solution was made a day prior to the first administration, and stored in a sealed glass container at 4 ^{o}C. NaB enemas were administered every other day for the entirety of the study. Mice were held inverted while NaB was injected into the distal colonic region with a 22G X 1.0" gavage needle with a 1.25 mm ball at the tip. Mice were kept inverted for 30 seconds after the injection. Experiment carried out by Janelle Jiminez, Tara Shelton, Kathaleen House, and Jenny Gusse (AAFC, Lethbridge).

A4.2.1.6 Mice Maintenance

Mice were maintained in individually ventilated cages under a 10:14 hour dark: light cycle. Shredded paper was provided for bedding, and mice were permitted to eat and drink *ad libitum*. Individual mouse health status was monitored daily using a quantitative scoring system[366]. Cage bedding, food, and water were replaced once a week. Body weights were measured at 7-day intervals and at the time of euthanization. Experiment carried out by Janelle Jiminez, Tara Shelton, Kathaleen House, and Jenny Gusse (AAFC, Lethbridge).

A4.2.1.6.1 Euthanization and Tissue Collection

14 days P.I., mice were anesthetised with isofluorane directly before euthanization by cervical dislocation. A lateral incision was made from the posterior to the anterior of the abdomen and the small and large intestines were extracted from the mouse. Colons were laid out and photographed, and gross pathological observations were made. A razor was used to cut the colon into 4 mm sections; biopsies from each intestine were taken for microbial DNA analysis and stored at -20 ^{o}C. The biopsy sections were measured for area and weight to enable qPCR correlation between intestinal sections. Experiment carried out by Janelle Jiminez, Tara Shelton, Kathaleen House, and Jenny Gusse (AAFC, Lethbridge).

A4.2.1.7 Histology

Within 5 mins of euthanization, colonic tissues were fixed in Surgipath® 10% neutral buffered formalin (Leica Biosystems, Concorde, ON) for 24 hours. Tissues were fixed using an Automated Leica TP1020 Fixing Machine with the program shown in Table A3.2. Fixed tissues were embedded in paraffin, and using a Leica Microtome, 5 µm sections were cut and attached to positively charged microscope slides. Experiment carried out by Janelle Jiminez (AAFC, Lethbridge).

Table A4.2 Fixing conditions for the Automated Leica TP1020 Fixing Machine.

Container	Solution	Vacuum	Time
1	Surgipath® 10% neutral buffered formalin	No	5 min
2	Alcoholic Formalin	No	1 hour
3	80% Ethanol	No	45 min
4	95% Ethanol	No	1 hour
5			
6			
7	100% Ethanol	No	1 hour
8			
9	Histo-clear (Diamed Lab Supplies, Mississauga, ON)	No	1.5 hour
10			
11	Paraffin	No	1 hour
12		Yes	1 hour

A4.2.1.8 Fluorescence *in situ* Hybridization (FISH)

The 16S ribosomal RNA oligonucleotide probes were chosen from probeBase (www.microbial-ecology.net/probebase/)[367] and are shown in Table A3.3. Oligonucleotide probes were commercially synthesized as 5'-labelled oligonucleotides with Alexa-fluors (Integrated DNA Technologies, Coralville, IA, USA) (Table A3.3). Oligonucleotide probes were optimized for FISH with pure and mixed cultures.

A4.2.1.9 FISH Controls

The EUB338 probe was optimized by modifying a previously described protocol[368]. *C. rodentium* was used for controls. For the microbial culture, one culture of 4mL was centrifuged at peak growth, for 5 min at 13,000 *g*. Pellets were re-suspended in anhydrous ethanol for 3 hours. After centrifugation at 13,000 *g* for 5 min, cells were air dried then re-suspended in FISH hybridization buffer (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl, and 0.01% SDS) containing the Alexa-fluor labelled probe at the concentrations shown in Table A3.3 at its respective incubation temperature. Cells were centrifuged again at 13,000 *g* for 5 min, and re-suspended in FISH wash solution (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl) for 15 mins. Cells were then washed in PBS, pelleted at 13,000 *g* for 5 min), and re-suspended in 100 µL PBS with 10 µL Prolong Gold Antifade Reagent (Life Science, St. Louis, MO). Circles were drawn using the ImmEdge™ Pen (H-4000) (Vector Labs, Burlington, ON), with 20 µL being added per sample. Coverslips were encased with clear nail polish to tack the coverslips together for imaging on the confocal microscope.

Intestinal section slides were subjected to fluorescence *in situ* hybridization (FISH) following the protocols in Table A3.3, modified from a protocol by Johansson and Hansson to maintain luminal content on the slides during deparaffinization[368]. For hybridization to intestinal sections, hybridizations were carried out in FISH hybridization buffer (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl, and 0.01% SDS) containing the Alexa-fluor labelled probes. Hybridization stringency was optimized for each probe over a range of incubation temperatures, with the optimal temperatures for each probe noted in Table A3.4. Slides with droplets of hybridization buffer and probe were incubated in aluminum foil covered 50 mL Falcon tubes containing hybridization buffer soaked paper towel, overnight at the indicated temperatures, in the Isotemp® Vacuum Oven Model 280A (Fischer Scientific, ON). During the first PBS wash, the nuclear stain 4'6-diamidino-2-phenylindole (DAPI) (Life Science, St. Louis, MO) was added (1/500). After hybridization, slides were washed (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl) for 10 mins. All slides were covered with Prolong Gold Antifade Reagent (Life Science, St. Louis, MO). Slides were visualized using an Olympus Fluoview FV1000 Confocal Laser Scanning microscope.

Table A4.3 Oligonucleotide sequence, target site, reference, and probe name in hybridization buffer for specific in situ hybridization with Alexa-Fluor labelled probe.

Probe	Probe Name	Target	Probe Sequence (5'-3')	Target Site (Gene, bp)	Alexa-fluor attached (nm)	Tm (^{o}C)
EUB338[369]	S-D-Bact-0338-a-A-18	Bacteria	GCTGCCTCCCGT AGGAGT	16S (338-355)	555	51.5

Table A4.4 Optimized FISH histology protocols for oligonucleotide Alexa-Fluor labeled probes including deparaffinization steps, dehydration of tissues, and hybridizing conditions for each probe.

		EUB338
Step	T_m (°C)	51.5
1	Deparaffinization 10 min	
2	**Xylene** 5 min x2	
3	Dehydration	
a	**95% Ethanol** 5 min x3	
b	100% Ethanol 5 min x1	
c	Air dry	
4	Circle sections with ImmEdge™ Pen (H-4000) (Vector Labs, Burlington, ON)	
5	Pre-warmed Slide 60°C and prepare 50mL humid tube	
6	Hybridization buffer	4 µg
7	Overnight Incubation (°C)	48 °C
8	FISH Wash Solution 10min	
9	**PBS** Wash 10min 3x	
10	Air dry	
11	Prolong Gold Antifade Reagent	

Appendix 5 Caco-2 Immune Responses

Table A5.1 NF-κB regulated pro-inflammatory PRRs, cytokines, and chemokines[240].

Inflammatory Family	**Marker**
Toll-Like Receptor (TLR)	TLR2[370], TLR9[371]
Cytokine	IL-12β[372], TNF [373]
Chemokine C-C Motif Ligand (CCL)	MCP-1[374], MEC[87]
Chemokine C-X-C Motif (CXCL)	ENA-78[375], IL-8[376], IP-10[377]