The Microbiota and NSAID-induced Enteropathy
THE INTESTINAL MICROBIOTA AND NONSTEROIDAL ANTI-INFLAMMATORY
DRUG-INDUCED SMALL INTESTINAL DAMAGE

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A Thesis

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### DESCRIPTIVE NOTE

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- ABSTRACT -

As one of the most common medications, it is reasonable to assume that the adverse effects from nonsteroidal anti-inflammatory drugs (NSAIDs) are well understood. While this is the case for NSAID-induced gastropathy, NSAID-induced enteropathy is often clinically overlooked and has a pathogenesis that is incompletely understood. The goal of this study was to determine how alterations in the microbiota impact NSAID-induced intestinal injury. Initial studies explore how gastric acid secretion suppression substantially decreases Bifidobacteria in the small intestine, and emphasize how replenishment of these bacteria results in an amelioration of NSAID-induced enteropathy. Follow-up studies involved pretreating rats with specific bacteria that have conferred protection in other models of small intestinal injury. We examined the role that acetate may play in reducing the damage by evaluating bacteria that had an acetate production gene knocked out via homologous recombination. Protection levels were similar between wildtype and knockout bacteria, and it did not appear that acetate had a key role in damage reduction. Moreover, we found that changes in intestinal damage were dependent not only on the strain of bacteria used but also on the NSAID administered. None of the bacterial pretreatments tested protected against indomethacin- or diclofenac-induced small intestinal injury, and pretreatment with one specific bacterial strain resulted in a significant worsening of damage. To gain further insight as to the potential role of the microbiota in exacerbation of injury, we conducted studies using single antibiotics and antibiotic cocktails. No single antibiotic treatment conferred protection against naproxen-induced small intestinal injury, but an
antibiotic cocktail decreased damage scores by ~46%. Furthermore we explored the effects of L-lactic acid supplementation of drinking water but this was unable to reduce naproxen-induced intestinal damage. Collectively, the work presented in this thesis provides novel insights on the relationship between alterations in the microbiota and susceptibility to NSAID-induced enteropathy.
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At 18-years-old, my world stopped spinning, albeit temporarily, when I was diagnosed with Crohn’s Disease. The realization hit me like a ton of bricks; the only thing certain in a life with Crohn’s is uncertainty. Could I take a full course load? Graduate on time? How many hospitalizations would I have? How many surgeries? How many days would I be so sick I couldn't get out of bed? How could I plan for anything? The questions come first, the game plan comes next.

“You suffer the blow, but you capitalize on the opportunity left in its wake.”

-Michael J. Fox

And although I may have passed out on my first day of grad school when I saw inside an animal the very first time, I came back for day two, and everyday following that as I slowly turned the blow of a diagnosis into a quest for answers and a PhD.

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Declaration of Academic Achievement
Experiments were conceived and designed by Stephanie D. Syer and John L. Wallace. Stephanie D. Syer and G. Webb McKnight performed all animal experiments. Jennifer Jury and Rebeca Martin helped grew bacteria used in experiments. Illumina sequencing and analysis was performed by Stephanie D. Syer, Michelle Shah, Laura Rossi, Fiona Whelan and Michael Surette. Rory W. Blackler contributed to the antibiotic cocktail studies. Stephanie D. Syer and John L. Wallace performed data analysis. Stephanie D. Syer and G. Webb McKnight performed all other experiments. Stephanie D. Syer wrote this thesis with contributions from John L. Wallace.
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- LIST OF ABBREVIATIONS AND SYMBOLS -

~  Approximately
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°C Degree(s) Celsius
15-HETE 15-Hydroxyeicosatetraenoic acid
AA Arachidonic acid
ABC ATP-binding cassette
ANOVA Analysis of variance
ATP Adenosine triphosphate
ASA Acetylsalicylic acid
BA  *Bifidobacterium* *adolescentis* JCM 1275
BL  *Bifidobacterium longum* subsp. *longum* JCM 1217
BN  *Bifidobacterium longum* subsp. *longum* NCC 1205
BNKO BN bacteria lacking the BL0033 gene
cAMP Cyclic adenosine-3',5'-monophosphate
CFU Colony forming units
cm Centimeters
CMC Carboxymethylcellulose
COX Cyclooxygenase
COX-1 Cyclooxygenase-1
COX-2 Cyclooxygenase-2
CRE cAMP response elements
DGGE Denaturing gradient gel electrophoresis
DMSO Dimethylsulphoxide
EGF Epithelial growth factor
ELISA Enzyme-linked immunosorbent assay
ER Endoplasmic reticulum
FPrau  *Faecalibacterium prausnitzii* A2-165 DSM 17677
FPrau SN FPrau supernatant
g G-force
G-proteins Guanosine nucleotide regulatory proteins
GF Germ-free
GI Gastrointestinal
g grams
h Hour(s)
H₂RA Histamine receptor antagonist
HCl Hydrochloric acid
HOX Hydroperoxidase
H&E Hematoxylin and eosin stain
i.p. intraperitoneal
Kₘ Michaelis constant – half-maximal rate of enzyme activity
KO Knockout
ICAM-1 Intercellular adhesion molecule-1
IL Interleukin
LPS Lipopolysaccharide
Kb kilobase
kDa kilodalton
mM Millimolar
μM micromolar
mmHg Millimetre(s) of mercury
MPO myeloperoxidase
mRNA Messenger ribonucleic acid
NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
NO Nitric oxide
NSAID Nonsteroidal anti-inflammatory drug
p Probability
p.o. Oral gavage
PBS Phosphate buffered saline, pH 7.4
PG Prostaglandin
PGD₂ Prostaglandin D₂
PGE₂ Prostaglandin E₂
PGF₂ Prostaglandin F₂
PGG₂ Prostaglandin G₂
PGG/HS Prostaglandin endoperoxide G/H synthase
PGH₂ Prostaglandin H₂
PGI₂ Prostacyclin
PGT Prostaglandin transporter
PPI Proton pump inhibitor
ROS Reactive oxygen species
RT-PCR Reverse transcriptase polymerase chain reaction
SEM Standard error of the mean
TLR-4 Toll-like receptor 4
TNFα Tumor necrosis factor-α
TXA₂ Thromboxane A₂
Vₘₐₓ Maximal rate of enzyme activity
- CHAPTER ONE -

INTRODUCTION
1.1 General Introduction

Due to their anti-inflammatory, analgesic and anti-pyretic properties, nonsteroidal anti-inflammatory drugs (NSAIDs) have become one of the most widely used classes of drugs in the world. NSAIDs are generally weak organic acids, making them well absorbed when taken orally, highly bound to plasma proteins and excreted by tubular or glomerular secretion (Burke 2006). NSAIDs are a heterogeneous group of compounds that can be categorized into six classes: 1. salicylic acid derivatives (aspirin), 2. acetic acids (indomethacin and diclofenac), 3. propanoic acid derivatives (naproxen and ibuprofen), 4. oxicams (piroxican), 5. pyrazolones (phenylbutazone), and 6. fenamic acids (mefenamic acid) (Burke 2006; Wallace 1992). Regardless of the categorization, NSAIDs exert their desired effects by inhibiting one or both of the cyclooxygenase (COX) enzymes, COX-1 and COX-2.

NSAIDs are the first-line therapy for a wide range of inflammatory conditions including, but not limited to, osteoarthritis, rheumatoid arthritis and chronic pain (Wallace 1992; Harris 1999; Ruoff 1999). In the US alone, prescription costs for NSAIDs in 2001 exceeded US$4.8 billion in addition to the estimated US$3 billion for over-the-counter NSAIDs (Laine 2001). With an aging population in first world countries, it is reasonable to assume that the prevalence of NSAID use will continue to expand as the incidence of inflammatory conditions, such as osteoarthritis rises. Approximately 70% of elderly (≥65 years of age) individuals consume NSAIDs at least once a week and up to half of those individuals take NSAIDs daily (Scarpignato and Hunt 2010). As new therapeutic niches for NSAIDs continue to be recognized,
for example, slowing the progression of Alzheimer’s disease or as a cancer chemo preventative agent, sales of NSAIDs are expected to grow.

Despite their frequent clinical use and proven effectiveness at mitigating pain and inflammation, NSAIDs have a relatively high incidence of adverse effects (Wallace 1992; Polisson 1996; Tannenbaum 1996). One of the more frequent detrimental effects of NSAID use is gastrointestinal ulceration. NSAIDs induce clinically significant gastroduodenal ulceration and bleeding while also causing symptoms of nausea, dyspepsia and abdominal pain (Silverstein 2000). In the late 1990s, GI complications associated with NSAID use were estimated to cost as much as US$3.8 billion annually. The limitations of NSAID use (adverse effects) as well as the associated economic burden have prompted clinical and experimental research into development of safer anti-inflammatory drugs (Bloom 1988; Smalley 1996; Griffin 1998). This chapter will focus on the pathogenesis of NSAID-induced GI damage including a brief history, a description of the biosynthesis of prostanoids and inhibition of cyclooxygenase enzymes, mucosal defense and the role of the intestinal microbiota in health and disease.

### 1.2 History of NSAIDs

The origins of aspirin, the pioneer drug of the NSAID family, can be traced back to the use of willow bark (*Salix alba vulgaris*) for treating inflammation (Insel 1990; Vane 1990). Hippocrates, often regarded as the father of modern medicine, left notes of a willow bark treatment for fever and pain (Vane 1990). In 1763, the first
clinical trial, conducted by Edmund Stone, confirmed the ability of the willow to cure fever (Stone, 1763). Over 60 years later, in 1829, the active ingredient in willow bark was isolated and crystallized. The bitter glycoside compound was named salicin by Leroux, the founding French pharmacist. In 1859, Kolbe first synthesized salicin and by 1884 synthetic salicins (for example salicylic acid and sodium salicylate) were in production in Germany. Synthetic salicins, quickly became the preferred form of the drug as they were more soluble and more effective at relieving fever and pain (Burke 2006). Despite salicylic acid's widespread use in Europe at the time, patients complained of the bitter taste. In the late 19th century chemists began to search for a derivative of salicylic acid that exhibited high efficacy, was safe to use and no longer had a bitter taste. Felix Hoffman, a young chemist employed by the Bayer Corporation at the time, was attempting to make a better tasting derivative of salicylic acid for his father who had arthritis and complained about the taste of his medication. By acetylation of the hydroxyl group of the benzene ring of salicylic acid, Felix was able to synthesize acetylsalicylic acid (Insel 1990; Wallace 1992). In 1899, Heinrich Dresser, Bayer's chief pharmacologist, introduced this new drug, aspirin, onto the market (Wallace 1997). Despite widespread use, the mechanism of action remained elusive for over 70 years. In 1971 Vane, Smith and Willis, and Ferreira discovered that aspirin and other NSAIDs worked by inhibiting prostaglandin synthesis (Vane 1971, Smith and Willis 1971, Ferreira 1971). Even before the mechanism was elucidated, there was evidence that NSAID administration could be damaging to the gastrointestinal tract, and in the late 1930s, gastroscopic observations led Douthwaite and Lintott to report that NSAIDs
could damage the stomach (Douthwaite and Lintott, 1938). In the ensuing years there was an increase in evidence for additional adverse effects related to NSAID use. This was likely attributable to higher potency of NSAIDs (i.e., indomethacin) as well as advanced visualization tools (i.e., flexible endoscopy) (Insel 1990).

1.3 Prostanoids

1.3.1 General Introduction: Prostanoids (prostaglandins and thromboxanes) belong to a hydroxyl fatty acid group, which, along with prostacyclin, lipoxins, hepoxylinis and leukotrienes make up the eicosanoid family of lipid mediators (Smyth 2006). Eicosanoids are ubiquitous within the body and act in an autocrine or paracrine fashion. They are short-lived and mediate their effects locally, not using the circulatory system to reach their site of action (Smith 1989; Campbell 1990; Smyth 2006; Funk 2011). All eicosanoids are derived from a 20-carbon polyunsaturated fatty acid. For mammals, the most common precursor is 5,8,11,14-eicosatetraenoic acid, better known as arachidonic acid (AA) (Smith 1989). AA is released from membrane lipids in response to various stimuli, which activate acyl hydrolases (most notably phospholipase A). Eicosanoids include many different mediators. The next section will focus on the biosynthesis of prostanoids, the key enzymes required for their biosynthesis and how NSAIDs inhibit the process.

1.3.2 Biosynthesis: Prostaglandins and thromboxanes are synthesized in a step-wise process (Figure 1): 1. AA is released from glycoprophospholipids by phospholipases; 2. cyclooxygenation and hydroperoxidation of AA by prostaglandin
endoperoxide G/H synthase (PGG/HS also referred to as cyclooxygenase; COX); 3. Metabolism of prostaglandin H₂ (PGH₂) by specific isomerases to biologically active end products. AA can be released from the membrane directly via phospholipase A and indirectly by phospholipase C and diacylglycerol lipase. The dominant pathway is direct release via phospholipase A (Davies 1984, Smyth 2006). The prostanoids are synthesized in response to a variety of different stimuli, which include hormones (i.e. vasopressin) (Garcia-Perez 1984), autacoids (i.e. bradykinin) (Neufield 1983), growth factors (i.e. epidermal growth factor) (Levine 1977) and mechanical stress (i.e. shear force on arterial endothelium) (Frangos 1985). The prostanoids maintain homeostasis as well as have a role in the inflammatory process, initiating and resolving inflammation (Ricciotti and Fitzgerald 2011).
Figure 1.1: Biosynthesis of Prostaglandins and Thromboxanes. Prostaglandins and thromboxanes are synthesized in a step-wise process initiated by the release of AA from membranes. Prostaglandins are synthesized in response to a number of triggers including, but not limited to, hormones and mechanical stress. Enzymes responsible for prostaglandin synthesis are denoted in violet italics. Adapted from Jacob de Wolff, 2007.

Triggers (e.g., cytokines) initiate the release of AA from the membrane within the vicinity of COX. Once AA is freed from the membrane is it rapidly metabolized via COX, converting it into endoperoxide prostaglandin H_{2} (Davies 1984). This conversion occurs in two steps, the first being a bis-dioxygenation reaction. This step adds two molecules of oxygen to AA, one at carbon 9 and the second at carbon 11 forming an unstable intermediary endoperoxide (prostaglandin G_{2}, PGG_{2}) (Davies 1984; Smith 1989). By adding the oxygen, two double bonds are broken resulting in the formation of a cyclopentane ring (Davies 1984; Smith 1989). This ring is common to all prostaglandins. The second step is a hydroperoxidase (HOX) reduction, which converts PGG_{2} to PGH_{2} by reducing the 15-hydroperoxyl group of PGG_{2} by two electrons (Smith 1989). PGH_{2} is the unstable precursor for the formation of all prostanoids (Funk 2001).

The final step in the biosynthesis of prostanoids can occur non-enzymatically or through specific isomerases (Funk 2001). This final step of determining which prostanoid will be formed is reliant on what specific synthases or isomerases are in the cell. For example, PGE_{2} and TXA_{2} from COX-2 are dominant in activated macrophages whereas platelets predominantly produce TXA_{2} derived from COX-1 (Smyth 2006). There are five groups of prostanoids, all of which are derived from
PGH₂. The five groups of prostanoids consist of four prostaglandins and the thromboxanes (Funk 2001). The prostaglandins differ by cyclopentane ring substitutions and are denoted by different letters (including, but not limited to, D, E, F and I) (Campbell 1990). This group is further subdivided by the number of double bonds the molecule has on its side chain. The number following the letter designation denotes the number of double bonds (for example, PGD₂ has two double bonds and is from the D class of prostaglandins) (Campbell 1990). Thromboxanes do not have a cyclopentane ring like prostaglandins do but rather possess an oxane ring. TXA₂ is the active compound, which is unstable and is non-enzymatically converted to TXB₂ (Smyth 2006). PGD and PGE contain hydroxyl and ketone groups on the cyclopentane ring and are formed by PGH-PGD isomerase and PDH-PGE isomerase respectively. Fα prostaglandins have two hydroxyl groups on the cyclopentane ring and formed from PGH₂ by PGFα reductase (Smyth 2006). Prostacyclins (PGIs) are formed from PGH₂ by the prostacyclin synthase enzyme. PGIs have an oxygen bridge between the 6th carbon on the side chain and the 9th carbon in the cyclopentane ring that gives a double ring structure unique from other prostaglandins (Smyth 2006). PGI₂ is an unstable active intermediate, which is non-enzymatically converted to inactive 6-keto-PGF1α (Smyth 2006).

The five classes of receptors are named according to the prostanoid letter they have the highest affinity for (example: PGI₂ binds with the greatest affinity to the IP receptor) (Smyth 2006). All eicosanoid receptors are G-protein coupled receptors (GPCRs) and thus determine adenylyl cyclase and phospholipase C activity by
interacting with three of the four G proteins (G_α, G_i and G_q) (Smyth 2006). When the GPCRs are activated they generate secondary messengers that mediate cellular effects. Prostanoid biosynthesis is complex and ubiquitous, emphasizing their physiological importance (Smyth 2006).

1.4 The Cyclooxygenase enzymes

1.4.1 Cyclooxygenase Isozymes: As noted, cyclooxygenase (COX) is the enzyme, which converts AA to PGH₂. The enzyme is found in two isoforms, termed COX-1 and COX-2. COX is responsible for oxygenation of AA and for reducing PGG₂ to PGH₂. COX-1 was first identified in the mid 1970s and cloned in 1988, although postulated by Flower and Vane in 1972, the existence of a second COX isoform was only confirmed in 1991 (Hemler 1976; DeWitt 1988; Kujubu 1991; O’Banion 1991; Xie 1991). The structure of the enzyme was determined via X-ray crystallography in 1994 (Picot 1994). There are a number of differences that exist between the two closely related enzymes. The genes encoding the different isoforms are located on different chromosomes, COX-1 is found on chromosome 1 and COX-2 is found on chromosome 9. The COX-1 gene is much larger than the COX-2 gene, (22.5 kb and 8.3 kb respectively) due to smaller introns in COX-2 as well as the merging of exons 1 and 2 from COX-1 into a single exon for COX-2 (Smith 2000). The mRNA for COX-1 is 2.8 kb, whereas it is 4.8 kb for COX-2. Within a species, the isoforms have 60% sequence homology and translational gene products are close in size, differing by only 11 amino acids (COX-1 has 576 and COX-2 has 587) (Smith 1996; Smith 2000). Biochemically, they have similar enzymatic function of AA with similar V_max and K_m.
values (Crofford 1997). The protein structures are very similar and have molecular weights of approximately 70 kDa (Vane 1998). Structurally, the two isozymes are extremely similar, differing by only one amino acid. Despite the similarity, the alteration results in a larger side pocket for COX-2 substrate access (Smith 2000). Both COX enzymes are comprised of two homodimer subunits that align in a ‘head-to-toe’ fashion (Smith 2000). Each subunit has an EGF-like domain, a membrane-binding domain and a catalytic domain (Smith 2000). Regardless of their structural similarities, the enzymes differ on substrate preferences. For example, COX-2 enzyme is able to more efficiently use alternative substrates such as eicosapentaenoic acid and linoleic acid as compared to COX-1 (Vane 1998).

Within a cell, COX-1 is associated with the endoplasmic reticulum membrane and COX-2 is localized more to the nuclear envelope (Crofford 1997). Historically and incorrectly, COX-1 has been regarded as the housekeeping enzyme, maintaining homeostatic PG levels. This notion was reinforced with the knowledge that COX-1 is constitutively expressed in most cell types (Funk 2001). The COX-1 gene is continuously transcribed and provides constant enzyme levels (Smith 1996; Crofford 1997). In contrast to this, COX-2 is an immediate-early gene as it has rapid up-regulation (up to 80-fold increases in expression within 3 hours) in response to cytokines (i.e. IL-1, IL-10), shear stress, mitogens (i.e. lipopolysaccharide; LPS) as well as growth factors (Smith 1996; Crofford 1997). The COX-2 gene includes a cAMP-response element binding protein, nuclear factor-κB, and CAAT-enhancer binding proteins (Crofford 1997). COX-2 mRNA is less
stable than COX-1 mRNA due to polyadenylation signals and instability sequences which make it easier to degrade (Crofford 1997).

In contrast to the initial dogma stating COX-1 was regulatory and COX-2 was inducible, further evidence emerged demonstrating COX-1 expression can be induced by bacterial endotoxin, cytokines and growth factors (Ferraz 1997; Diaz 1998; Bryant 1998). Moreover, the COX-2 enzyme is expressed constitutively in some tissues having roles in development and function (for example, the kidney) (DuBois 1998). These findings make it difficult to classify COX-1 and COX-2 based on constitutive and inducible tissue expression, but they do provide evidence against over-generalizing the distinct functions of the two isoforms.

1.4.2 Mechanisms of NSAID inhibition of Prostaglandin biosynthesis via COX:

NSAIDs exert their therapeutic effects by inhibiting PG synthesis as first described by Vane and colleagues in 1971 (Vane 1971). All NSAIDs inhibit PG synthesis by interacting with the bis-oxygenase subunit of COX. This interaction prevents the introduction of an oxygen moiety and cyclization of AA (Burke 2006). How the NSAID will inhibit the bis-oxygenase subunit depends on both the NSAID itself as well as the COX isoform. With the exception of aspirin, NSAIDs reversibly inhibit COX. NSAIDs directly compete with AA for the COX binding site but have little effect on the peroxidase activity of the enzyme (Smith 2000).
There are three classifications of inhibition mechanisms that NSAIDs can be categorized into, often denoted as class I, II or III. Class I inhibitors inhibit COX in a reversible, competitive manner (Smith 2000). They compete with AA for the active site but do not alter the conformation of the enzyme. This type of inhibition can be overcome by an increase of substrate (AA) availability (Burke 2006). Naproxen and ibuprofen are examples of this rapid and reversible competition inhibition.

Class II inhibitors are reversible, competitive and time-dependent. When a class II NSAID binds a COX active site, an enzyme-inhibitor complex is formed, changing the COX protein (Smith 2000). As this conformational alteration is not a covalent interaction, the COX protein will revert to its original state. Indomethacin and diclofenac are both examples of drugs exhibiting class II modes of NSAID inhibition. The carboxyl groups of AA and indomethacin bind at tyrosine 355 and arginine 120 (Palmer, 2012). Interestingly, the carboxyl group of diclofenac is hydrogen-bonded to serine 530 and to the catalytic tyrosine 385 (Palmer, 2012). The interaction of the carboxylic acid group found on all class II inhibitors and arginine 120 of COX-1 is required to suppress enzyme activity (Palmer 2012).

Class III inhibition drugs suppress COX irreversibly and in a time-dependent manner. A conformational covalent bond change occurs over seconds to minutes. The covalent bond makes the inhibition irreversible and is due to the acetylation of serine 530 on COX-1 and 516 on COX-2 (Palmer 2012). AA is prevented from accessing the active site due to steric hindrance. Aspirin is a class III drug and due
to the irreversible inactivation, once the drug is removed, the COX enzyme remains unable to synthesize PGs, regardless of an increase in AA. Cells exposed to class III NSAIDs must synthesize new COX protein to be able to once again produce PGs. Class III NSAIDs modify COX-1 differently than COX-2. At concentrations that completely abolish COX-1 enzymatic activity, the COX-2 enzyme retains functionality (Palmer 2012). Under class III inhibition, the COX-2 enzyme is able to oxygenate and cyclize AA but in an adapted manner. The oxygenation occurs at the 15th carbon instead of the usual 11th carbon, which results in the formation of 15R-hydroxyeicosatetraenoic acid (15R-HETE), opposed to the usual PGH2 (Lecomte 1994). The 15R-HETE is able to undergo 5-lipoxygenase metabolism, which results in the anti-inflammatory 15-epilipoxin (Serhan and Oliw 2001).

1.4.3 COX and mucosal defense: The advent of selective COX-2 inhibitors was based on the theory that by selectively inhibiting COX-2, pain, fever, and inflammation would be reduced while prostaglandin synthesis in the gastrointestinal tract would be spared, so damage would not be induced. This theory was based, incorrectly, on the hypothesis that COX-2 generates PGs that mediate pain, fever and inflammation whereas COX-1 generates the PGs responsible for maintaining gastrointestinal homeostasis (Wallace 1999a). In fact, COX-1 and COX-2 make the exact same prostaglandin: PGH2. The prostaglandins which are synthesized from PGH2 are determined by alternative enzymes. Once the selective COX-2 inhibitors were used clinically, it was evident that the COX injury thesis was incorrect, and that selective COX-2 inhibitors could reduce but not eliminate gastroduodenal damage (Laine
2003; Lanas 2007). It is now known that COX-1 and COX-2 each have roles in regular GI function. Although many of the PGs produced by a healthy stomach are derived from COX-1, there is evidence indicating PGs derived from COX-2 are increased after mucosal damage and when COX-1 is inhibited (Wallace et al., 2000a). Post-insult up-regulation of COX-2 derived PGs plays an important role in mucosal defense mechanisms, such as increasing blood flow, as well as in injury repair, such as ulcer healing (Ma 2002). Because of their overlapping roles, selectively inhibiting one COX enzyme is unlikely to cause injury (Wallace 2000a). The concept that mucosal defense is mediated by COX-1 and COX-2 was exemplified in rat and mouse experiments using pharmacological agents and genetic knockouts respectively. COX-1 deficient mice have low levels of gastric mucosal PGs but do not spontaneously develop gastric ulcers (Langenbach 1995). Additionally, COX-2 deficient mice had an impaired ability to resolve inflammation, indicating that COX-2 is an important contributor to anti-inflammatory mediators (Wallace, 2006). These enzymes have an important role in mucosal defense, but their inhibition by NSAIDs does not fully explain their GI toxicity. The following section will address alternative factors, which have important roles in NSAID-induced gastropathy and enteropathy.

1.5 Pathogenesis of NSAID-induced injury

1.5.1 Pathogenesis of NSAID-induced gastropathy: The focus of this thesis is on the small intestinal damage caused by NSAIDs. This section on gastropathy has been included to allow for comparisons to be made between the conditions.
Because one of the therapies used to treat gastropathy (suppression of gastric acid secretion) appears to influence susceptibility to NSAID-enteropathy, it is important to highlight that the pathogenic mechanisms differ and are not merely related to the inhibition of PG synthesis.

The two central factors affecting NSAID-induced gastropathy are topical irritation and the inhibition of the COX isoforms (Figure 1.2).

**Figure 1.2: Factors contributing to NSAID gastropathy.** By disrupting the phospholipids on the mucosal surface of the stomach and duodenum, NSAIDs can result in epithelial damage from acid exposure. Additionally, NSAIDs can result in epithelial cell death by direct contact (see text). Reduction of mucosa blood flow is the result of a decrease in potent vasodilator PGs (such as PGE$_2$ and PGI$_2$), mainly associated with inhibition of the COX-1 enzyme. Adherence of leukocytes, mainly neutrophils, to the vascular endothelium is a contributing early-factor in mucosal injury and a result of inhibition of the COX-2 enzyme.

Arguably the most widely recognized adverse event from repeated NSAID administration with regards to frequency and clinical impact is ulceration of the stomach. Superficial erosions often occur in the corpus region, but more
importantly, deep ulcers that penetrate through the muscularis mucosae form primarily in the antrum (Sostres 2010). These are more clinically relevant due to their increased likelihood of bleeding and potential for perforation (McCarthy 1990). As the stomach is an acidic environment, platelet aggregation is inhibited, contributing to gastric bleeding (Green 1978). Despite this, small intestinal damage is most likely due to difficulties in detection and the lack of effective treatments.

NSAIDs disrupt phospholipids on the mucosal surface, which can lead to an increased susceptibility of the mucosa to acid damage (Giraud 1999; Lichtenberger 2006). Additionally, since NSAIDs are organic acids, they can kill epithelial cells by direct contact (Tarnawski 1988). The theory behind this is that NSAIDs can become charged from stomach acid. Charged NSAIDs can induce osmotic lysis when they are trapped within epithelial cells leading to the uncoupling of oxidative phosphorylation and eventually cell death (Somasundaram 1995). Moreover, NSAIDs interfere with epithelial repair by interrupting the epithelial growth factor (EGF) signal pathways, which are important for epithelial cell proliferation and thus repair (Pai 2001; Kajanne 2007). Although topical effects contribute to gastric damage, results from peritoneal administered NSAIDs confirm that these factors are not required for ulcer formation (Estes 1993; Wallace and McKnight 1993b). Further evidence supporting this comes from studies with pro-drug NSAIDs, which have comparable frequencies of ulceration to traditional NSAIDs (Graham 1985; Wallace 2008).
There is substantial evidence that the primary mechanism of NSAID-induced gastropathy is the suppression of mucosal PG synthesis. The ability for an NSAID to induce gastroduodenal injury correlates strongly with the extent to which it inhibits mucosal PG synthesis (Whittle 1981; Rainsford and Willis 1982). It is known that NSAIDs interfere with the host’s natural protective elements such as gastric mucus and bicarbonate secretions, blood flow and epithelial repair. Moreover, when PGs are inhibited, the mucosa is susceptible to damage from luminal agents such as gastric acid, ethanol and the NSAID itself (Wallace 2000a; Wallace 2008). The impact of gastric acid secretion on NSAID-induced gastropathy is highlighted by how effective pharmacologically suppressing the secretion reduces upper GI damage. Additionally, compelling evidence suggests a role for neutrophils in NSAID-induced gastroduodenal ulceration, as seen in an increase in leukocyte adherence to the vascular endothelium (Wallace 1993).

Neutrophil adherence in the microcirculation may further mucosal susceptibility to NSAID-induced damage by obstructing mucosal blood flow (Wallace and Granger 1999b). Adherence of neutrophils to the vascular endothelium is accompanied by activation of the cells, leading to the release of oxygen-derived free radicals and proteases (Vaananen 1991). A reduction in gastric mucosa blood flow has been demonstrated following NSAID administration as shown in antibody studies in rats; rats made neutropenic via anti-neutrophil serum were not at risk for hemorrhagic lesions following NSAID administration (Wallace 1990). Moreover, administering
specific monoclonal antibodies that prevent leukocyte adherence to the vascular endothelium attenuates NSAID-induced gastropathy (Wallace 1993c).

The pathogenesis of NSAID-induced gastropathy and leukocyte adherence has been linked to tumour necrosis factor-α (TNFα). This cytokine is a potent stimulus for gastric vascular endothelium intercellular adhesion molecule-1 (ICAM-1) on the gastric vascular endothelium. Administration of the NSAID indomethacin resulted in a significant increase in serum TNFα that was consistent with a significant increase in adherent leukocytes in the gastric microcirculation (Miura 1991). Rats treated with a TNFα synthesis inhibitor (pentoxifylline) exhibited a reduction in gastric damage, leukocyte adherence and serum TNFα levels (Appleyard 1996). Although TNFα has an important role in leukocyte adherence, other mediators can be attributed to the adhesive interactions of neutrophils. Prostacyclin (PGI2) is an important inhibitor of neutrophil activation and adherence (Wallace 1992). The suppression of prostacyclin following NSAID-administration may be in part attributable to adhesive interactions between neutrophils and the vasculature endothelium. Mediators that are able to reduce leukocyte-endothelial cell adhesion in the gastric vasculature include nitric oxide and hydrogen sulfide (MacNaughton 1989; Wallace 1994; Zanardo et al., 2006). Both are endogenously produced gaseous mediators that can prevent NSAID-induced gastroduodenal damage. The aforementioned mechanisms explain NSAID-induced injury, but despite the proximity of the stomach and the small intestine, enteropathy induced by NSAIDs has a distinct pathogenesis from gastropathy.
1.5.2 Pathogenesis of NSAID-induced enteropathy: It is well understood and recognized that repeated NSAID administration causes significant bleeding and ulceration in the stomach and in the duodenum (Wallace 2008). Suppression of gastric acid secretion substantially reduces the incidence of gastropathy, but does not appear to diminish injury in the more distal areas of the GI tract. NSAID damage in the small intestine is under appreciated, clinicians often will overlook the condition mainly because it takes longer to develop and often does so asymptotically (Wallace 2012). Enteropathy has a higher incidence than gastropathy, a troubling fact given how often small intestinal damage is neglected in a clinical setting (Scarpignato and Hunt 2010).

Clinical trials using video capsule endoscopy investigated the incidence of small intestinal injury in young, healthy volunteers taking PPI and NSAID over a 2-week period. The incidence of small intestinal injury was 55-75% (compared to 7-11% in the control group) (Goldstein 2005; Fujimori 2010). In these clinical trials, the subject population was not a realistic patient population, who are most likely elderly, will have underlying disease, and will be on long term NSAID administration. Thus, it is reasonable to assume that in a clinical setting, the incidence of small intestinal injury would be greater than values reported above. Furthermore, there has been a decrease in upper GI NSAID-related complications in the last 20 years, although it has come along side an increase in the number and severity of NSAID-related complications is areas distal to the ligament of Treitz (Lanas 2009). The understanding of the mechanism of lower GI injury from NSAID
administration has come largely from animal studies where the mechanism appears multifactorial as well as clearly distinct from that of the upper GI tract (Wallace 2012).

Gastropathy and enteropathy share pathological similarities, for example the inability to undergo cellular repair from topical damage due to mucosal PG synthesis inhibition. Where the pathologies differ is in the COX inhibition. While a major player in gastropathy, COX inhibition does not have a primary role in lower GI injury (Reuter 1997). Evidence for this comes from the lack of correlation between intestinal PG levels and the degree of intestinal injury. NSAID-induced intestinal damage is also not temporally synchronized with suppression of intestinal PG synthesis, like it is in gastropathy (Reuter 1997). There are several important contributing factors to NSAID-induced small intestinal injury: altered permeability, epithelial cell damage, neutrophil infiltration, an increase in luminal gram-negative bacteria, TNFα release and enterohepatic recirculation of NSAIDs (Wallace 2012).

Human and rat studies confirm that intestinal epithelial permeability increases within 12 hours of NSAID administration (Bjarnason 1986; Reuter 1997). In rats, severe damage from NSAID administration can be seen throughout the small intestine. Within 12-24 hours of NSAID administration this damage is macroscopically visible (Whittle 1981; Bertrand 1998). Morphological changes can be seen via light microscopy within 4-6 hours of NSAID administration and occur before marked ulceration develops (Anthony 1993; Somasundaram 1997). The
hallmarks of the morphological changes are villous shortening, microvasculature
damage and eosinophil/neutrophil infiltration (Anthony 1993; Somasundaram
1997; Kelly 1998). It has been postulated that damage occurs in response to
uncoupled oxidative phosphorylation within mitochondria, which causes a drop in
ATP levels and leads to tight junction deficiencies (Somasundaram 1995). Increased
permeability facilitates the entry of harmful agents such as bacteria and bile acids
into the lamina propria (Somasundaram 1995). It is difficult to determine how
instrumental a role intestinal permeability plays in NSAID-induced enteropathy as
NSAIDs display variable effects on permeability while initiating significant intestinal
damage.

In addition to changes in permeability the NSAID moiety itself can contribute to
intestinal epithelial cell damage by disrupting oxidative phosphorylation of the lipid
bilayer (Somasundaram 2000; Zhou 2010). It is possible that the damage is due to
both injury mechanisms occurring simultaneously.

The epithelial cell damage causes an infiltration of neutrophils into the inflamed
mucosa. When activated neutrophils are in the mucosa they release reactive oxygen
species (ROS) in addition to proteases, which are detrimental to surrounding cells
(Antoon and Perry 1997). In contrast to gastropathy, leukocyte adherence to the
vascular endothelium is not a critical factor in enteropathy and although it has a
role, TNFα is not as important in lower GI incidences of NSAID-induced damage
(Wallace 2012). For enteropathy, increases in serum TNF-α levels may be a
consequence of damage and not a driving force (Appleyard 1996; Watanabe 2008a). Despite this, anti-TNFα antibodies prevented indomethacin-induced damage by 67% (Watanabe 2008a). Drawing conclusions from the literature is difficult, as induction of NSAID-induced damage is not a standardized model. Research groups use methods, which differ in drug, dose, route of administration, and duration of treatment (Table 1.1). Many groups use a single-dose method to induce damage, although the clinical relevance of this procedure is questionable. As NSAID treatment is often long-term and high-dose, a good laboratory model will mimic this type of therapy. In this thesis, a 4.5-day twice-daily administration of high dose (20 mg/kg) of naproxen is used to simulate what often occurs in clinic.

Table 1.1: Laboratory models of NSAID-induced injury

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Administration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diclofenac</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>Orally Evaluated 24 hours later</td>
<td>Satoh et al. 2013</td>
</tr>
<tr>
<td></td>
<td>8 mg/kg</td>
<td>Intragastrically twice daily Evaluated 7 or 14 days later</td>
<td>Fornai et al. 2013</td>
</tr>
<tr>
<td></td>
<td>7.5 mg/kg</td>
<td>Intragastrically twice daily Evaluated 24 hours or 5 days later</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td></td>
<td>7.5 mg/kg</td>
<td>Intragastrically single dose Evaluated 24 hours or 5 days later</td>
<td>Chao et al. 2012</td>
</tr>
<tr>
<td><strong>Indomethacin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-40 mg/kg</td>
<td>Intragastrically Evaluated 24 hours later</td>
<td>Kent et al. 1969</td>
</tr>
<tr>
<td></td>
<td>3.5-15 mg/kg</td>
<td>Orally Evaluated 24 hours later</td>
<td>Sigthorsson et al. 2002</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>Orally Evaluated 24 hours later</td>
<td>Hayashi et al. 2014</td>
</tr>
<tr>
<td></td>
<td>18 mg/kg</td>
<td>Orally Evaluated 6 hours later</td>
<td>Shi et al. 2014</td>
</tr>
<tr>
<td><strong>Naproxen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>Orally twice daily Evaluated 4.5 days later</td>
<td>Wallace et al. 2011</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>Orally twice daily Evaluated 4.5 days later</td>
<td>Blackler et al. 2012</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>Subcutaneously</td>
<td>Takeuchi et al.</td>
</tr>
</tbody>
</table>
Evidence for bacteria’s critical role in NSAID-induced enteropathy is exemplified from the results of antibiotic and germ free studies. While broad-spectrum antibiotics have been reported to prevent small intestinal injury from NSAIDs in rats, it is particularly interesting that germ-free mice are not susceptible to NSAID-induced small intestinal damage (Robert and Asano 1977; Konaka 1999). The ulcerations resulting from NSAIDs appear to be dependent on gram-negative bacteria in rodents. The bacteria may be cleaving the drug or bile conjugates that may initiate drug-facilitated microbial damage to the mucosa as suggested by Wax et al (1970). Antibiotics that eliminate or reduce enteric bacteria may reduce cleavage of drug or bile conjugates and the ensuing mucosal injury. What is more likely is that bacteria have a role in perpetuating pre-existing mucosal injury initially established via NSAID administration since germ-free mice are not completely devoid of intestinal injury, just as antibiotic treatment does not completely prevent the injury (Bjarnason 1990; Davis 1990; Sartor 1994). The exact role of the intestinal microbiota in NSAID-induced small intestinal damage has yet to be firmly established.

Enterohepatic recirculation is arguably the most critical factor of NSAID-induced small intestinal injury (Figure 1.3)(Wallace 2012). Small intestinal injury from NSAIDs that exhibit extensive enterohepatic recirculation is significantly worse when compared to small intestinal injury from NSAIDs which do not undergo enterohepatic recirculation (Reuter 1997). When NSAIDs are enterohepatically
recirculated, intestinal epithelial cells have increased exposure to the damaging topical effects of the drug. Additionally, NSAIDs and bile form micelles, which have a synergistic damaging effect, as they are more toxic than NSAIDs or bile on their own (Yamada 1993; Petruzzelli 2007). Lastly, enterohepatic recirculation seems required to alter the intestinal bacteria, promoting the growth of gram-negative bacteria (Reuter 1997). A plethora of information is available on the microbiota alterations, but how this change correlates or manifests into intestinal damage remains unknown.

Figure 1.3: Pathogenesis of NSAID enteropathy. NSAIDs produce their effects during their initial pass-through to the small intestine. NSAIDs that are enterohepatically recirculated are absorbed in the distal intestine and glucuronidated in the liver, where they subsequently produce effects as they are secreted back into the proximal small intestine. Subsequent exposure of NSAIDs occurs in conjunction with bile. The repeated exposure the intestinal epithelium has to NSAIDs and bile results in damage. It is likely that this damage is exacerbated by changes in the intestinal microbiota (example: increased gram-negative bacteria). These are key mechanisms suggested to be involved in NSAID-enteropathy. Adapted from Wallace 2013.
1.6 Microbiota in Health and Disease

1.6.1 General introduction: The mucosal surface is colonized by a dense and complex microbial ecosystem (microbiota) immediately after birth. Although the majority of the constituents of the microbiota are bacterial, this ecosystem also includes eukarya, archaea and viruses (Backhed 2005; Eckburg 2005; Lozupone 2012). The microbiota is extremely diverse, housing an estimated 100 trillion microbes (Eckburg 2005; Gill 2006; Whitman 1998). The genes of the intestinal microbiota outnumber the total number of human genes by at least a factor of 1000 (Savage 1977). As more information about the microbiota is uncovered, it is becoming clear that it has an essential role in health and disease.

1.6.2 The intestinal microbiota: The gastrointestinal tract can be divided into distinct sections, each with its own digestive function and microbial composition (Figure 1.4). Due to its highly acidic nature, the stomach has a relatively low biomass of bacteria, approximately 10 bacteria per gram of contents (Monstein 2000). Bacterial density increases distally through the GI tract. In the duodenum the density increases to $10^3$ per gram of contents and reaches $10^4$-$10^7$ per gram of contents in the jejunum and ileum (O’Hara and Shanahan 2006; Walter and Ley 2011). The colon houses over $10^{12}$ cells per gram of content (Dethlefsen 2007; Whitman 1998). It is not only the number of bacteria that changes through different areas of the gastrointestinal tract, but also the types of microbes. Bacteria in the oral cavity are mostly aerobes from Streptococcus and Lactobacilli (Stearns 2011). In contrast, the majority of the microbes in the intestines are anaerobes. The Bacilli
class of *Firmicutes* and *Actinobacteria* dominate the small intestine while the large intestine is enriched mainly with *Bacteroidetes* and *Lachnospiraceae* family of the *Firmicutes* (Frank 2007).

**Figure 1.4 Spatial aspects of the intestinal microbiota.** The diversity and number of bacteria increase from proximal to distal GI tract. The numbers and classes of bacteria that colonize a specific area are due to micro-environmental pressures related to oxygen content and nutrient availability among other factors.

The symbiotic relationship between a host and its intestinal microbiota has resulted from years of co-development where microbes have adapted to survive in distinct regions of the gut. The host provides intestinal microbes with a steady supply of nutrients and, in return, the host is provided with several benefits. Beneficial microbes protect against infection by competing with potential pathogens for nutrients and space and are stimuli for host physiological development (Stecher and Hardt 2008; Hooper 2002; Benson 2009; Roun and Mazmanian 2009; Smith 2007).
1.6.3 Intestinal homeostasis: In a healthy state, trillions of bacteria live within a host and do not elicit a detrimental immune response. Due to this, the intestinal microbiota can be described as homeostatic under healthy conditions. As the microbes exist in close proximity to a host, they do present a potential threat. The intestinal microbiota has the potential to induce chronic inflammation. Several mechanisms, including the innate and adaptive immune systems, help to maintain a balance between the host and the intestinal microbes. A loss of homeostasis can result in detrimental effects, some of which can be permanent, such as disease development. Driving forces for the breakdown of homeostasis are not completely understood or characterized but it is known that genetics, environment, comorbidities, or a combination of any of these factors can play a role.

Early environmental factors strongly influence the microbial composition of the intestinal microbiota. Examples include vaginal or caesarian birth or whether infants are breastfed or bottle-fed. These foundational factors for intestinal microbial composition can impact disease development later in life (Gareau 2007; Varghese 2006). Although in adulthood the microbiota is relatively stable, factors such as stress, diet, antibiotic use and hygiene habits can lead to compositional shifts (Nicholson 2012). Dysbiosis remains poorly defined but the term is used to describe an alteration in microbiota composition. A microbiota that is dysbiotic may result in disease such as Inflammatory Bowel Disease (Tamboli 2004; Szilagayi 2004). There are many unanswered questions regarding dysbiosis, and the implications of microbial shifts are poorly understood. In this thesis, how PPIs
induce intestinal dysbiosis and how this influences the severity of NSAID-induced small intestinal damage is explored.
2.1 Thesis Introduction and Relevance

Due largely to the clinical focus on the gastroduodenal injury caused by NSAIDs, priority has been given to development of therapies to prevent upper GI toxicity. PPIs are drugs that inhibit gastric acid secretion. When PPIs are co-administered with an NSAID, the incidence of gastroduodenal ulceration is substantially reduced. NSAID-associated intestinal damage is not dependent on gastroduodenal pH. By virtue of this, co-administration of a PPI would be unlikely to prevent lower GI toxicity of NSAIDs.

Clinical studies have shown that PPI and NSAID co-treatment does not prevent small intestinal injury (Goldstein 2005; Maiden 2005). The aforementioned studies reported an incidence of enteropathy of up to 75% and an additional study noted that a marker of intestinal inflammation (calprotectin) was significantly increased in volunteers taking both drugs (Poullis 2003). Because clinical trials have largely focused on the stomach/duodenum (where the damage is prevented) and ignored the distal small intestine (where the damage is exacerbated), the safety profile may not accurately reflect the risk. The advent of PPI and NSAID combination tablets (i.e., Vimovo) should be cautiously examined due to the risk of small intestinal damage.

In the present study, we examined the effects of co-administration of a PPI and an NSAID on small intestinal damage. By evaluating the small intestinal microbiota, we characterized alterations resulting from co-administration of these drugs. In
addition to examining changes in the intestinal microbiota due to administration of PPIs, we subsequently observed how manipulation of the intestinal microbiota influences intestinal damage in rats.

2.2 Thesis Objectives

The following primary objectives are addressed in this thesis:

1. To evaluate the severity of naproxen-induced small intestinal injury in rats co-administered a gastric acid secretion suppressant (PPI or H₂RA).

2. To establish how the co-administration of naproxen and omeprazole influences the small intestinal microbiota in rats.

3. To assess the effectiveness of modulating the microbiota by probiotic or antibiotic administration in altering the severity of naproxen-induced enteropathy in rats.

4. To determine how NSAIDs other than naproxen respond to modulation of the intestinal microbiota with respect to induction of small intestinal injury.
- CHAPTER THREE -

GENERAL MATERIALS AND METHODS
3.1 Animals
Male Wistar rats weighing 180-220 g were obtained from Charles River (Montreal, QC, Canada) and were housed in the Central Animal Facility at McMaster University. The rats were fed standard chow and water *ad libitum*. The animals were bred under specific pathogen-free (SPF) conditions. Germ-free National Institutes of Health (Bethesda, MD) Swiss mice (male, 8 weeks of age) were raised in the Farncombe Institute Anexic Gnotobiotic Facility. The mice had never encountered a bacterium nor bacterial products. The Animal Care Committee of the Faculty of Health Sciences at McMaster University approved all experimental procedures described herein. The studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

3.2 Administration of NSAIDs
Rats received naproxen (20 mg/kg; Sigma-Aldrich, ON), indomethacin (7.5 mg/kg, Sigma-Aldrich, ON), diclofenac (15 mg/kg; Sigma-Aldrich, ON) or vehicle orally for one dose for acute studies or in 12-hour intervals for a total 9 doses for chronic studies. Naproxen and diclofenac were solubilized in 1% carboxymethylcellulose (CMC; Sigma-Aldrich, ON). Indomethacin was solubilized in 5% sodium bicarbonate (Sigma-Aldrich, ON). The total volume administered was 0.1 mL/kg body weight.

3.3 Assessment of Small Intestinal Macroscopic Damage
Macroscopic damage was quantified starting from the ligament of Treitz until the end of the terminal ileum. The rats were euthanized by an overdose of isoflurane
prior to cervical dislocation. The small intestine was then removed and rinsed with 20 mL of water. The small intestine was cut along the mesenteric border and opened for evaluation. An individual who was blinded to the treatment groups scored the intestine for damage. The damage score was the cumulative length in millimeters of all lesions within the defined area.

3.4 Assessment of Small Intestinal Microscopic Damage

Histological staining of small intestinal tissues was performed for microscopic damage evaluation. Samples for staining were taken 20 cm distal to the ligament of Treitz. A tissue sample 2 cm in length was removed and pinned flat to a wax block and fixed in 10% neutral-buffered formalin (ACP Montreal, QC). After 24 hours, tissue was removed from formalin and immersed in 70% ethyl alcohol (Sigma-Aldrich, ON). After 24 hours in ethyl alcohol, the tissue was processed for blind histological evaluation (hematoxylin and eosin staining). Tissue staining was examined under a light microscope (Nikon Instruments, NY, USA).

3.5 Genomic Tissue Preparation

Plastic screw top 2 mL tubes had 0.2 g of 0.1 mm beads (Mo Bio, CA, USA) and 300 μL of sample added to 800 μL of 200 mM NaPO₄ (pH 8; Sigma-Aldrich, ON) and a 100 UL mixture of guanidine thiocyanate (60 g; Sigma-Aldrich, ON), 0.5M ethylenediaminetetraacetic acid (20 mL, pH 8; Sigma-Aldrich, ON), 20 mL of sterile double distilled water and 1 g N-lauroylsarkosine (Sigma-Aldrich, ON). The tubes were homogenized by a bead beater for 3 minutes. Samples were incubated at 37°C
for 1.5 hours with 50 μL of lysozyme (100 mg/mL in H₂O; Sigma-Aldrich, ON), 50 μL of mutanolysin (10U/μL; Sigma-Aldrich, ON) and 10 μL of RNase A (10 mg/mL in H₂O; Sigma-Aldrich, ON). The samples were incubated a second time for 1 hour at 65°C with 25 μL of 25% sodium dodecyl sulfate (Sigma-Aldrich, ON), 25 μL of proteinase K (Sigma-Aldrich, ON) and 62.5 μL of 5M NaCl (Sigma-Aldrich, ON). Samples were then centrifuged (Eppendorf, ON) for 5 minutes at 6000 x g and 900 μL of the supernatant was removed and transferred to a 2 mL tube. Equal volume of 25:24:1 phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, ON) was added and the sample was centrifuged at 6000 x g for 10 minutes.

Following centrifugation, the top layer was removed from the tube and 200 μL of DNA binding buffer (Zymo Research, CA, USA) was added prior to being transferred to a DNA column 500 μL at a time. Tubes were centrifuged at 6000 x g for 1 minute, after which the flow-through was discarded. Once the entire top layer and DNA binding buffer had been transferred through the DNA column, 200 μL of wash buffer (Zymo Research, CA, USA) was added to the tube. The tube was spun at 6000 x g for 1 minute and flow-through was discarded. The DNA was eluted in 50 μL of pre-headed (65°C) sterile DNase and RNase-free ddH₂O pre-heated 65°C water.
3.6 DNA Extraction and 16S rRNA gene Amplification

DNA extraction was conducted as previously described using a custom protocol and sequence amplification of the 16S rRNA gene variable 3 region (Bartram 2011). Briefly, the sample was resuspended in 800 μL of 200 mM NaPO4 and 100 μL of guanidine thiocyanate-EDTA-Sarkosyl and homogenized with glass beads (0.2 g of 0.1 mm, Mo Bio, California, USA). Enzymatic lysis was conducted by incubating the sample at 37°C with 50 μL lysozyme (100 mg/mL), 50 μL mutanolysin (10 U/μL) and 10 μL RNaseA (10 mg/mL) for 1 h. This was followed by adding 25 μL of 25% sodium dodecyl sulfate, 25 μL of Proteinase K and 62.5 μL of 5 M NaCl, and incubating at 65°C for 1 h. Samples were centrifuged at 12,000 x g after which the supernatant was transferred to a new microcentrifuge tube. An equal volume of Phenol-chloroform-isoamyl alcohol was added to the sample, which was then centrifuged for 10 min. The lowest density solution was transferred to a new tube to which 200 μL of DNA binding buffer (Zymo, California, USA) was added. The solution was transferred to a DNA column, washed and the DNA was eluted using sterilized water.

The 16S rRNA gene v3 region amplification was conducted after the DNA extraction, as previously described. Briefly, 341F and 518R rRNA primers were modified to include the addition of barcodes to the reverse primer for use in the Illumina platform, allowing for multiplex amplification. PCR amplification, separation by gel electrophoresis and gel extraction steps were completed as described previously12 with the following modifications. Primer volume was 5 picomoles each. A Taq polymerase (Life Technologies) was used for amplification and cycling
times were modified to 30 s for each step. The products of this step were sequenced on the Illumina MiSeq platform.

3.7 Statistical Analysis

All data are presented as the mean plus-or-minus standard error of the mean. For comparisons between two experimental groups, data were analyzed by using the Student’s $t$ test. Comparisons of more than two experimental groups were performed with a one-way analysis of variance and an appropriate post-hoc test (see specific experiments for details). An associated probability (p value) of less than 0.05 was deemed significant.
– CHAPTER FOUR –

EXACERBATION OF NSAID-INDUCED SMALL INTESTINAL INJURY BY GASTRIC ACID SUPPRESSION: A CONSEQUENCE OF DYSBIOSIS
4.1 Introduction

The ability for nonsteroidal anti-inflammatory drugs (NSAIDs) to induce damage in the stomach and duodenum is well documented, but recently, animal models and subsequent human video capsule endoscopy studies have documented their ability to elicit damage in more distal areas of the intestine. Chronic NSAID use is associated with intestinal inflammation, which occurs in approximately 70% of all users (Bjarnason 1993). Intestinal bleeding, strictures, as well as the risk of perforation are all associated with NSAID-induced intestinal inflammation (Bjarnason 2009). Similar to the pathogenesis of gastropathy, inhibition of prostaglandin synthesis leaves the intestinal mucosa at an increased risk for injury and renders it less efficient at repair (Reuter 1997; Tanaka 2002). Despite this, there does not appear to be a primary role for the COX enzymes in the pathogenesis of NSAID-enteropathy.

The enterohepatic recirculation of NSAIDs and their secretion in bile are both important contributing factors to intestinal damage. These two factors directly contribute to intestinal injury and have direct cytotoxic ramifications on enterocytes (Reuter 1997; Somasundaram 2000; Zhou 2010). Recent studies have uncovered the detrimental impact of gram-negative bacteria in the induction of intestinal damage and the inhibition of mucosal repair. Part of these observations comes from studies with germ-free mice, which do not develop ulcers following NSAID administration. Additional studies with broad-spectrum antibiotics reported significantly less ulceration after NSAID administration when compared to controls (Kent 1969; Konaka 1999). Moreover, NSAID administration does not result in
ulceration in mice lacking the bacterial endotoxin receptor (Toll-like receptor 4; TLR-4) (Watanabe 2008).

It is widely known that co-administration of a proton pump inhibitor (PPI) significantly decreases the incidence of NSAID-induced gastroduodenal injury (Scheiman 2006). However, acid does not appear to contribute significantly to NSAID-induced damage occurring distal to the ligament of Treitz (i.e., in the jejunum and ileum). Clinical trials that used video capsule endoscopy in young, healthy humans to investigate the effects of NSAID and PPI administration for two weeks reported a higher incidence of damage with PPI than without (55%-75% vs. 7%-11% in placebo treated) (Graham 2005; Goldstein 2005). These results suggest that PPI administration does little, if anything, to protect major sites of NSAID-induced injury (mid- and distal small intestine).

Gastric acid kills the majority of the bacteria that we ingest (Verdu 1994; Lombardo 2010). When gastric acid is chronically suppressed, individuals can exhibit bacterial overgrowth in the stomach and in the small intestine (Lombardo 2010; Williams 2006). It is possible that the chronic suppression of gastric acid secretion by PPIs could exacerbate NSAID-induced injury by perturbing homeostatic bacterial levels. This is especially important given the major role gram-negative bacteria may play in the induction of NSAID-induced gastrointestinal injury.
4.2 Specific Methods and Materials

4.2.1 Suppression of gastric acid secretion: Rats (180-220 g) were given famotidine (an H₂RA; 20 mg/kg), omeprazole (a PPI; 10 mg/kg) or vehicle (CMC) orally, twice daily for five days. The rats were fasted during the fourth night and a pyloric ligation was performed in the morning of the fifth day, one hour after the final administration of drug or vehicle. Three hours post-ligation, rats were euthanized by an overdose of isoflurane prior to an overdose of pentobarbital sodium.

4.2.2 Measurement of inhibition of acid secretion: Rats treated for 5 days with an acid suppressing drug or vehicle were anaesthetized with isoflurane and underwent a pyloric ligation. For 18 hours prior to ligation rats were fasted but had free access to water. Three hours after rats had recovered from anaesthetic, the stomach was excised and contents were emptied into a 2 mL tube. Volume and pH of the collected gastric samples were measured (Accument AB 15, Fisher Scientific, ON) and milliequivalents of hydrogen ions were calculated.

4.2.3 Pharmacokinetics of naproxen: Rats were administered omeprazole (10 mg/kg) orally twice daily for 5 days prior to a single dose of naproxen (10 mg/kg) orally. Rats were anesthetized with isoflurane 3 or 6 hours after naproxen administration. The bile duct was cannulated and bile was collected for one half hour. After this, a blood sample was taken from the inferior vena cava. The plasma
and biliary levels of naproxen were determined by high-performance liquid chromatography (HPLC).

### 4.2.4 Microbiota analysis: denaturing gel gradient electrophoresis:

Extracted DNA from biological samples had the hypervariable v4 region of the bacterial 16S ribosomal DNA gene amplified by polymerase chain reaction (PCR) or reverse-transcription PCR with universal bacterial primers HDA1-CG (5’-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGA CCG GGG GGG GAC TCC TAC GGG AGG CAG CAG T-3’) and HDA-2 (5’-GTATTACCGCGGCTGCTGGCAC-3’]). PCR reactions were done using 50 μL volumes with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 100 μM each dNTP, 0.6U HiTaq (Life Technologies, Burlington, ON). Amplification used a program of 94°C for 3 minutes followed by 35 cycles of 94°C (30 seconds), 56°C (30 seconds), 68°C (30 seconds) and then was held at 68°C.

Gels were 6% polyacrylamide and 16 cm by 16 cm by 1 mm. They were electrophoresed with 1xTAE buffer (2M tris base, 1M glacial acetic acid, 50 mM EDTA). The denaturing gradient was formed with two 6% acrylamide (acrylamide/bisacrylamide ratio 37.5:1) stock solutions. The gels had a 22% to 55% gradient of urea and formamide, which increased down the gradient. A 100% denaturing solution was used which consisted of 40% (vol/vol) formamide and 7.0 M urea. Electrophoresis was conducted at 130V (constant voltage) and 60°C for approximately 4.5 hours. When the xylene cyanol dye reached the bottom of the gel,
the electrophoresis was terminated. Gels were stained with an ethidium bromide solution (5 μg/mL) for 20 minutes prior to being washed with deionized water.

Quantity One software was used to measure staining intensity from a scanned image of an electrophoretic gel. The intensity of fragments was expressed as a percent relative to the sum of the intensities of all fragments in the same lane. By comparing the 16S rDNA sequences with the National Centre for Biotechnology Information GenBank databases through the BLAST2 program, we identified bacterial phylogenies.

Additionally, RT-PCR primers were used to detect Actinobacteria and *Bifidobacteria*. The primers were used to detect the target bacterial groups in samples of jejunal tissues and in the broth containing selected commensal bacteria. For all primers, the annealing temperature was 55°C.

4.2.5 *Commensal bacteria isolation:* After being euthanized, mid-jejunum contents from healthy rats were added to 1 mL of sterile saline in a sterile microcentrifuge tube and were vortexed for 10 seconds. The contents were streaked onto Man, Rogosa and Sharpe (MRS) agar plates with l-cysteine HCl (0.5 g/L) and the antibiotic mupirocin (50 mg/L) was incubated anaerobically at 37°C for 48 hours.

A quarter of inoculating loop was scraped from the agar plates and inoculated into 13 mL tubes of MRS broth and incubated for 48 hours. The concentration was
determined via optical density measurements. Rats were gavaged 1 mL per day of $10^9$ CFU/mL commensal bacteria. PCR analysis of the broth confirmed which bacteria were present by using the primers in Table 4.1.

In studies with germ-free mice, the protocol described above was used for commensal bacteria isolation, but contents from the mid-jejunum of rats treated with omeprazole or from vehicle-treated rats were used. The samples from each group were pooled respectively for bacterial isolation.

<table>
<thead>
<tr>
<th>Target bacterial group/species</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria phylum</td>
<td>CGCGGCCTACGCTTGGTTG</td>
</tr>
<tr>
<td></td>
<td>CCGTACTCCCAAGCGGGG</td>
</tr>
<tr>
<td>Bifidobacterium group</td>
<td>CTCCCTGGAACGCGTTGG</td>
</tr>
<tr>
<td></td>
<td>GTGGTCTCCCGATATCTACA</td>
</tr>
<tr>
<td>B. longum group</td>
<td>TTTTGTTGATCGCATGTC</td>
</tr>
<tr>
<td></td>
<td>TCAGCGCTCGTCGCCGAT</td>
</tr>
<tr>
<td>B. bifidum</td>
<td>CCACATGACTCGATGTAGTG</td>
</tr>
<tr>
<td></td>
<td>CGGAAGGCTTGCTCCCAA</td>
</tr>
<tr>
<td>B. breve</td>
<td>CCGGATGCTCCCATCACAC</td>
</tr>
<tr>
<td></td>
<td>CAAAAGTCCTTGCTCCT</td>
</tr>
<tr>
<td>B. adolescentis</td>
<td>TCAGCGCTGATCGATGTC</td>
</tr>
<tr>
<td></td>
<td>TCCAGTGGACCGATGTT</td>
</tr>
<tr>
<td>B. catenulatum group</td>
<td>CGGATGCTCCGACTCCT</td>
</tr>
<tr>
<td></td>
<td>CGAAGGCTTGCTCCCGAT</td>
</tr>
<tr>
<td>B. angulatum</td>
<td>CAGCCATGCAATGGGTGTT</td>
</tr>
<tr>
<td></td>
<td>GAAGGCTTGCTCCCAAC</td>
</tr>
<tr>
<td>B. lactis</td>
<td>GTGGAGACACGCGTTTCC</td>
</tr>
<tr>
<td></td>
<td>CACACCAACACAATCCATAC</td>
</tr>
</tbody>
</table>

Table 4.1 Reverse-transcription PCR primers used to detect Actinobacteria and Bifidobacteria. For each primer, the annealing temperature was 55°C. The primers were used for detection of target bacterial groups/species in samples of jejunal tissue and select commensal bacteria broth.
4.2.6 Administration of isolated bacteria: (a) specific pathogen-free rats: Rats (180-220 g) were treated with omeprazole twice daily for 9 days. Subgroups of 6 rats were selected at random to be administered either selected commensal bacteria from healthy rats or sterile MRS broth once daily for 4 days.

In a secondary experiment, rats received omeprazole or vehicle twice daily for a total of 9 days and received naproxen (10 mg/kg) twice daily beginning on the final 4 days. Subgroups of 6 rats were assigned randomly to select commensal bacteria or with sterile MRS broth administration three hours after the morning naproxen administration each day.

For each experiment, rats were euthanized three hours after the final naproxen dose, samples were collected and damage was blindly evaluated.

4.2.7 Administration of isolated bacteria: (b) germ-free mice: Two groups of germ-free mice (20-25g) were administered jejunal contents orally. Each mouse in the first group was gavaged 0.1 mL of jejunal contents from PPI-treated rats and each mouse in the other group received 0.1 mL of jejunal contents from vehicle-treated rats. After one week, three mice from each group were euthanized via isoflurane overdose and blindly examined for damage. The remaining mice were administered naproxen (10 mg/kg) orally twice daily for 4 days. The mice were euthanized via isoflurane overdose and blindly evaluated for damage.
4.2.8 Assessment of myeloperoxidase activity (MPO): Tissue samples (100-200 g) were placed in microcentrifuge tubes, flash frozen in liquid nitrogen and stored at -80°C. The assay was performed within one week of the tissue being removed.

Tissues were taken from the freezer and placed in plastic test tubes with 0.5 mL of HTAB buffer. The tissue was then homogenized with a polytron tissue homogenizer for 15 seconds. Additional HTAB buffer was added to give 1 mL of buffer per 50 mg of tissue. The test tubes were vortexed and about 1 mL of solution was decanted into a microcentrifuge tube. This was centrifuged for two minutes at 6000 x g in a bench top centrifuge. After centrifugation, 7 μL of each sample was added to individual wells in a microplate. Immediately prior to reading the plate at 460 nm, 200 μL of 0-dianisidine solution was added. Absorbance was measured over 30 second intervals (SpectraMax M3, Molecular Devices Corp., Sunnyvale, CA, USA) and converted to a $K_{max}$ value using SoftMax Pro 5.4 software. These values were converted to average units of MPO activity per mg of tissue. One unit of MPO is defined as the quantity of enzyme required to convert 1 μmol of H$_2$O$_2$ into water in 1 minute at room temperature.

4.2.9 Prostaglandin E$_2$ assay: A Cayman chemical kit was used for all PGE$_2$ assays. Prior to the assay, enzyme immuno assay (EIA) buffer was diluted with 90 mL of UltraPure water. Wash buffer was made by diluting the stock to a final volume of 2 L and adding 1 mL of Polysorbate 20. The vial of tracer was reconstituted with 6 mL of EIA buffer. The antibody vial was also reconstituted with 6 mL of EIA buffer. The standard (10 ng/mL starting concentration) was serially diluted to 8 standards with
concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/mL respectively. Samples were tested at the following dilutions: neat, 1:10, 1:50, 1:100 and 1:200.

50 µL of each standard or sample was loaded into a microplate, and 50 µL of each antibody were added. The plate was covered and incubated for one hour at 22°C on an orbital shaker.

After incubation, wells were rinsed with ddH$_2$O three times and washed with the wash buffer five times. 200 µL of Ellman’s reagent was added to each standard and sample. The plate was covered and incubated at room temperature on an orbital shaker. Every 30 minutes the plate was read at 420 nm. The plate was fully developed at an absorbency of 0.7.

**4.2.10 Serum thromboxane B$_2$ assay:** Blood (3 mL) was drawn from the inferior vena cava, transferred to a glass tube and placed in a 37°C water bath for 45 minutes. The samples were then centrifuged at 1000 x $g$ for 10 minutes and the serum was transferred to microcentrifuge tubes and frozen at -20°C. Within one week, the thromboxane B$_2$ concentration in the samples was measured using a specific enzyme-linked immunosorbent assay (ELISA). Cayman Chemical kits were used for all TXB$_2$ assays (Cayman, MI, USA). Prior to the assay, EIA buffer (1M phosphate solution containing 1% BSA, 4M sodium chloride, 10 mM EDTA and 0.1% sodium azide), wash buffer, tracer and antibody were prepared as described in the PGE$_2$ assay protocol above. The standard (200 ng/mL starting concentration) was
diluted (100 μL of standard in 900 μL of UltraPure H₂O) to make a 20 ng/mL bulk standard. The bulk standard (500 μL) was serially diluted in 900 μL of EIA buffer to yield concentrations of standards 1-8 to be 2000, 1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL respectively. Samples were tested at 1:50, 1:100, 1:200 and 1:3000 dilutions.

The plate was incubated and washed as described in the PGE₂ assay above. The concentration of each sample was calculated from the equation obtained from the standard curve. %B/B₀ between 20 and 80% were accepted as they fell within the linear range of the standard curve.

4.2.11 Cytokine and chemokine analysis: Blood was collected from rat tail snips (1.5 mL). The samples were spun at 1000 x g for 10 minutes. The serum was collected from the centrifuged samples. 300 μL of each sample was sent to Quansys Biosciences (Logan, UT). Quansys Biosciences tested the serum samples for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, IFNγ, TNFα, GM-CSF and RANTES. Each sample was tested in triplicate.

4.2.12 Hydrogen sulfide assay: Small intestinal samples from the mid-jejunum of rats were removed and immediately snap frozen and stored at -80°C. The tissue was homogenized in ice-cold 50 mmol/L potassium phosphate buffer (pH 8; 4% w/v). The homogenate (0.5 mL) and buffer (0.4 mL) were cooled on ice for 10 minutes before L-cysteine (10 mmol/L) and pyridoxal 5'-phosphate (2 mmol/L)
were added for a final volume of 1 mL. A smaller 2 mL microcentrifuge tube containing a piece of 0.5 x 1.5 cm filter paper soaked with zinc acetate (1%; 0.3 mL) was put inside the larger vial. The vials were flushed with nitrogen gas for 20 seconds and capped with an airtight serum cap. Vials were incubated in a shaking water bath at 37°C for 90 minutes. Vials were removed and cooled on wet ice for 15 minutes before 0.5 mL of trichloroacetic acid (TCA; 50%) was injected through the cap membrane of each vial. The vials were incubated at room temperature for 5 minutes before being incubated for 1 hour in a shaking water bath at 50°C. The serum cap was removed and N, N-dimethyl-p-phenylenediamine sulfate (20 mmol/L; 50 μL) in 7.2 mol/L HCl and FeCl₃ (30 mmol/L; 50 μL) in 1.2 mol/L HCl were added to the inner tube. After 20 minutes a microplate reader measured absorbance (670 nm). The calibration curve of absorbance vs. H₂S concentration was obtained by using NaHS solutions of varying concentrations as NaHS releases HS⁻ when dissolved in water and HS⁻ forms H₂S with H⁺. H₂S concentration in the standards is considered to be 30% of the NaHS concentration in the calculation.

4.3 Results

4.3.1 Effective inhibition of gastric acid secretion: Twice-daily administration of famotidine, omeprazole, or lansoprazole resulted in significant suppression of gastric acid secretion by the fifth day when twice-daily administration of naproxen was initiated in subsequent experiments. Famotidine, a H₂RA, suppressed gastric acid secretion by ~85% (data not shown). Omeprazole, a PPI, suppressed gastric acid secretion by >99%. A second PPI, lansoprazole, was also tested, and was found
to suppress gastric acid secretion as effectively as omeprazole (>99%). Due to the higher percentage suppression compared to famotidine, the remaining experiments used omeprazole to suppress gastric acid secretion. The mean pH of the vehicle-treated group was 1.6, and that in the omeprazole group was 3.6.

Figure 4.1 PPIs significantly suppress gastric acid secretion. Omeprazole (red bar) lansoprazole (green bar) at 10 mg/kg twice daily for five days reduced gastric acid secretion significantly. Bars represent the mean ± standard error of the mean. * p<0.05 vs. the vehicle group; Student’s t-test. (Figure credit: Wallace 2011)

4.3.2 Effective target enzyme inhibition with naproxen: Naproxen inhibited systemic COX-1 activity (whole blood thromboxane synthesis) by >90% after a single dose and by >99% after twice-daily dosing for 4 days. Intestinal PGE₂ synthesis was inhibited >85% after a single dose and >95% after 4 days of twice-daily dosing. Co-administration of a PPI did not significantly alter the degree of thromboxane or PGE₂ suppression.
4.3.3 PPIs exacerbate NSAID-induced small intestinal damage and bleeding:

Twice-daily naproxen administration for 4 days resulted in low levels of hemorrhagic damage in the stomach and small intestine. When a PPI was co-administered, no gastric damage was observed, but damage in the small intestine was significantly worsened. Blood was evident in the lumen of rats that were treated with naproxen and a PPI and ulcers were evident. Consistent with this was a significant decrease in hematocrit in rats treated with naproxen and PPI. No change in hematocrit was observed with naproxen alone or PPI alone.

Exacerbation of small intestinal damage was also observed when rats were administered the selective COX-2 inhibitor celecoxib in place of naproxen. The mean small intestinal damage score in rats treated with celecoxib alone was 0.5 ± 0.3 in contrast to rats treated with omeprazole and celecoxib where the mean damage score was 35.1 ± 4.6 (p < 0.001). Blood was observed in the lumen of the small intestine in rats treated with both celecoxib and a significant decrease in hematocrit was observed (-6.2% vs. 2.2% vs. -0.5% vs. 0.3% in rats treated with vehicle + celecoxib; P < 0.05).
**Figure 4.2 PPIs exacerbate small intestinal injury induced by naproxen.**

Exacerbation of 10 mg/kg naproxen damage (oral four day dosing) from PPI administration. Bars represent the mean ± standard error of mean. * p<0.05 vs. the vehicle group; Student’s t-test. (Figure credit: Wallace 2011)

4.3.4 **Omeprazole did not cause intestinal injury or inflammation:** The jejunum of rats treated only with omeprazole for 9 days did not exhibit histological signs of inflammation or damage. Tissue myeloperoxidase levels were comparable between vehicle- and omeprazole-treated rats (50.0 ± 6.8 vs. 48.9 ± 4.6 U/mg tissue respectively). Omeprazole treatment did not affect PGE₂ synthesis, although it did increase hydrogen sulfide synthesis by ~100%.

Expression of mRNA for COX-1, COX-2 (the enzymes responsible for PG synthesis) and hydrogen sulfide synthesis enzymes was unchanged after omeprazole administration. Similarly, omeprazole treatment did not change expression of the mRNA for endothelial nitric oxide synthase (1.38 ± 0.24-fold change vs. vehicle treated), TNF-α (1.55 ± 0.51-fold change vs. vehicle treated) or serum levels of IL-2, IL-17, IFNγ, FM-CSF and RANTES.
Figure 4.3 Treatment with omeprazole significantly increased H₂S synthesis but did not alter PG synthesis. Omeprazole treatment did not influence PG synthesis, but did significantly increase H₂S synthesis (Panels A and B; **P< 0.01 vs. vehicle-treated group). Treatment with omeprazole did not affect the expression of messenger RNA for key enzymes involved in PG or H₂S synthesis (Panels C-F; **P< 0.01 vs. vehicle-treated group). Panels C-F show expression of messenger RNA for COX-1, COX-2, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), respectively. (Figure credit: Wallace 2011)
4.3.5 *Omeprazole did not alter naproxen pharmacokinetics:* The exacerbation of naproxen-induced small intestinal damage by omeprazole is not due to altered absorption of naproxen. Omeprazole did not alter the concentration of naproxen in plasma. Additionally, the exacerbation is not due to an increased biliary excretion of naproxen. There were slightly lower levels of naproxen in bile in rats treated with omeprazole and naproxen compared to those treated with naproxen alone.

![Graph](image)

**Figure 4.4 Omeprazole administration does not alter the pharmacokinetics of naproxen.** Rats were given a single dose of naproxen (10 mg/kg) following five days of twice-daily omeprazole (10 mg/kg). Rats were anesthetized with isoflurane 3 or 6 hours following naproxen administration. Bile was collected for 30 minutes after which a vena cava blood sample was taken. No significant difference was
observed in plasma or biliary concentrations of naproxen. (Figure credit: Wallace 2011)

**4.3.6 Omeprazole treatment resulted in dysbiosis:** When treated for 9 days with omeprazole, rats had significant increases in the number of gram-positive and gram-negative aerobes in the jejunum. PCR-DGGE analysis revealed that omeprazole-treated rats had a significantly lower proportion of Actinobacteria (~75%, P < 0.05) in the jejunum as compared to vehicle-treated rats. Based on the 16S ribosomal DNA database, operational taxonomic units are attributed to Actinobacteria at 97% identity. In our samples, *Bifidobacteria* corresponded to a 94% identity threshold, thus it is likely that *Bifidobacteria* accounted for a significant component of the Actinobacteria. Consistent with this, RT-PCR analysis of jejunal tissue confirmed a significant reduction (80%, P<0.001) of Actinobacteria and *Bifidobacteria* spp in omeprazole-treated rats.
Figure 4.5 Administration of omeprazole resulted in significant intestinal dysbiosis. Rats treated with omeprazole (10 mg/kg) twice daily for 9 days had key changes in their jejunal microbiota compared to vehicle (control) as revealed by PCR-DGGE. Each lane in panel A represents 1 rat. In panel B, the lines represent the mean of four or five rats (Rf, retention factor). Rats treated with omeprazole had reductions in the Actinobacteria phyla, but no significant differences in any of the other bacterial subgroups when compared to control. (Figure credit: Wallace 2011)
4.3.7 Recolonization with Bifidobacteria-enriched commensal bacteria

Prevented intestinal damage and bleeding: Administration of selected commensal bacteria to rats receiving only omeprazole partially reversed the PPI-induced dysbiosis. When rats treated with naproxen and omeprazole had a daily administration of $10^9$ CFU of selected commensal bacteria, the mean intestinal damage score was significantly lower than what was observed in rats treated with naproxen and omeprazole but had daily administration of sterile control MRS broth (mean damage scores of $2 \pm 2$ and $17 \pm 5$ respectively). This reduction in damage score is consistent with the abolition of the decrease in hematocrit observed with naproxen and omeprazole administration.
Figure 4.6 PPI-induced dysbiosis and increase in naproxen-induced bleeding were reversed after administration of selected *Bifidobacteria*-enriched commensal bacterial. (A) Jejunal Actinobacteria (black bar) was increased after selected bacterial administration but there was no increase in any other subgroups of bacteria (Rf, retention factor). (B) The decreased hematocrit associated with omeprazole and naproxen was abolished after commensal bacteria administration. (Figure credit: Wallace 2011).
4.3.8 PPI-induced exacerbation of NSAID enteropathy is transferable via the 
microbiota: Germ-free mice are resistant to NSAID-induced enteropathy. When 
NIH Swiss mice were colonized for a week with jejunal bacteria from vehicle-treated 
rats prior to being treated for 4 days with naproxen, they exhibited mild hyperemia 
with mucosal structure largely intact. In contrast, NIH Swiss mice colonized for a 
week with jejunal contents from PPI-treated rats prior to being treated for 4 days 
with naproxen developed significant enteropathy. The mid-jejunum of the mice was 
friable, exhibited mucosal damage and had extensive subepithelial edema. 
Histologically there was no evidence of granulocyte infiltration, as confirmed by 
little myeloperoxidase activity in germ-free mice in any group. Germ-free mice did 
not have any intestinal injury without the administration of naproxen.
Figure 4.7 PPI-induced exacerbation of naproxen small intestinal damage is transferable via the intestinal microbiota. (A) Germ-free mice administered intestinal bacteria from PPI-treated rats developed more severe naproxen-induced small intestinal damage compared to germ-free mice administered intestinal bacteria from vehicle-treated rats (*P < 0.05). (B) Germ-free mice administered intestinal bacteria from vehicle-treated rats had mucosal structure which was mostly intact after twice-daily naproxen treatment for 4 days. (C) Germ-free mice administered intestinal bacteria from PPI-treated rats had epithelial damage in the small intestine after twice-daily administration of naproxen for 4 days and some mice (D) had extensive subepithelial edema.
- CHAPTER FIVE -

THE HETEROGENEOUS PROTECTIVE EFFECTS OF BACTERIAL ADMINISTRATION FOR NAPROXEN-INDUCED ENTEROPATHY
5.1 Introduction

There is emerging evidence that the small intestinal injury caused by NSAIDs is more serious than that which occurs in the upper GI tract due to higher rates of mortality and relapse, longer periods of hospitalization and higher associated healthcare costs (Adebayo & Bjarnason 2006). The serious outcomes related to NSAID-enteropathy are similar to those caused by NSAIDs in the upper GI tract with regards to prevalence and damage, including ulceration, bleeding and perforations, (Adebayo & Bjarnason 2006). There is therefore a great need for therapies that can treat or prevent NSAID-enteropathy.

The pathogeneses of NSAID-induced damage in the upper and lower gastrointestinal tracts are distinct (Wallace 2011). PPIs are the most commonly used drugs for prevention and treatment of NSAID-induced gastroduodenopathy, however, PPIs do not prevent NSAID-induced damage in the more distal small intestine, where gastric acid does not contribute significantly to the generation of tissue injury. Indeed, there is emerging evidence from animal and human studies, which indicate that PPIs exacerbate NSAID-induced ulceration in the distal small intestine (Wallace 2011). Moreover, a possible underlying mechanism for the PPI-induced exacerbation of NSAID-enteropathy in rats may be due to a marked depletion of Bifidobacteria following administration of a PPI (Wallace 2011).
Fukuda et al. reported differences in protection from enteropathogenic *E. coli* (EHEC) infection amongst different *Bifidobacteria* strains (Fukuda 2011). Treatment with some *Bifidobacteria* subspecies protected mice from EHEC infection, whereas treatment with others did not. The Fukuda group attributed protection due to the production of acetate, a short-chain fatty acid (SCFA). SCFAs are the product of colonic fermentation and have been associated with a reduced risk of many conditions, including inflammatory bowel disease, irritable bowel syndrome, cancer and cardiovascular disease (Hijova 2007). The diverse effects that SCFAs have are testament to how their benefits are not restricted to the immediate area where they are fermented.

The *Bifidobacteria* species used in the Fukuda paper were *Bifidobacterium longum* subsp. *longum* JCM 1217 (BL), *Bifidobacterium adolescentis* JCM 1275 (BA), *Bifidobacterium longum* subsp. *longum* NCC 1205 (BN), and a BN mutant where the gene involved in increased fructose consumption (BL0033) was knocked out via homologous recombination (BNKO) (Fukuda 2011). Two specific loci were found in the genomes of the preventative bacteria that contained ATP-binding cassette (ABC)-type carbohydrate transporters. *In vitro* metabolic profiling determined that preventative bacteria had higher rates of fructose consumption and acetate production due to specific carbohydrate transporters (Fukuda 2011). The expression of the BL0033 gene is highly induced by fructose and was expressed by preventative *Bifidobacteria* in the colon of mice (Fukuda 2011). BNKO, which no longer expressed BL0033, gave mechanistic insight in the EHEC study. Given that a
loss of *Bifidobacteria* was a contributing factor to naproxen-induced small intestinal damage in our previous study (Wallace 2011), we explored the potential for the specific aforementioned species of bacteria to confer protection from naproxen-induced damage, including the *Bifidobacteria* that no longer expressed BL0033. We observed these bacteria to determine what role, if any, acetate may play in naproxen-induced small intestinal damage. Acetate, the principal SCFA, is highly produced by BL and BN compared to lower amounts produced by BA, and no acetate produced from BNKO. To further investigate the role of SCFAs in our model, we treated naproxen-administered rats with the commensal bacteria *Faecalibacterium prausnitzii* (FPrau). FPrau is an acetate consumer and butyrate producer (Duncan 2002).

As lower gastrointestinal damage induced by naproxen appears to be gastroduodenal pH-independent, research has been focusing on the key role bacteria may play (Lanas 2006). Sufficient evidence of a bacterial role in NSAID enteropathy has accumulated since initial experiments reported germ-free rats are resistant to indomethacin-induced small intestinal damage (Robert & Asano 1997). Among their diverse roles, commensal bacteria generate varying levels of SCFAs and other organic acids, which have been implicated in a variety of beneficial host effects (Fukuda 2011). Here we investigate five commensal bacteria specifically chosen for their ability to protect against an alternative enteropathy model and examine their influence on NSAID-induced small intestinal damage.
We hypothesized that treatment with bacteria producing high levels of acetate would protect the small intestine from naproxen-induced damage through altering the intestinal microbiota.

5.2 Specific Methods and Materials

5.2.1 Treatment with bacteria: Rats were treated orally each day with 1 mL of a bacteria strain (10^9 CFUs) five days prior to initiation of naproxen treatment. Bacterial administration continued during the period of naproxen treatment. Five species of bacteria were studied, of which four belong to the Bifidobacteria genus (BA, BL, BN and BNKO), the fifth bacterium was the commensal bacteria, FPrau. Due to the oxygen intolerance of FPrau, FPrau supernatant (FPrau SN) was evaluated in some damage models. FPrau SN was recovered from an overnight culture following centrifugation. It was then filtered with a 0.45 mM filter and frozen at -80°C until use.

5.2.2 Bacterial strains and growth conditions: BA, BL, BN and BNKO were grown in de Man, Rogosa and Sharpe (MRS) broth (Difco, MI, USA) supplemented with cysteine (0.5 mg/ml; Sigma-Aldrich, ON). For BNKO, selective spectinomycin (75 µg/ml, Sigma-Aldrich, ON) and 1.5% (w/v) agar (Difco, MI, USA) were added to the liquid medium. The bacterial strains were incubated in anaerobic jars using the AnaeroGen Compact system (Biomerieux) or in an anaerobic chamber. All the bacterial strains were incubated anaerobically at 37°C overnight for ~16h.
FPrau was grown as previously described (Duncan 2002). Briefly, the bacteria was isolated from human fecal stool and grown in LYHBHI (brain-heart infusion medium with 0.5% yeast extract (Difco, MI, USA) and 5 mg/L hemin) medium with cellobiose (1 mg/mg; Sigma-Aldrich, ON), maltose (1 mg/ml; Sigma-Aldrich, ON) and cysteine (0.5 mg/ml; Sigma-Aldrich, ON) at 37°C under anaerobic conditions overnight for ~16h.

5.3 Results

5.3.1 BA attenuates naproxen-induced small intestinal damage: Daily oral administration of naproxen for 4.5 days resulted in the consistent formation of extensive bleeding ulcers in the small intestine of each rat. Damage was centralized to the mid-jejunum region, approximately 20 cm distal to the ligament of Treitz. Damaged area consisted of erosions, ulcerations and marked hyperemia.

Treatment with BA (10⁹ CFU) significantly reduced the extent of intestinal damage induced by naproxen (by ~82%; p<0.01; Fig. 1). In contrast, treatment with BL (10⁹ CFU) did not significantly affect the severity of small intestinal damage induced by naproxen (Fig. 5.1). When tested at doses of 10⁷ CFU and 10⁸ CFU, neither strain of Bifidobacteria significantly affected the severity of intestinal damage induced by naproxen (data not shown).
Figure 5.1: Treatment with *Bifidobacteria adolescentis* (BA) reduced naproxen-induced small intestinal damage. When administered to rats once daily for five days prior to initiating twice-daily treatment with naproxen, BA significantly reduced the severity of intestinal damage by ~82%. In contrast, treatment with *Bifidobacterium longum* subsp. *longum* JCM 1217 (BL) did not affect the severity of naproxen-induced intestinal damage. Each bar represents the mean ± SEM of 9 rats per group. **p<0.01 versus the vehicle-treated group; one-way ANOVA and Dunnett’s Multiple Comparison test.

Small intestinal samples taken from the mid-jejunum, 20 cm distal to the ligament of Treitz from vehicle-treated rats that were not treated with naproxen appeared macroscopically and histologically normal (Figure 5.2A).

Vehicle-, BA- and BL-treated rats that were co-administered naproxen had histological abnormalities in areas of macroscopic damage, as expected. The villi were denuded with a high number of cell infiltrates (Figure 5.2B-2E). Small intestinal histological samples from vehicle-treated rats exhibited a thickened muscularis layer, increased number of crypts and villus blebbing. When treated with BL, there was an increased thickness of the muscularis and increased crypt
numbers. Conversely, small intestinal samples from rats treated with BA showed normal muscularis thickness and crypt numbers, as well as no villus tip blebbing (Figure 5.2A). The most severe damage was from samples from rats treated with vehicle and naproxen. Samples had extensive ulceration, hyperemia and edema (Figure 5.2B).

**Figure 5.2: Microscopic differences among treatment groups were observed distal to the ligament of Treitz.** All panels show samples taken from the mid-jejunum 20 cm distal to the ligament of Treitz at 10X magnification. A. Histological sample from a naïve rat. B. A sample from a rat that had vehicle and naproxen administered. There is extensive ulceration, loss of villi and lymphocyte infiltration. C. A sample from a rat that had BA and naproxen administered. There is no increase in crypt number, no villus tip blebbing and the muscularis is reduced as compared to panel B. D. A sample from a rat that had been treated with BL and naproxen. There is ulceration and loss of villi architecture.
5.3.2 BN and BNKO attenuate naproxen-induced small intestinal damage: The production of acetate via the fructose consumption gene BL0033 in the BN genome accounts for protection against lethal EHEC-induced enteropathy. To evaluate the potential role of the BL0033 gene, we treated rats with BN and BNKO. Vehicle treatment with naproxen administration resulted in an average macroscopic damage score of 253 mm² where ulceration and hyperemia were observed from the mid-jejunum to the ileum.

As was the case for BA, treatment with BN and BNKO significantly reduced the macroscopic damage score. Treatment with bacteria resulted in fewer erosions and ulcerations throughout the small intestine. Damage started in the more proximal small intestine compared to where the damage started in vehicle-treated rats. Treatment with BN and BNKO resulted in a 62% and 69% reduction in small intestinal macroscopic damage, respectively (Figure 5.3). There was no significant difference in macroscopic damage scores between rats treated with BN and BNKO.
Figure 5.3: *Bifidobacterium longum* supsp *longum* NCC2705 (BN) and its BL0033 gene knockout (BNKO) ameliorate naproxen-induced small intestinal injury. Similarly to BA treatment, when administered to rats once daily for five days prior to naproxen administration, BN and BNKO significantly reduced the intestinal damage severity by ~62% and ~69% respectively. Each bar represents the mean ± SEM 6 rats per group. **p < 0.01 versus the vehicle-treated group; one way ANOVA and Dunnett’s Multiple Comparison test.

5.3.3 *FPr*au diminishes naproxen-induced small intestinal damage: NSAID-induced enteropathy has some similarities to Crohn’s disease, including, but not limited to, increases in permeability and bile acid malabsorption (Bjarnason 1989). A decrease in *FPr*au has been associated with disease activity in Crohn’s disease patients (Sokol 2008; Galecka 2013). These facts were the foundation for the examination of *FPr*au administration in our model. Vehicle treatment resulted in an average macroscopic damage score of 230. We observed that treatment with *FPr*au reduced the macroscopic damage score by ~75% (Figure 5.4). As with other bacteria treatment, macroscopic damage began more proximally compared to where damage started in vehicle-treated rats. Ulcers and erosions were of similar size in *FPr*au-treated rats as in vehicle-treated rats. There were significantly fewer ulcerations and erosions in the small intestine of *FPr*au-treated rats.

Due to the oxygen intolerance of *FPr*au, the supernatant was tested as a treatment (*FPr*au SN). Treatment with the bacteria-free *FPr*au SN was unable to significantly decrease the number of erosions and ulcerations from the naproxen treatment compared to vehicle treatment. We did observe that the damage with *FPr*au SN
treatment did begin more proximately in the small intestine compared to where the damage initiated in vehicle-treated rat.

**Figure 5.4: Faecalibacterium prausnitzii (FPrau) significantly reduced naproxen-induced intestinal damage.** When administered to rats once a day for five days prior to naproxen administration, FPrau significantly decreased macroscopic intestinal damage scores by over 75%. FPrau supernatant was unable to significantly decrease macroscopic damage scores. Each bar represents the mean ± SEM of at least 4 rats per group. **p<0.01 versus the vehicle-treated group; one-way ANOVA and Dunnett’s Multiple Comparison test.

**5.3.4 Bacterial treatment altered the mid-jejunum microbiota profile:** To determine if microbiota changes correlate with differences in macroscopic damage scores, we profiled the small intestine of rats treated with vehicle, BA and BL (Figure 5.5).

Illumina sequencing revealed that mid-jejunum samples from rats treated with vehicle on average had ~96% of their total microbiota made up from bacteria in the Firmicutes phylum, ~1.5% of the bacteria were in the Bacteroides phylum and
~0.2% from the Actinobacteria phyla. The remaining phyla were grouped together and labeled as *non-dominant phyla*. The non-dominant phyla made up about 1.8% of the total bacteria found in the samples. In order of relative abundance, the non-dominant phyla subsection from vehicle-treated rats is made up of: Proteobacteria (~73%), Tenericutes (~21%), Plantomycetes (~3%) and Verrucomicrobia (~1%).

Five days of treatment with BA before naproxen administration resulted in different microbial profiles compared to those rats treated with BL or vehicle. BA-treated rats had the highest overall percentage of Firmicutes bacteria (~99%). In order of relative abundance, the non-dominant phyla subsection from BL-treated rats is made up of Tenericutes (~54%), Proteobacteria (~23%) and Verrucomicrobia (~3%).

Illumina revealed that small intestinal samples from BL-treated rats had almost 92% of the bacteria derived from the Firmicutes phyla and fewer than 3% were from the Bacteroides phyla. The non-dominant phyla made up slightly less than 4.5% of the bacteria in the sampled area. In order of relative abundance, the non-dominant phyla subsection from BL-treated rats is made up of: Proteobacteria (~80%), Tenericutes (~17%), Plantomycetes (~1%) and Fusobacteria (~0.5%).

Focusing on the non-dominant phyla (see bar graph insert in Figure 5.5A-C), the greatest differences occurred with the Tenericutes phyla among vehicle-, BA-, and BL-treated small intestinal contents. In BA-treated rats, the Tenericutes phyla
accounted for 54% of the non-dominant phyla, in contrast to ~20% in vehicle-treated and ~17% in BL-treated.
**Figure 5.5: Small intestinal bacterial Illumina sequencing data reveals alterations in non-dominant phyla.** Tissue and contents were sequenced from small intestinal sections of treated rats. **A.** Vehicle-treated rats had ~96.5% of the total bacteria assigned to the Firmicutes phylum, ~1.4% assigned to the Bacteroides phylum and ~0.2% assigned to the Actinobacteria phylum. The remaining ~2% of bacteria were categorized as non-dominant phyla. Over 73% of the non-dominant phyla in vehicle-treated rats belong to the Proteobacteria phylum and ~20% belong to the Tenericutes phylum. **B.** *Bifidobacterium adolescentis* (BA)-treated rats had over 99% of the total sequenced bacteria assigned to the Firmicutes phylum and ~0.65% to the Bacteroides phylum. With respect to non-dominant phyla, BA-treated rats had a decrease in the Proteobacteria phylum compared to vehicle-treated rats. Bacteria assigned to the Proteobacteria phylum made up less than 24% of the total non-dominant phyla. BA-treated rats had an increase in non-dominant phyla bacteria assigned to Tenericutes. Bacteria assigned to this phylum represented ~54% of non-dominant phyla. **C.** *Bifidobacterium longum* supsp *longum* (BL)-treated rats had the lowest level of bacteria assigned to Firmicutes (~92%), the highest level of bacteria assigned to Actinobacteria (~1%) and Bacteroides phylum (~2.5%). Of the non-dominant phyla, BL-treated rats had bacteria assigned to different phyla in a similar fashion to vehicle-treated rats. BL-treated rats had ~80% of non-dominant bacteria assigned to the Proteobacteria phylum, ~16% assigned to the Tenericutes and less than 0.5% assigned to Verrucomicrobia.

5.3.5 **BL exacerbates diclofenac-induced small intestinal injury:** One single dose of diclofenac (15 mg/kg) resulted in consistent formation of bleeding ulcers in the small intestine of rats 24 hours post-administration. The damaged area had marked generalized hyperemia and ulcerations in the mid-jejunum.

Treatment with BL (*10⁹ CFU) exacerbated the intestinal damage induced by diclofenac by ~238% (Figure 5.6). In contrast, pretreatment with other bacteria (BA, BN, BNKO, FPrau or FPrau SN) did not alter the diclofenac-induced intestinal damage.
Figure 5.6: *Bifidobacterium longum* subsp. *longum* JCM 1217 (BL) exacerbates diclofenac induced small intestinal damage. A single oral administration of diclofenac (15 mg/kg, in 1% CMC) was given to rats after the same pretreatment protocol from the naproxen-enteropathy study. Animals were euthanized 24 hours after the administration and damage was assessed. No bacterial pretreatment was able to protect against diclofenac-induced small intestinal damage. Pretreatment with BL significantly exacerbated the injury. \( n \geq 7 \) per group, **\( p \leq 0.001 \) versus the vehicle-treated group; one-way ANOVA and Dunnett’s Multiple Comparison test.

5.3.6 Indomethacin-induced enteropathy is unaltered following bacterial administration: A single administration of indomethacin (7.5 mg/kg) resulted in extensive small intestinal damage after 24 hours, consisting of marked hyperemia, ulcerations, and bleeding in the small intestine. None of the bacterial pretreatments resulted in alterations aggravating or mitigating indomethacin-induced enteropathy (Figure 5.7).
Figure 5.7: None of the bacterial pretreatments influenced small intestinal damage induced by a single administration of indomethacin. After the bacterial pretreatment protocol, 7.5 mg/kg of indomethacin was orally administered and rats were euthanized 24 hours later. No bacterial pretreatment altered the macroscopic intestinal damage from indomethacin treatment. n ≥ 7 per group. Treatment groups are not significantly different compared to the vehicle-treated group as confirmed by one-way ANOVA and Dunnett’s Multiple Comparison test.
- CHAPTER SIX -

ANTIBIOTIC COCKTAIL TREATMENT ALTERS NAPROXEN-INDUCED SMALL INTESTINAL INJURY
6.1 Introduction

It is known that the intestinal microbiota plays a contributing role in NSAID enteropathy, but in what capacity remains unknown. The microbiota may have a primary role in initiating injury or it may have a secondary role where it exacerbates existing tissue damage or impedes repair. Key observations regarding the intestinal microbiota and NSAID-induced injury have been made using germ-free animals (Robert 1977, Uejima 1996; Wallace 2011). Although there is currently no clear evidence of an initiating role of the intestinal microbiota in NSAID-enteropathy, bacteria do colonize sites of ulceration and are able to interfere with ulcer healing (Elliott 1998, 2000). In experiments in which antibiotics were administered along with and NSAIDs, ulceration was not prevented, but was reduced in severity, likely due to an enhancement of healing (Koga 1999; Yamada 1993). In addition, studies conducted with LPS administration have shown an impairment of ulcer healing (Hagiwara 2004). In this study, LPS did not cause ulceration in the absence of an NSAID, but it did cause a worsening of ileal ulceration. Other studies examined mice lacking TLR4, the LPS receptor. After NSAID administration, TLR4-deficient mice developed ~80% less intestinal damage than wild-type controls, reinforcing the importance of the intestinal microbiota in NSAID intestinal damage (Watanabe 2008).

Bacteria play a significant role in the pathogenesis of NSAID-induced injury, as does bile. This is evident by the resistance of germ-free animals to developing NSAID
injury, as well as in the protection from NSAID damage in wild type animals with a ligated bile duct (Somasundaram 1997; Wax 1970; Jackob 2007).

The exacerbation of NSAID-induced intestinal damage by bile is due mainly to secondary bile acids, which are derived from primary bile acids through the actions of bacterial enzymes. Secondary bile acids, alone or in conjunction with NSAIDs, have been shown to cause damage to cultured intestinal epithelial cells (Uchida 1997; Zhou 2010).

The relationship between the intestinal microbiota and bile goes beyond the conversion of primary to secondary bile acids. Re-absorption of NSAIDs in the distal small intestine is dependent on the de-conjugation of NSAID-glucuronides by bacteria β-glucuronidase (Boelsterli 2011). In the liver, NSAIDs are conjugated with glucuronic acid and form a water-soluble 1-β-O-acyl glucuronide. The acyl glucuronide (AG) is secreted across the hepatocanalicular membrane into the biliary tree where it is delivered into the distal small intestine. The NSAID-AG is then cleaved, a required step for the NSAID to be transported across the epithelium to be enterohepatically recirculated (LoGuidice 2012). Germ-free mice, which lack bacterial β-glucuronidase, show a reduction in NSAID-induced injury, as do mice administered a bacterial β-glucuronidase inhibitor (LoGuidice 2012).

Animal and human studies have reported beneficial effects of antibiotics against NSAID-induced enteropathy, specifically with metronidazole, an antibiotic agent against anaerobic bacteria (Yamada 1993; Bjarnason 1999). Bjarnason and
colleagues suggested that damage was caused mostly by neutrophils and that a metronidazole-sensitive microbe may be the main chemoattractant. Rifaximin is an semi-synthetic anaerobic antibiotic, limited to having effects in the gut. Due to this, rifaximin has a better safety profile, as the development of resistance to rifaximin occurs with lower frequency (Adachi 2006). Recently, rifaximin was shown to prevent indomethacin-induced small intestinal damage in guinea pigs. Here we examine the effect of the three different antibiotics on naproxen-induced small intestinal injury. We observed the effects of a broad-spectrum antibiotic (rifaximin), a gram-negative antibiotic (kanamycin) and a gram-positive antibiotic (vancomycin) to determine if they would affect the severity of naproxen-induced small intestinal damage.

6.2 Specific Materials and Methods

6.2.1 Administration of single antibiotics: Rats were treated with rifaximin (100 mg/kg in 1% CMC; Sigma-Aldrich, Canada) or vehicle once daily for a total of 9 days. Half of the rats were subsequently treated orally with naproxen twice daily for the final 4 days (as described in chapter 3).

In another experiment, rats were treated with kanamycin or vancomycin (100 mg/kg in ddH2O and 50 mg/kg in 1% CMC respectively; Sigma-Aldrich, Canada) once daily for 7 days.
(Koga 1999). Following this, half of the rats were treated orally with naproxen for an additional four days and the other half treated with vehicle (as described in chapter 3).

6.2.2 Administration of antibiotic cocktail:

Male, Wistar rats were given antibiotics in drinking water or by oral gavage (twice daily) for 9 days. Beginning on the evening of the 7th day, naproxen was co-administration twice-daily for 4 doses total. One hour following the final dose of naproxen, the rats were euthanized, the intestinal damage blindly scored and samples collected for Illumina sequencing.

Rats that received the antibiotic cocktail orally were gavaged twice daily for 7 days prior to 4 doses of naproxen, given 12 hours apart. The cocktail consisted of neomycin trisulfate salt (100 mg/kg; Sigma-Aldrich, ON), streptomycin sulfate salt (100 mg/kg; Sigma-Aldrich, ON) and bacitracin (100 mg/kg; Sigma-Aldrich, ON) (Bhowmik 2012).

The antibiotic treatment administered in drinking water was made up of four antibiotics: ampicillin sodium salt (1 g/L; MP Biomedicals OH, USA), vancomycin hydrochloride (500 mg/L; MP Biomedicals OH, USA), neomycin trisulfate salt hydrate (1 g/L; Sigma-Aldrich, ON) and metronidazole (1 g/L; Sigma-Aldrich, ON).
6.3 Results

6.3.1 Antibiotic administration did not affect the severity of naproxen-induced small intestinal damage: Rifaximin is a broad-spectrum antibiotic reducing the numbers of gram-positive and gram-negative bacteria in the intestine. It has been suggested to be beneficial in reducing the severity of NSAID-enteropathy (Scarpignato 2008). We found that rifaximin treatment did not affect the severity of naproxen-induced small intestinal damage (Figure 6.1A). Ulceration, erosions and hyperemia were observed throughout the small intestine in both groups.

In contrast to the broad-spectrum antibiotic properties of rifaximin, kanamycin and vancomycin specifically target gram-negative and gram-positive bacteria, respectively (Tana 2010; Bartram 2011). Neither kanamycin nor vancomycin attenuated naproxen-induced small intestinal damage (Figure 6.1B-C). As with rifaximin treatment, ulcerations, erosions and hyperemia were visible throughout the small intestine in both treatment groups.
Figure 6.1: Antibiotic treatment did not significantly alter the severity of naproxen-induced small intestinal damage. A. When administered to rats once daily for five days prior to initiating a twice-daily naproxen treatment, rifaximim did not significantly alter the severity of intestinal damage. B. When pretreated with kanamycin before naproxen administration, rats exhibited no reduction in severity of intestinal damage. C. Vancomycin pretreatment prior to naproxen therapy did not attenuate the severity of naproxen-induced small intestinal injury. Each bar represents mean ± SEM of 6 rats per group. Groups are not significantly different based on a two-tailed unpaired t-test.

6.3.2 Oral administration of an antibiotic cocktail did not protect against naproxen-induced small intestinal damage: An antibiotic cocktail comprised of neomycin, streptomycin and bacitracin can elicit changes in the intestinal microbiota without being absorbed into the systemic circulation (Bohnhoff 1962; Xing 2005; Xu 2007). By giving the antibiotic cocktail to rats orally, it allows for a simple, easy administration and, most importantly, an exact dose is known. The orally administered antibiotic cocktail did not convey protection against naproxen-induced intestinal damage (Figure 6.2). The intestine from rats receiving naproxen and those receiving naproxen and antibiotics both had marked hyperemic areas. Treatment with antibiotics alone did not induce small intestinal injury.
Figure 6.2: An oral antibiotic cocktail administration failed to protect against naproxen-induced small intestinal damage. Rats pretreated orally with an antibiotic cocktail of neomycin, streptomycin and bacitracin were not protected from naproxen-induced enteropathy. Antibiotic treatment alone did not induce any intestinal damage. Bars represent the mean ± standard error of the mean of 6 rats per group.

6.3.3 An antibiotic cocktail administered in drinking water reduced the severity of naproxen-induced small intestinal damage in rats: Administration of antibiotics in drinking water has conveyed protection in an array of experimental models including colitis, small intestinal ulcers, bacterial overgrowth and fructose-induced hepatic lipid accumulation (Videla 1994; Rath 2001; Kent 1969; Bergheim 2008). The goal of the present experiments was to reduce the severity of naproxen-induced small intestinal damage. As the above antibiotic cocktail did not convey protection, a secondary antibiotic cocktail was made from ampicillin (1 g/L), vancomycin (500 mg/L), neomycin (1 g/L) and metronidazole (1 g/L) and given to rats in drinking water. This method is less stressful to the rats and would likely result in a more continuous exposure to the antibiotics throughout the study period.
When administered in drinking water, the antibiotic cocktail significantly reduced the severity of naproxen-induced small intestinal injury, decreasing macroscopic damage ~46% (Figure 6.3). Water consumption was measured to ensure there were no differences between treatment groups (Figure 6.4)

**Figure 6.3: Antibiotic treatment in drinking water ameliorated naproxen-induced small intestinal damage.** An antibiotic cocktail administered to rats via drinking water significantly decreased the small intestinal macroscopic damage induced by naproxen. Bars represent the mean ± standard error of the mean of 6 rats per group. **p<0.01 vs. the naproxen group.
Figure 6.4. Water consumption by rats in antibiotic drinking water study. Regardless of their treatment groups, rats consumed equal amounts of drinking water throughout the duration of the study. Bars represent the mean ± standard error of the mean. p=0.2827; one-way ANOVA.

6.3.4 Changes induced by antibiotic administration may not be predictive of intestinal damage: Often, studies will correlate a change in the intestinal, cecal or fecal microbiota with a change in disease state. This correlation can be seen in a recent study of elderly adults where changes in fecal microbiota significantly correlated with measures of frailty (Claesson 2012). Compositional changes in the gut microbiota have also been associated with obesity, inflammatory bowel disease and irritable bowel syndrome (Ley 2006; Frank 2007; Qin 2010; Kassinen 2007; Jeffery 2012). Despite this, changes can occur in the microbiota that have no correlation to clinical responses (Kump 2013). Here we see that Illumina sequencing of rats treated only with naproxen revealed a promotion of gram-negative bacteria. Similarly, sequencing of cecal contents of rats treated only with vancomycin had an increase of gram-negative bacteria. Co-administration of
naproxen and vancomycin produced a reduction of gram-negative bacteria and an increase in gram-positive bacteria, specifically Firmicutes. Despite similar changes in intestinal microbiota, the macroscopic intestinal damage scores from naproxen-treated rats and vancomycin treated rats were drastically different (Figure 6.5A, taxonomy legend Figure 6.5B).

Figure 6.5: Taxa summary of cecal microbiota contents from rats treated with vancomycin. A. Taxa summaries from cecal samples of naproxen-treated rats are similar to those from vehicle-treated rats. They are categorized by a large proportion of Firmicutes bacteria. Their levels of Firmicutes are not statistically different (85.00% ± 4.11% Firmicutes in naproxen-treated rats vs 90.48% ± 2.75% Firmicutes in vehicle-treated rats). The two groups also had a similar low-level of
Bacteroides (13.72% ± 4.21 in naproxen-treated rats and 8.26% ± 2.63% in vehicle-treated rats). The damage scores were significantly different, with naproxen-treated rats having a mean macroscopic damage score of 135 ± 22 and vehicle-treated rats having a mean macroscopic damage score of 0. The taxa summaries from vancomycin- and vancomycin+naproxen-treated rat cecal samples show a decrease in Firmicutes (13.04% ± 2.49% in vancomycin-treated rats and 8.92% ± 1.53% in vancomycin+naproxen-treated rats) and a significant increase in Bacteroides (57.62% ± 4.98% in vancomycin-treated rats and 56.29% ± 4.20% in vancomycin+naproxen-treated rats). B. Taxonomy legend to identify phyla in columns.

Similar changes in cecal microbiota are seen in rats treated via oral gavage with naproxen, an antibiotic cocktail or both (Figure 6.6A, taxonomy legend Figure 6.6B). Although there are dramatic losses of Firmicutes bacteria, and significant increases of Bacteroides, this did not alter the intestinal macroscopic damage scores. Rats treated with naproxen had intestinal damage scores of 214 ± 60, which was not significantly different from the intestinal damage scores for rats treated orally with naproxen and the antibiotic cocktail (121 ± 42, p = 0.2361).
Figure 6.6: Taxa summary of changes in phyla in cecal microbiota in naproxen- and/or antibiotic cocktail-treated rats by oral gavage. A. Taxa summaries from cecal samples of naproxen-treated rats are similar to those from vehicle-treated
rats, defined by a high proportion of Firmicutes bacteria (95.24% ± 0.80% and 96.99% ± 0.63% in naproxen- and vehicle-treated rats respectively). Both treatment groups had low levels of Bacteroides (3.38% ± 0.57% and 2.06% ± 0.52% in naproxen- and vehicle-treated cecal samples, respectively). The mean macroscopic damage scores for the groups were 215 ± 60, 5 ± 2, 121 ± 42 and 0.33 ± 0.33 for naproxen-, antibiotic cocktail-, naproxen+antibiotic cocktail- and vehicle-treated rat cecal samples respectively. The taxa summaries show a dramatic shift in the cecal microbiota after treatment, with a loss of Firmicutes bacteria and a subsequent expansion of Bacteroides in both antibiotic cocktail- and naproxen+antibiotic cocktail-treated rats. There is a 36-fold increase in Bacteroides in antibiotic cocktail-treated rats compared to vehicle-treated rats and ~9-fold decrease in Firmicutes bacteria. B. Taxonomy legend to identify phyla in columns.

Similar changes were seen when an antibiotic cocktail was administered in drinking water. In this experiment, like others, naproxen- and vehicle-treated rats had cecal microbiota profiles that were similar, with high levels of Firmicutes and low levels of Bacteroides (Figure 6.7A, taxonomy legend Figure 6.7B). When treated with the antibiotic cocktail in drinking water, regardless of whether naproxen was administered, there was a decrease in Firmicutes bacteria and an expansion of Cyanobacteria and Proteobacteria. There were no Cyanobacteria detected in samples from naproxen- or vehicle-treated rats, but in antibiotic cocktail- and naproxen+antibiotic cocktail-treated rats, Cyanobacteria made up ~16% ± 7% and ~29% ± 10% respectively. Proteobacteria, which comprises, on average, less than 1% of the cecal microbiota from vehicle-treated rats (0.71% ± 0.31%) makes up a large proportion of naproxen+antibiotic cocktail-treated rat cecal samples (41% ± 20%). Two rats from the naproxen+antibiotic cocktail group had over 99.5% of their cecal contents belong to the Proteobacteria phyla. Both of these rats had low intestinal damage scores (16 and 36 mm²).
B.

<table>
<thead>
<tr>
<th>Legend</th>
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<td>Root;p_Tenericutes</td>
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<td>Root;p_Verrucomicrobia</td>
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Figure 6.7: Taxa summary of changes in phyla in cecal microbiota in naproxen-and/or vehicle-treated rats given antibiotic cocktail or vehicle in drinking water. A. Naproxen- and vehicle-treated rats have similar taxa summaries, defined by high levels of Firmicutes and low levels of Bacteroides. In contrast, treatment with the antibiotic cocktail in drinking water resulting in a decrease of Firmicutes bacteria, and an increase in Cyanobacteria (16% ± 7% of total cecal microbiota; antibiotic cocktail only and ~29% ± 10% of total cecal microbiota; antibiotic cocktail and naproxen). Cyanobacteria were not detected in cecal samples from naproxen- or vehicle-treated rats. Proteobacteria was detectable at low levels in vehicle-treated rats (0.71% ± 0.31%) but expanded in naproxen+antibiotic-treated rats (41% ± 20%), with two rats (SS 56.13 and SS56.15) having over 99% of their cecal microbiota comprised of Proteobacteria. B. Taxonomy legend to identify phyla in columns.
- CHAPTER SEVEN -

AMELIORATION OF NAPROXEN-INDUCED DAMAGE BY PROBIOTICS OCCURS INDEPENDENTLY OF LACTIC ACID
7.1 Introduction

For over 100 years, the benefits of probiotics have been recognized and examined. Metchnikoff, a Russian biologist, zoologist and protozoologist is considered, amongst other things, a founding father of probiotic research. He can be credited for the modern hypothesis that certain bacteria can play a positive role in the body, specifically in the gut. In the century following his proposed hypothesis, scientist and consumers have accepted the probiotic concept all over the world (Fuller 1992). The term lactic acid bacteria (LAB), was accepted in the beginning of the last century, first appearing in a publication by Orla-Jensen in 1919 (Orla-Jensen 1919; Carol 2010; Axelsson 1989). Amongst other things, LAB are characterized by their production of lactic acid as a major end product of glucose catabolism. LAB are nonpathogenic and can enhance immune responses, control infections of the gut, and have anti-carcinogenic roles (Link-Amster 1994; De Simone 1993; Majamaa 1995). Lactobacillus casei strain Shirota (LcS) was shown to exhibit a strong protective effect against indomethacin-induced small intestine injury (Watanabe 2008). Repeated administration of LcS was necessary to observe the amelioration of the NSAID-induced intestinal damage, as a single dose and heat-killed LcS failed to do so. Watanabe et al. (2009) showed an elevated lactic acid concentration correlating with a decrease in indomethacin-induced enteropathy. Administration of L-lactic acid in drinking water reduced the severity of NSAID-induced intestinal damage independently of changes in pH.
There have been conflicting reports in the literature with respect to the relationship between lactic acid and inflammation. Lactic acid has been reported to inhibit expression of mRNA for iNOS, IL-6 and IL-10, as well as NFκB DNA binding activity in RAW 264.7 cells stimulated with LPS (Kellum 2004). Moreover, lactic acid can decrease TNFα mRNA expression and NFκB DNA binding activity in human blood-derived macrophages incubated with lactated dialysis solution (Douvdevani 1995). Additionally, increased TNFα expression has been observed in macrophages cultured with lactic acid (Jensen 1990). In a recent study, L-lactic acid prevented NFκB activation, phosphorylation of IκBα and TNFα expression in LPS-stimulated THP-1 cells (Watanabe 2009). Earlier reports by the same group stated that TNFα neutralizing antibodies prevented indomethacin-induced intestinal injury in rats (Watanabe 2008). Together these studies suggest that L-lactic acid production by LAB may be a key mechanism of the positive effect seen on NSAID-induced intestinal injury.

Although Bifidobacteria are genetically distinct and thus are not defined within LAB parameters, they have an overlapping habitat with LAB as well as produce lactic acid as a major metabolic product (Fukuda 2011). For these reasons, we examined Bifidobacteria in conjunction with LAB. Moreover, Bifidobacteria are one of the beneficial intestinal bacteria that have health-promoting effects in humans and, as previous chapters of this dissertation have reported, can ameliorate naproxen-induced small intestinal damage. Given the similarities between Bifidobacteria and LAB, we hypothesized that the beneficial effects from some Bifidobacteria may be
due to the production of lactic acid. In this chapter we explore the effects of L-lactic acid supplemented in drinking water on naproxen-induced small intestinal damage.

### 7.2 Specific Materials and Methods

**7.2.1. Lactic acid supplementation of drinking water**

Lactic Acid was administered through drinking water as previously described (Watanabe 2009). Briefly, drinking water for rats was supplemented with 15 mM of lactic acid or 2.5 mM HCl. The 2.5 mM HCl in drinking water was administered as it has a pH which is similar to that of 15 mM L-lactic acid (pH 2.7). Rats were given the water ad libitum starting three days prior to and continuing throughout naproxen administration. Water bottles were filled daily and the change in water bottle weight was used as a surrogate marker of water consumption. Small intestinal damage was assessed as described in section 3.3.

### 7.3 Results

**7.3.1 Lactic acid supplementation in drinking water does not influence naproxen-induced small intestinal damage**

We examined the potential anti-ulcerogenic effects that it may have in our naproxen-induced small intestinal injury model (Figure 7.1). Rats supplied with tap water before naproxen administration had macroscopic small intestinal damage scores averaging 165 mm² (Figure 7.1). 15 mM L-lactic acid supplied in drinking water did not affect the severity of naproxen-enteropathy. Similarly, HCl-
supplemented drinking water did not affect the severity of naproxen-induced intestinal injury.

![Bar chart showing macroscopic damage scores for tap water, lactic acid water, and HCl water.](image)

**Figure 7.1:** Lactic acid-supplemented water did not influence naproxen-induced small intestinal damage.

Rats given water supplemented with lactic acid did not significantly change the naproxen-induced small intestinal damage severity. Similarly, rats given HCl-supplemented water did not affect the severity of naproxen-induced small intestinal damage. Each bar represents mean ± SEM of 6 rats per group. No differences versus the tap water group; one-way ANOVA and Dunnett’s Multiple Comparison test.
- CHAPTER EIGHT-

DISCUSSION
8.1 Summary

NSAIDs and PPIs are two of the most commonly used classes of drugs. The former are taken to relieve inflammation, while the latter are taken to reduce NSAID-associated gastroduodenal bleeding and ulceration (Scarpignato 2010). The co-administration of these drugs reduces gastroduodenal injury, but there is no reason to believe this strategy would benefit the small intestine (Scheiman 2006; Scarpignato 2010; Yeomans 1998; McCarthy 2009; Lanas 2006). Although the pathogenesis of NSAID-induced enteropathy is not fully understood, it is clear that it is distinct from the pathogenesis of NSAID-induced gastropathy. This fact has been the driving force for the study of how these drugs affect more distal areas of the small intestine. Results from animal and human studies confirm that the small intestine, as a distinct organ, requires discrete protection strategies from that of the stomach and duodenum. This dissertation was undertaken in an attempt to clarify the changes that occur in the small intestine after PPI and NSAID administration (Chapter 4), how changes in the microbiota influence intestinal toxicity (Chapters 5 and 6) and explore possible mechanisms of action (Chapter 7).

8.2 PPIs exacerbate naproxen-induced enteropathy via induction of dysbiosis

The mid-jejunum area of the small intestine, like the stomach and duodenum, is susceptible to injury from the use of NSAIDs. Despite this similarity, the pathogeneses of the damage to these sites are distinct, and thus their treatment is also expected to be distinct. The pathogenesis of NSAID gastropathy is well
understood and prevention therapies (suppression of gastric acid secretion) have been successfully developed. In contrast, the small intestine has been largely ignored in clinical settings. Post-mortem studies first revealed NSAID use caused damage in the small intestine and recently, video capsule endoscopy studies have demonstrated a high incidence of intestinal damage in subjects taking NSAIDs and PPIs (Bjarnason 1993; Goldstein 2005; Graham 2005; Maiden 2005). Moreover, NSAIDs induce enteropathy in rodents similar to that seen in humans, and these models have proven useful for furthering our understanding of the pathogenesis. There is no evidence that suppression of gastric acid secretion will decrease the incidence of NSAID-induced enteropathy (Hunt 2009). In this dissertation, we explored the effects on the small intestine of concurrent administration of an NSAID (naproxen) and a PPI (omeprazole).

Co-administration of naproxen and omeprazole at doses that efficiently inhibited their target enzymes resulted in a reduction in gastric damage, as expected. However, this treatment also resulted in an exacerbation of small intestinal injury and bleeding. The pharmacokinetics of naproxen were not altered from the co-administration of omeprazole and thus, an increase in intestinal toxicity cannot be attributed to higher plasma concentration of the NSAID. The results from rodent studies presented in this thesis warrant further evaluation of these two classes of drugs within a clinical setting. Human studies evaluating the small intestine have been conducted on healthy volunteers taking an NSAID and a PPI. The incidence of damage ranged from 55%-70% as detected by video capsule endoscopy (Graham
While this significant small intestinal damage was reported in studies of healthy volunteers receiving treatment with an NSAID and a PPI for up to two weeks, a realistic patient population using this treatment would most likely be seniors (>65 years of age) and would likely have underlying diseases (such as osteoarthritis), which may increase their susceptibility to intestinal damage. Most importantly, a patient would be chronically on concurrent NSAID and PPI therapy, far longer than the two weeks in the clinical studies. Because of these factors, it is reasonable to hypothesize that small intestinal damage in an older, chronically treated patient population would be more severe.

The pathogenesis of NSAID-induced gastropathy and enteropathy are distinct (Wallace 2012). Inhibition of COX enzymes are required for gastroduodenal injury, but this does not appear to play as central of a role with regards to distal to the ligament of Treitz. Moreover, gastric acid secretion contributes significantly to NSAID-induced gastropathy, which is why patients being treated chronically with an NSAID are often co-prescribed a PPI or H$_2$RA. Indeed, the results from Chapter 4 revealed that concurrent administration of an NSAID and a PPI to a rodent resulted in substantially more ulceration and bleeding than naproxen alone. In NSAID-enteropathy, enterohepatic recirculation results in repeated exposure of the mucosa to the drug (mixed with bile). Damage from NSAIDs is worse when bile is present, and intestinal damage is prevented if the bile duct is ligated (Konaka 1999; Zhou 2010). In addition to enterohepatic recirculation, the microbiota plays a role in
small intestinal injury. The importance of the microbiota, specifically gram-negative enteric bacteria, has been observed in studies with antibiotics, GF rodents and TLR-4 deficient mice (Hagiwara 2004; Uejima 1996; Konaka 1999; Robert 1977; Watanabe 2008). From our DGGE analysis, it is clear that treatment with omeprazole resulted in significant dysbiosis, where the pronounced change in the jejunal microbiota was the significant reduction in Actinobacteria. Sequencing and RT-PCR revealed that the most prominent member within the lost phyla were *Bifidobacter ssp.*. When a *Bifidobacteria*-enriched suspension of bacteria was administered to rats treated with naproxen and omeprazole, the omeprazole-associated decrease in Actinobacteria, as well as the increased intestinal damage, were both reversed. Furthermore, the study with GF mice revealed that an increased susceptibility to NSAID-enteropathy is transferrable from rats to mice via the microbiota. As a whole, the data strongly suggest that the PPI-induced alterations in the intestinal microbiota are a significant contributing factor to the increased susceptibility to NSAID-induced small intestinal damage.

Human studies that directly examined the effects of PPIs on the intestinal microbiota have not been reported, though there are studies that document prominent gastrointestinal symptoms in PPI users (Compare 2011, Lombardo 2010). Moreover, microscopic colitis has an increased incidence in PPI and NSAID consumers, with the strongest association in older adults on both medications (Wilcox 2009; Keszthelyl 2010; Pardi 2011). These studies support our hypothesis that PPIs can worsen NSAID-induced small intestinal damage by initiating a shift in
the number and type of bacteria in the intestine. In addition to these shifts, two
groups have reported an association between higher fecal calprotectin (a marker of
gut inflammation) levels in patients taking a PPI (Poullis 2003; Andreasson 2011).
Studies evaluating PPIs and the gastrointestinal tract are important due to the
common practice of combining the use of these two drugs, as well as the
introduction of fixed-dose combination tablets. Vimovo is a combination tablet with
an enteric-coated NSAID and esomeprazole. The FDA approved it in 2010 based on
two major studies that evaluated the cumulative incidence of endoscopic gastric
ulcers (Anderson 2010). The approval did not take into consideration any impact on
the small intestine (indeed, even the proximal duodenum was excluded from
endoscopic examination), despite the aforementioned rodent and human studies
reporting exacerbation of naproxen-induced injury. The effect on the small
intestinal bacterial composition and density are of paramount importance, since
elevated numbers of these bacteria has been linked to increases in small intestinal
permeability, which is widely accepted as an early event in the pathogenesis of
NSAID-induced enteropathy (Riordan 1997; Bjarnason 2009; Reuter 1997).

Despite changes in the microbiota from administering PPI alone, there was minimal
detectable impact on the intestinal mucosa. There was no tissue damage or
inflammation when the tissue was evaluated histologically and the gene expression
of key mucosal defence enzymes were unchanged. TNFα is implicated in the
pathogenesis of NSAID enteropathy, but its mRNA expression remained unchanged
after omeprazole administration (Appleyard 1996). Additionally, mucosal hydrogen
sulfide synthesis was increased after administration of omeprazole. An increase in mucosal hydrogen sulfide synthesis has been observed in the stomach and colon following induction of injury and has been reported to contribute to injury healing and inflammation resolution (Wallace 2007, 2009). Moreover, reduction of mucosal hydrogen sulfide synthesis has correlated with higher levels of mucosal inflammation, impairment of tissue repair and a higher level of susceptibility to NSAID-induced gastropathy (Wallace 2007, 2009; Fiorucci 2005). The impact of this on NSAID-induced enteropathy is currently unclear.

The lack of clinical studies evaluating the effect of PPI and NSAID administration on the small intestine is surprising given the widespread use of these drugs. This may be due to the difficulty in evaluating the small intestine with conventional endoscopy. Clinical trials examining the concurrent use of PPIs and NSAIDs with video capsule endoscopy will aid in determining if humans exhibit the same exacerbation from co-treatment as observed in the rat.

*Future Perspectives:* While the animal models presented in Chapter 4 present a proof-of-concept for the role of the small intestinal microbiota in the protection from NSAID-induced enteropathy, they do not provide a link between how the changes in the microbiota are impacting NSAID-induced small intestinal damage. With Illumina technology, it is possible to obtain species-level resolution. By running the *Bifidobacteria*-enriched bacteria through the Illumina platform, species
would be identified and the list of potential responsible bacterium or bacteria would be shortened.

In addition to species resolution, metabolomic profiling would identify changes in the metabolite composition, which may be responsible for protection from NSAID-induced small intestinal injury. As a complete metabolite list is unknown, it would be advantageous to conduct both gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) as the detection of compounds varies between the two techniques (Figure 8.1). By performing both GC-MS and LC-MS, a complete metabolic profile would be obtained.

**Figure 8.1: Classes of chemicals and the analytical techniques with which they are most compatible.** Although the two analytical techniques do have cross over with respect to chemical classes they detect, there are distinct metabolites which are better suited for one of the two analyses.
Although LC-MS is often used and best suited during a discovery-based approach when researching unknown metabolites, if this technique alone is used, less polar metabolites will be missed (Figure 8.1).

8.3 Select bacteria confer protection against naproxen-induced enteropathy independently of acetate

Many diseases of the gastrointestinal tract are thought to be a result of microbial imbalance (Guarner 2003). Without a healthy and stable microbiota, detrimental bacteria may overpopulate, creating a cascade of negative effects that may include inflammation, toxin production, changes in pH, and susceptibility to pathogens (Guarner 2003; Quigley 2006). Probiotics are being used in a clinical setting for disorders such as irritable bowel syndrome to help restore balance in a host’s microbiota with the hope of mitigating symptoms. In theory, the probiotics will be able to regulate immunity (Sartor 2005), modify the inflammatory response (Fedorak 2004), decrease permeability (Boyle 2006) and inhibit pathogenic bacteria colonization (Freitas 2003). There are many different proposed mechanisms of action, which include stimulation of host cells, production of exogenous beneficial proteins, enhanced biological activity, and changes in the host microbiota composition (Meier 2005).

The understanding of underlying mechanisms associated with the beneficial effects of probiotics is still in its infancy, and clinicians face a difficult road ahead. They must navigate between patient perceptions of benefits of bacterial administration as
well as the ever-changing face of literature. While some studies are at the stage of randomized control trials, many, like this one, are only exploring animal models. Current progress would suggest that we are only at the beginning of probiotic research and it is becoming increasingly evident that generalizations about bacterial strains cannot be made, since administration may result in adverse effects, depending on the host’s condition as well as concurrent medications.

The studies from Chapter 5 exemplify how the same bacteria can result in different effects depending on the conditions. We evaluated BA and BL in a naproxen-induced small intestinal injury model. In BA-treated rats, macroscopic small intestinal damage was reduced by ~82%, whereas BL did not significantly decrease damage scores. Histologically, alterations could be seen in vehicle- and BL-treated rats, such as muscularis thickening, epithelial blebbing, increased crypt number and loss of villus architecture, whereas these changes were not seen in BA-treated rats. These results were somewhat surprising since previous studies showed that BL-treated rats were protected against EHEC-induced death, and BA-treated rats were not (Fukuda 2011). Microbiota from BL-treated rats had significantly higher levels of acetate production \textit{in vivo} as compared to \textit{in vivo} acetate production from microbiota of BA-treated rats (Fukuda 2011). As BL-treated rats were protected from EHEC-related deaths, and BA-treated rats were not, the protection was attributed to the production of acetate via fructose consumption. To further investigate how acetate production via fructose consumption might influence naproxen-induced small intestinal damage, we treated rats with a mutant \textit{B. longum}
that did not produce acetate via fructose consumption due to a knockout of the BL0033 gene (BN or BNKO respectively). Both BN and BNKO treatment protected against naproxen-induced small intestinal damage (62% and 69% respectively). The BL0033 gene codes for a fructose-binding protein and is a part of an ABC transport system (Wei 2012). This gene is imperative as the movement of sugars across the cytoplasmic membrane is required for carbohydrate metabolism. This transport system is thought to have an important role given that fructose is the preferred substrate of Bifidobacteria (Wei 2012). Like BNKO, the BA strain does not endogenously express the ABC-type carbohydrate transporter BL0033-BL0036. In contrast, BN, like BL, endogenously expresses the aforementioned ABC-type carbohydrate transporter. If acetate did play a role in the reduction of naproxen-induced small intestinal injury, it is reasonable to assume that BN-treated rats would have similar small intestinal damage scores to those of BL, likewise, it would be expected that BA-treated rats would have similar macroscopic damage scores to BNKO-treated rats. In fact, we observed that BN- and BNKO-treated rats had similar levels of small intestinal enteropathy after naproxen administration, suggesting that bacterial treatment induces protection in our model of naproxen enteropathy independently of bacterial acetate production.

To further examine if acetate may influence naproxen-induced small intestinal injury, we conducted experiments with FPrau. FPrau is an acetate consumer and butyrate producer (Duncan 2002). Moreover, FPrau is highly metabolically active and is found in a healthy microbiota of humans, calves, pigs, mice and cockroach
It also has a low prevalence in many intestinal disorders, such as Crohn’s disease (Miquel 2013). Following some Crohn’s disease treatments, for example high-dose cortisol therapy or Infliximab infusions, levels of FPrau can be restored from concentrations of approximately zero to higher than $1.4 \times 10^{10}$ bacteria/mL within days (Swidsinski 2008). FPrau supernatant is often used in place of FPrau due to the severe oxygen intolerance of the bacteria, and in many cases the supernatant is just as beneficial as the bacteria. For example, FPrau and FPrau SN attenuate 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice (Miquel 2013). The similarities between Crohn’s disease and NSAID-induced enteropathy (Sokol 2008) make FPrau supplementation a suitable choice to examine as a therapy in our model. Interestingly, the NSAID indomethacin has been used to model Crohn’s disease in rodents, as it results in inflammatory injury with similar severity and location to that in humans (Washington Biotechnology Inc. 2009). In our studies, treatment with FPrau reduced the number of erosions and ulcerations in the small intestines of naproxen-treated rats by over 75%; however, FPrau SN was unable to reduce the number of ulcerations or erosions. Given that protection was observed in our naproxen enteropathy model with treatment from bacteria that produced acetate (BN), bacteria that did not produce acetate (BA, BNKO) as well as bacteria that consumed acetate (FPrau), evidence to suggest protection may be correlated with acetate levels is absent at this time.
Future Perspectives: The administered bacteria were genetically similar, but varied greatly with respect to protection from naproxen-induced small intestinal injury. The bacteria may have differed greatly in their released metabolites. Although having a protective role in *E.coli* enteropathogenic infection, acetate does not appear to confer protection against naproxen-induced enteropathy. By analyzing the selected bacteria by GS-MS and LC-MS, a full list of differences in metabolites can be established and used to drive future studies, potentially identifying a protective mechanism.

Given the importance of permeability in the pathogenesis of naproxen-induced small intestinal injuries, a logical next step would be to evaluate changes in intestinal permeability after administration of the selected bacteria. Modulation of the gut bacteria has been reported to increase intestinal permeability by reducing the expression of the tight junction proteins ZO-1 and Occludin (Cani 2008). These changes occurred in mice fed a high-fat diet, which coincided with a loss of *Bifidobacteria* (Cani 2008, 2007a, 2007b). Some *Bifidobacter1eria* ssp. do not degrade intestinal mucus glycoproteins (Ruseler-van Embden 1995; Caplan 1999). Modulation of the intestinal microbiota by *Bifidobacteria* ssp. has led to increases in villus depth as well as a thicker mucosal layer in the jejunum and colon (Kleessen 2003). *In vivo* permeability assays of small intestinal samples from rats treated with selected bacteria and NSAIDs may provide insight as to the mechanism by which some *Bifidobacteria* ssp. protect against naproxen-induced small intestinal damage. Additionally, TNFα, IL-1β, IL-1α, INF-γ are cytokines which are well known to
promote tight junction disruption (Yang 2003; Adams 1993; Coyne 2002; Nusrat 2000). Cytokine profiling may aid in determining how the specific bacteria suspensions are influencing intestinal permeability.

8.4 Co-administration of bacteria and naproxen induces changes in the small intestine microbiota

Illumina sequencing is the most widely used sequencing technology, being cited in more than 5,400 publications as of April 2014 (Illumina 2014). The sequencing by synthesis chemistry used in this technology has the highest yield of error-free reads with fewer false negatives and positives compared to sequencing alternatives (Illumina 2014). For these reasons, we used Illumina sequencing to evaluate if naproxen-induced macroscopic damage scores corresponded to changes in the microbiota profile.

Illumina results from intestinal samples of BA- and BL-treated rats showed the highest percentage of Firmicutes bacteria in BA-treated samples. Low levels of Firmicutes have been associated with poorer health in human studies (Monira 2011). Specifically, elderly patients chronically taking NSAIDs have a significantly lower number of intestinal Firmicutes and a higher level of intestinal Bacteroides (Makivuokko 2010).

Additionally, BA treatment had a striking effect on the non-dominant phyla. In BL- and vehicle-treated rats, the Tenericutes phylum accounted for 17% and 21% of the
non-dominant phyla, respectively. This is in stark contrast to BA-treated rats, in which the Tenericutes phylum made up 70% of the non-dominant phyla. At this time, we are unable to say if this is an effect of the BA treatment or if it is a result of lower intestinal damage scores. Interestingly, the phylum Tenericutes contains only one class: Mollicutes (Brown 1984). Initially, Mollicutes was classified as a Firmicutes bacterium, but in the 2004 and 2009 revisions of Bergey’s Manual of Systematic Bacteriology, it was classified as Tenericutes based on its unique phylogenetics (Brown 1984). A murine diet study associated an increase in Mollicutes bacteria with a higher capacity to import and metabolize carbohydrates into SCFAs, namely butyrate and acetate (De Bandt 2011). SCFAs have been associated with the inflammatory process. In vivo, SCFAs bind the G-protein-coupled receptor 43 (GPR43), sometimes referred to as FFAR2 (Maslowski 2009). Stimulation of GPR43 by SCFAs is required for resolution of certain normal inflammatory processes (Maslowski 2009) and may play a role in protection from naproxen-induced small intestinal damage.

It is plausible that BA is protective against naproxen-induced enteropathy by altering the microenvironment, allowing the Tenericutes phylum and thus bacteria in the Mollicutes class, to bloom. If this were the case, the resulting higher SCFA levels would induce a larger downstream effect through GPR43 signaling, regulating the immune system and inflammatory process. BL and vehicle treatment did not result in increased Tenericutes bacteria, which may account for the lack of protection against naproxen-induced enteropathy.
It is well documented that the intestinal microbiota can be altered due to stress, food and medication, and other factors (Bailey 2011). It is reasonable to assume that NSAIDs can also alter the intestinal microbiota in varying ways. For this reason, it may be necessary to consider the prescribed drug and the specific bacteria in a clinical setting. The importance of this is exemplified by the inability of BA treatment to decrease small intestinal injury in rats treated with indomethacin or diclofenac, two alternative NSAIDs (Figures 5.6 and 5.7). Although bacterial intervention may be a promising avenue for protection against naproxen-induced small intestinal damage, further research of NSAID and bacterial treatment combinations is imperative.

*Future Perspectives:* Administration of bacteria as potential therapies has limitations with respect to storage requirements and related viability. These strict requirements may impact their clinical applications and decrease their ease of use. To circumvent these issues, prebiotics, defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth of one, or a limited number, of bacterial species in the colon, such as Bifidobacteria and Lactobacilli, which have the potential to improve host health” may be a more appropriate treatment (Ashwell 2002).

Inulin is a dietary fructan that stimulates LAB and *Bifidobacteria* spp within the gut microbiota following ingestion (Ranurez-Farias 2009). Cross-feeding of fructose
and fructo-oligosaccharides in vivo from bacteria which are able to degrade inulin may lead to Bifidobacteria stimulation (Rossi 2009). A study with human volunteers had results which indicated that B. adolescentis has a major role in the response to inulin in vivo. Moreover, F. prausnitzii was significantly stimulated by inulin in vivo. Both of these bacteria were found to be protective in our rat model of naproxen-induced small intestinal injury.

In a recent study examining the effects of inulin of dextran sodium sulfate (DSS) colitis, researchers found that oral inulin, but not inulin enemas were able to reduce inflammation as indicated by a reduction in MPO and a reduced release of inflammatory mediators (Videla 2001). Daily administration of inulin by oral gavage (400 mg/kg) or in drinking water (1%) produced the desired anti-inflammatory effect. If inulin administration induced protection from naproxen-induced enteropathy, it may, as a prebiotic, offer an easier clinical solution.

8.5 Antibiotics and the modulation of naproxen-induced small intestinal injury

A microbial influence in the pathogenesis of NSAID-induced small intestinal damage is often hypothesized but specifics of mechanisms remain unknown. Studies examining how antibiotic treatment influences NSAID-induced small intestinal damage have reported amelioration of injury, but have not identified specific bacteria or shifts in microbial composition responsible for this (Evans 2003; Fang 1997; Kent 1969; Yamada 1993). Using culture-independent techniques, we found that the cecal microbiota after naproxen administration was not significantly different from the cecal microbiota composition of controls. After oral
administration of single antibiotics (vancomycin) and an antibiotic cocktail (neomycin, streptomycin and bacitracin) by gavage, the microbiota was significantly changed, but this did not influence the small intestinal damage score after naproxen treatment. When a second antibiotic cocktail (ampicillin, vancomycin, neomycin and metronidazole) was administered in drinking water, the microbiota was significantly changed and the small intestinal damage score was significantly reduced. Whereas changes in cecal microbial composition from vancomycin or the oral gavage antibiotic cocktail resulted in a change in dominant bacteria (vehicle-treated rats had a cecal microbiota dominated by Firmicutes bacteria whereas rats receiving antibiotic treatment in the aforementioned groups had microbiota dominated by Bacteroidetes bacteria), rats receiving an antibiotic cocktail in drinking water (comprised of ampicillin, vancomycin, neomycin and metronidazole) had changes in their intestinal microbiota as well an overall increase in diversity.

Low bacterial diversity has been associated with disease state, as in obesity and IBD (Greenblum 2012; Ott 2004; Manichanh 2012). An increase in diversity, as seen in cecal microbiota samples from rats treated with an antibiotic cocktail in drinking water, may be a protective factor from naproxen-induced small intestinal damage. Commensal anaerobic gut bacteria can attenuate inflammation, thus it is reasonable to conclude that a loss of anaerobic bacteria, like those residing in the cecum, may be associated with mucosal inflammation (Kelly 2003). In our samples here, we see an increase in diversity of anaerobic bacteria, which may be associated with the decrease in naproxen-induced intestinal damage.
The group of rats that received both antibiotic cocktail in drinking water and naproxen saw an increase in diversity, but also an increase in Proteobacteria and Cyanobacteria. Proteobacteria and Cyanobacteria phyla have been detected in the intestinal microbiota in humans and murine animals, although in much lower numbers than observed in our studies (Lupp 2007). The increase in Proteobacteria we saw was specifically an increase in the family Enterobacteriaceae. This family includes harmless symbionts as well as common pathogens (Husnik 2011). Due to species-level resolution limitations, at this time it is unclear what species were expanded after antibiotic treatment.

It is imperative to note that it is unclear why decreases in macroscopic damage scores were observed after antibiotics were administered in drinking water. For instance, the decrease in macroscopic damage scores could be attributed to smaller damaged areas. This could be due to increased protection from an altered microbial composition, immunological changes or alterations in mucus thickness.

Alternatively, the decrease in macroscopic damage scores could be due to an accelerated rate of wound healing. “Cross-talk” is a theoretical process through which the mucosal immune system can communicate with the microbiota about growth, survival and inflammatory control of the intestines (Camp 2009; Wick 1991). A disruption in the microbiota may influence the cross-talk with the intestinal mucosa and interfere with pattern recognition receptors (PRRs) and microbial-associated molecular patterns (MAMPs), changing the signals being transmitted throughout the cells. Disruption of the ligand process of PRRs with
MAMPs has been linked to Crohn’s disease (Neish 2009). Toll-like receptors (TLR) are a classification of PRRs, which have played a role in NSAID-induced small intestinal injury, specifically TLR4, the LPS receptor. Recent studies have focused on the exacerbation of NSAID-enteropathy after increased TLR4 expression as well as amelioration of NSAID-enteropathy following suppression of the LPS/TLR4 signaling pathway (Kato 2007, Watanabe 2009). Altering the microbiota via antibiotics would change the microbial composition; in turn changing this signaling pathway.

It is also possible that the microbial shift observed after treatment with antibiotic cocktail in drinking water was not responsible for the amelioration of naproxen-induced small intestinal damage. If the decrease in naproxen-induced small intestinal injury were due to an increase in wound healing time, the cecal microbiota we were analyzing would be representative of a post-healing composition, and would not necessarily be representative of the microbial composition responsible for the accelerated wound healing. To address this, shorter term time-point experiments that would correlate changes in damage and changes in microbial composition would aid in the elucidation of what is responsible for the decrease in intestinal damage.

Lastly, the antibiotic cocktail may have been manifesting microbe-independent, which led to the decrease in naproxen-induced small intestinal damage. In the case of metronidazole, some of the beneficial effects on intestinal inflammation may be
due to its ability to inhibit leukocyte-endothelial cell adhesion and emigration elicited by indomethacin (Arndt 1994). Further investigation of the effects of antibiotics on immune modulation independent of the changes of the microbiota may help determine potential mechanisms of naproxen-induced enteropathy score decreases.

_Future Perspectives:_ Using the antibiotic cocktail originally administered in drinking water (ampicillin, vancomycin, neomycin and metronidazole) and repeating the study administering the antibiotics by oral gavage will help to determine if the route or the makeup of the antibiotic cocktail was most important in lowering naproxen-induced small intestinal damage. Moreover, it would be important to repeat the study and euthanize rats at earlier time points, correlating intestinal damage scores with perturbations in the microbiota. This would give clarity as to why the naproxen-induced small intestinal damage scores were lower; i.e., if it was due to a general decrease in damage, or if to increased rates of wound healing.

Additional follow-up work examining changes in the microbiota throughout the gastrointestinal tract would be of interest. Sampling throughout the small intestine, large bowel and cecum, as well as monitoring fecal changes, would allow one to correlate changes in a distinct area to changes in intestinal damage, analyzing for patterns. This evaluation would also help to discern the validity of examining cecal contents and correlating this with small intestinal damage.
Beyond antibiotic treatment and macroscopic damage, it would be of interest to evaluate mucin and cytokine expression. Goblet cell secretion of mucins is induced by inflammatory signals in an attempt to protect the intestinal mucosa (Dharmani 2009). Additionally, bacteria in the gut microbiota can improve mucosal barrier integrity (Willemsen 2003). An increase in mucoprotection may be what is driving the decrease in naproxen-induced macroscopic damage. SCFAs have been shown to dose-dependently induce MUC-2 expression \textit{in vitro} and \textit{in vivo} in the rat colon through activation of cholinergic nerves (Shimotoyodome 2000). Moreover, prolonged incubation with SCFAs induces MUC-2 expression in epithelial cells independently of cholinergic activation, skewing the balance towards mucoprotection (Willemsen 2003). SCFAs, as bacterial fermentation products, support mucosal barrier integrity, and by changing the microbial landscape, it is reasonable to conclude that bacterial fermentation products, specifically SCFAs, will change as well.

It would be interesting to compare the SCFA profile from antibiotic cocktail treatments, single antibiotic treatment and bacterial treatment (BA, BL, BN and BNKO). Evaluating SCFA profiles and corresponding mucin secretion may lead to a potential mechanism of action for protection against naproxen-induced small intestinal damage.

8.6 \textit{Lactic acid administered in drinking water did not confer protection in a naproxen-induced small intestinal injury model}
The pathogenesis of NSAID-induced small intestinal injury involves key mechanisms, including bile, enterohepatic recirculation and the intestinal microbiota. After the first pass through the small intestine, NSAIDs are absorbed in the distal intestine, glucuronidated in the liver and secreted with bile into the proximal small intestine. Repeated exposure results in more severe damage and is mediated, in part, by bacteria. Specifically, increases in gram-negative bacteria have been associated with NSAID-induced enteropathy (Kent 1969; Dalby 2006; Hagiwara 2004).

Lactic acid, a small water-soluble molecule, is able to inhibit the growth of many gram-negative species of bacteria due in part to its ability to penetrate the cytoplasmic membrane (Salminen 1998). By transporting through the water-filled porin proteins of the cytoplasmic membrane, undissociated lactic acid reduces intracellular pH and disrupts the transmembrane proton motive force, inhibiting the ability for the cell to generate energy (Ray 2005). Moreover, lactic acid from bacteria may be able to inhibit harmful gram-negative bacterial strains by a combined action of lactic acid and the presence of bile salts which would act as detergents (Nikaido 1996; Alakomi 2000). Despite this, the effects of lactic acid on inflammation have been controversial. Lactic acid has been shown to inhibit the expression of iNOS, IL-6, IL-10 and NFkB DNA binding activity in the mouse macrophage cell line RAW 264.7 (Kellum 2004). Conversely, in macrophages cultured with lactic acid there has been a reported increase in TNFα expression (Jensen 1990). More recently, Watanabe et al. have reported that L-lactic acid
prevented NFkB activation, phosphorylation of IκBα and TNFα expression in THP-1 cells stimulated with LPS (Watanabe 2009).

NFkB, a transcription factor responsible for regulating numerous genes, including some inflammatory mediators, is a heterodimeric complex inactive in resting cells and bound to IκB (Ghosh 2002). When activated, IκB is phosphorylated and undergoes the ubiquitination process, enabling NFkB to translocate to the nucleus (Ghosh 2002). Watanabe and colleagues suggested that L-lactic acid might inhibit the phosphorylation of IκB, preventing NFkB activation and TNFα expression in vitro. Interestingly, this group found intestinal protection against indomethacin-induced small intestinal damage in vivo after L-lactic acid was administered in drinking water. In our naproxen model of small intestinal injury, drinking water that was supplemented with lactic acid prior to and during naproxen administration conferred no small intestinal injury protection. Variance in protection from lactic acid administered in drinking water may be due to the different NSAID model used. Although it is important to note that during these preliminary studies we did not decipher how much lactic acid reached the site of injury.

In the present study, we used an NSAID model in which rats were administered naproxen (20 mg/kg) twice daily for four-and-a-half days. This schedule of administration is more clinically relevant than a single dose of NSAID. Because of this, inferences about therapies or potential treatments in acute NSAID models may not be as clinically relevant.
8.7 The Pathogenesis of NSAID-induced Enteropathy

While NSAID-induced gastropathy is strongly related to the ability of these drugs to suppress COX-1 and -2, how the drugs induce enteropathy is not as straightforward. It is understood that the inhibition of COX contributes to the injury which develops following administration of an NSAID, but bile, the intestinal microbiota and enterohepatic circulation are also all driving forces for injury. When NSAIDs are glucuronidated in the liver and excreted into bile, they synergistically increase intestinal epithelial cell damage. The rationale behind this is incompletely understood, but hypotheses include NSAID-induced changes in the microbiota. These changes may be due to a loss of protective bacteria (for example a loss of Actinobacteria observed in this thesis), a dysbiosis which a. increases concentrations of cytotoxic secondary bile acids, or b. increases in bacteria that have β-glucuronidase activity (required for enterohepatic recirculation). The effect of intestinal microbial changes on enteropathy may be from a combination of these listed reasons. The experiments in this study have highlighted the intestinal microbiota as being the primary driving force in NSAID-induced enteropathy, and further studies elucidating the mechanistic details will aid in guiding therapeutic options.

8.8 The Intestinal Microbiota in Health and Disease

The symbiotic relationship between a host and its intestinal microbiota has resulted from years of co-evolution. Within a healthy host, microbes are able to live without
eliciting an immune response. The intestinal microbiota is responsible for several essential aspects of host physiology and health. The ability for bacteria to enhance the digestive capabilities of a host is believed to be a primary driving factor for the intestinal microbiota’s co-evolution with a host (Hooper 2010). From this relationship, mammals have an adaptable *metagenome*, which is able to digest a vast breadth of saccharides. The results presented in this thesis suggest that further investigation into the specific *Bifidobacteria* subspecies degradation capabilities specifically in an NSAID-treatment environment may hold key information as to their protective capabilities.

Despite this importance, the relationship between a host and its microbiota goes beyond the metabolic capabilities of the bacteria. The bacteria that reside in the intestine deliver intestinal development signals, including those for angiogenesis (Stappenbeck 1999). The prophylactic effects of bacterial administration on NSAID-enteropathy may be due to the bacteria's ability to stimulate angiogenesis, similar is what is observed in the prophylactic effects of prostaglandin E2 and monosodium glutamate (Takeuchi 2014, Amagase 2014). Both prophylactic monosodium glutamate and prostaglandin E2 stimulate angiogenesis via the up-regulation of vascular-derived growth factor through the activation of EP4 receptors. Bacteria that conveyed protection from naproxen administration in these studies may have done so by increasing the healing capabilities of intestinal tissue through granulation and re-epithelization by VEGF. Preliminary studies should be conducted to see if the protective bacteria from these experiments do upregulate
VEGF or alternative growth factors. Additional studies investigating how the upregulation may occur (through MAP kinase activation or epidermal growth factor receptors, for example) would provide a potential mechanism of protection.

8.9 Conclusions

The work presented in this thesis reveals novel insights into PPIs, NSAIDs and the intestinal microbiota in regards to small intestinal injury. Further studies will aid in delineating their implication in the pathogenesis of NSAID-induced enteropathy, with the aim to develop optimized or customized therapeutic approaches, which may be based on microbial modulation strategies.
- APPENDIX I -

ENVIRONMENTAL AND NSAID ENTEROPATHY:
DYSBIOSIS AS A COMMON FACTOR
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A2: Environmental and NSAID-Enteropathy: Dysbiosis as a Common Factor
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A.3 Abstract

At first sight, environmental enteropathy and NSAID-enteropathy may appear to have little in common. One occurs almost exclusively in poor countries and the other primarily in rich countries. One is the consequence of unhygienic living conditions, while the other is a consequence of use of a drug for relief of pain and inflammation. However, there may be a common pathogenic link between these two conditions, namely a significant alteration in the microbiome (dysbiosis), and this raises the possibility of common approaches to treatment. Correction of the dysbiosis that is central to the intestinal tissue injury and dysfunction observed in environmental and nonsteroidal anti-inflammatory drug (NSAID)-induced enteropathies is a logical approach to bringing about restoration of intestinal function. For both conditions, removal of the trigger for dysbiosis is the simplest approach, but it is not always feasible. Correcting of the underlying dysbiosis through the use of probiotics or prebiotics may be a viable option.

Keywords: Intestine; Malabsorption; Bleeding; Ulcer; Dysbiosis; Bacteria; Microbiome; Probiotic; Proton pump inhibitor; Nonsteroidal anti-inflammatory drug
A.4 Environmental Enteropathy

Malnutrition and compromised physical and cognitive development are major concerns in the developing world. A significant factor contributing to childhood malnutrition is environmental enteropathy. This disorder occurs among inhabitants of regions with poor hygiene and sanitation. Chronic exposure to fecal pathogens leads to dysbiosis (a significant imbalance of the intestinal microbiome), which in turn is hypothesized to cause inflammation and structural changes in the small bowel, ultimately resulting in functional changes and malnutrition (Bartram and Cairncross 2011; Lin 2013) (Figure A1). In a normal state, the intestine fully absorbs nutrients, but acts as a line of defense against entry of luminal pathogens. The intestinal barrier is an integral component of the gastrointestinal tract, with an outer layer of mucus, epithelial cells sealed with tight junctions and a complex mucosal immune system in the lamina propria. Epithelial damage leads to increased permeability and decreased surface area for absorption. The resulting bacterial translocation leads to mucosal inflammation, which can impair mucosal immune function and further contribute to malabsorption. Chronic exposure to high levels of fecal bacteria will keep the intestines in an inflamed state, which increases susceptibility to infections, thus initiating the cycle of a chronic inflammatory response. It is noteworthy that this condition is characterized by malnutrition, but not by diarrhea. Malabsorption of nutrients results in growth faltering, increased risk of other morbidities, and may also account for failure of oral vaccines (Sullivan 2006; Ramakrishna 2010). Most dangerous of all is environmental enteropathy in children aged 0 to 5, as the intestinal tract, immune system and microbiota are
immature at this time and interruptions to a normal developmental course may result in long term consequences such as physical and cognitive abnormalities (Calder 2006).

**Figure A1: Common elements of pathogenesis of environmental and NSAID-induced enteropathy.** Intestinal dysbiosis is a common feature that may drive the mucosal inflammation and injury in both disorders. In the case of environmental enteropathy, mucosal injury primarily results in malabsorption and growth failure, while with NSAIDs, ulceration and bleeding are the most recognized outcomes. Both conditions are also largely asymptomatic.

Studies from the 1960s reported that almost all seemingly healthy adults and children in developing countries exhibited morphological abnormalities and/or functional signs of environmental enteropathy (Humphrey 2009). These abnormalities were deemed environmental rather than genetic when further evidence emerged from Peace Corps volunteers in Pakistan who contracted the
same enteropathy after only a few months of residence (Lindenbaum 1968). Environmental enteropathy will resolve shortly after sanitation standards are improved, as evidenced by the healthy intestinal status in Westerners shortly after returning home from affected areas, as well as in individuals from affected areas who had emigrated to developed countries (McKay 2010).

A.5 Treatment Options

Once the underlying hygiene issues that trigger environmental enteropathy have been addressed, as best as possible, attempts can be made to accelerate the recovery of intestinal function. Nutritional interventions have been strongly promoted, but these face significant challenges, since this disorder is characterized by diminished intestinal absorption. After correction of the dysbiosis, there needs to be healing of the intestine, so as to improve intestinal barrier function, before nutritional interventions can be fully effective. Vitamin A and zinc have been reported to reduce small intestinal permeability and improve height-for-age $Z$ scores, though a reduction of intestinal permeability to levels seen in developed countries was not achieved (Thurnham 2000; Chen 2003). Treatment with glutamate has been shown to improve intestinal permeability, but not growth (Williams 2007). The use of ‘anti-inflammatory’ probiotics has been suggested as a treatment for environmental enteropathy (Sartor 2005; McKay 2010). Treatment with the probiotic *Lactobacillus rhamnosus* GG was assessed by Galpin *et al.* (2005), but was found not to significantly affect intestinal permeability or height-for-age $Z$ scores (Galpin 2005). Antibiotic approaches have also been explored,
including the use of rifaximin. However, this non-absorbable antibiotic failed to 

improve gut barrier function (Trehan 2009).

A.6 NSAID-Enteropathy

The ability of NSAIDs to cause ulceration and bleeding in the stomach and 
proximal duodenum has been recognized since the early part of the 20th century. 
The damaging effects of these drugs in the more distal small intestine was not 
reported until the 1980s (Bjarnasson 1988), and only in recent years, with the 
advent of video capsule endoscopy and double-balloon enteroscopy, has it become 
clear that the damaging induced by these drugs in the distal small intestine is a 
common occurrence, with marked clinical significance (Graham 2005; Maiden 
2005). Small intestinal damage induced by NSAIDs has been overlooked in part 
because it is largely asymptomatic, and in part because of the greater difficulty 
in visualizing that damage in the more distal intestine. With the introduction of 
potent suppressors of acid secretion, particularly proton pump inhibitors (PPIs), 
there has been a significant decrease in the frequency of upper GI events, but with a 
corresponding rise in the frequency of lower GI events (Lanas 2009). The latter are 
associated with greater costs of treatment, longer hospital stays and higher rates of 
mortality than the former (Lanas 2009). Co-prescription of low-dose aspirin aimed 
at reducing the incidence of NSAID-related cardiovascular adverse events also 

further exacerbates NSAID-enteropathy, particularly when enteric-coated aspirin is 
used (Endo 2012). There are no proven-effective preventative therapies or 
treatments for NSAID-induced small intestinal damage (Wallace 2012). Diagnosis
also remains a significant challenge despite the above-mentioned advances in endoscopic techniques.

The pathogenesis of NSAID-enteropathy is incompletely understood, but there is convincing evidence for an important role of intestinal bacteria in the induction of intestinal ulceration and impairment of healing (Kent 1969; Uejima 1996; Reuter 1997; Kinouchi 1998; Hagiwara 2004) (Figure A1). NSAID-enteropathy occurs predominantly, if not exclusively, with NSAIDs that undergo enterohepatic recirculation (Brune 1997). Animal and human studies have documented dramatic shifts in the types and numbers of bacteria in the intestine following NSAID administration (Reuter 1997; Kinouchi 1998; Hagiwara 2004). In general, there is an increase in the numbers of gram-negative bacteria and a decrease in gram-positive bacteria (Wallace 2013). Correction of this dysbiosis, via administration of antibiotics or certain probiotics, appears to reduce the severity of NSAID-enteropathy in some animal studies (Kinouchi 1998; Mizoguchi 2001; Syer 2012), although very few such interventions have yet been shown to consistently provide benefits in humans (Table A1).
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<thead>
<tr>
<th>Treatment</th>
<th>Species</th>
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**Table A1: Interventions Affecting the Severity of NSAID-Enteropathy.** This table compares treatments from 1993-2012 across species and highlights the effect on small intestinal damage. *small open-label trial. Note: VSL #3 is a probiotic combination of 8 bacteria.

While potent suppressors of acid secretion, such as proton pump inhibitors (PPIs) and histamine H-2 receptor antagonists, are very effective at reducing the gastroduodenal damage caused by NSAIDs, these drugs are known to cause small intestinal bacterial overgrowth (Williams 2006; Lombardo 2011) and inflammation (Poullis 2004; Andreasson 2011). Moreover, there is emerging evidence that these drugs exacerbate NSAID-enteropathy (Wallace 2011; Satoh 2012), consistent with
the findings of Lanas et al. (2009) that lower intestinal adverse effects of NSAIDs have been steadily increasing since the introduction of PPIs. Thus, in animal studies, PPIs caused a dramatic exacerbation of intestinal damage caused by naproxen or celecoxib, with increased levels of intestinal bleeding (Wallace 2011). The mechanism underlying this effect of PPIs was a dramatic shift in types of bacteria colonizing the intestine. Specifically, treatment with a PPI led to a marked loss of Bifidobacteria species (Wallace 2011). Prevention of the PPI-induced decrease Bifidobacteria, through daily administrations of medium enriched with Bifidobacteria, resulted in a correction of the dysbiosis and prevention of the increase in intestinal damage and bleeding (Wallace 2011). Proof that it was the dysbiosis that was producing the dramatic increase in severity of injury came from studies of germ-free mice (Wallace 2011). These mice are resistant to NSAID-induced intestinal damage.

When the mouse intestine was colonized with bacteria from healthy rats, a small level of intestinal damage could be elicited by NSAID administration. However, when the mouse intestine was colonized with bacteria from PPI-treated rats, subsequent administration of an NSAID resulted in severe intestinal injury.

As in the case of environmental enteropathy, the dramatic shifts in bacterial numbers and types in the intestine during chronic NSAID use leads to increased susceptibility to mucosal injury. The anemia that often accompanies
NSAID-enteropathy is usually attributed to the ability of most NSAIDs to inhibit platelet aggregation, but it could also be in part due to malabsorption of iron and vitamins.

**A.7 Conclusions**

Environmental and NSAID-induced enteropathies are initiated by completely different factors, but they share some important common features of pathogenesis. Particularly striking is the importance of dysbiosis in the initiation of mucosal injury and inflammation in both settings. Both disorders can be prevented by removing the ‘environmental’ triggers (poor hygiene/sanitation for environmental enteropathy, and use of NSAIDs for NSAID-enteropathy). However, this is not always simple or possible. There are significant challenges for the treatment of these conditions, as is evident by the lack of proven-effective remedies. However, targeting the dysbiosis that characterizes both conditions may represent a particularly promising approach, possibly through the use of probiotics or prebiotics. The subclinical nature of these two distinct disorders emphasizes the need for improvement of diagnostic tools for detection of injury and inflammation in the small intestine.
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APPENDIX II
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