The Microbiota and NSAID-induced Enteropathy

THE INTESTINAL MICROBIOTA AND NONSTEROIDAL ANTI-INFLAMMATORY

DRUG-INDUCED SMALL INTESTINAL DAMAGE

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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
©	Copyright
°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)
H_2RA	Histamine receptor antagonist
HCl	Hydrochloric acid
HOX	Hydroperoxidase
H&E	Hematoxylin and eosin stain
i.p.	intraperitoneal
K_m	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
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGG/HS	Prostaglandin endoperoxide G/H synthase
PGH_2	Prostaglandin H_2
PGI_2	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
TXA_2	Thromboxane A_2
V_{max}	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 acetylating 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, hepoxylins 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 glycophospholipids 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 H_2 (PGH_2) 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).
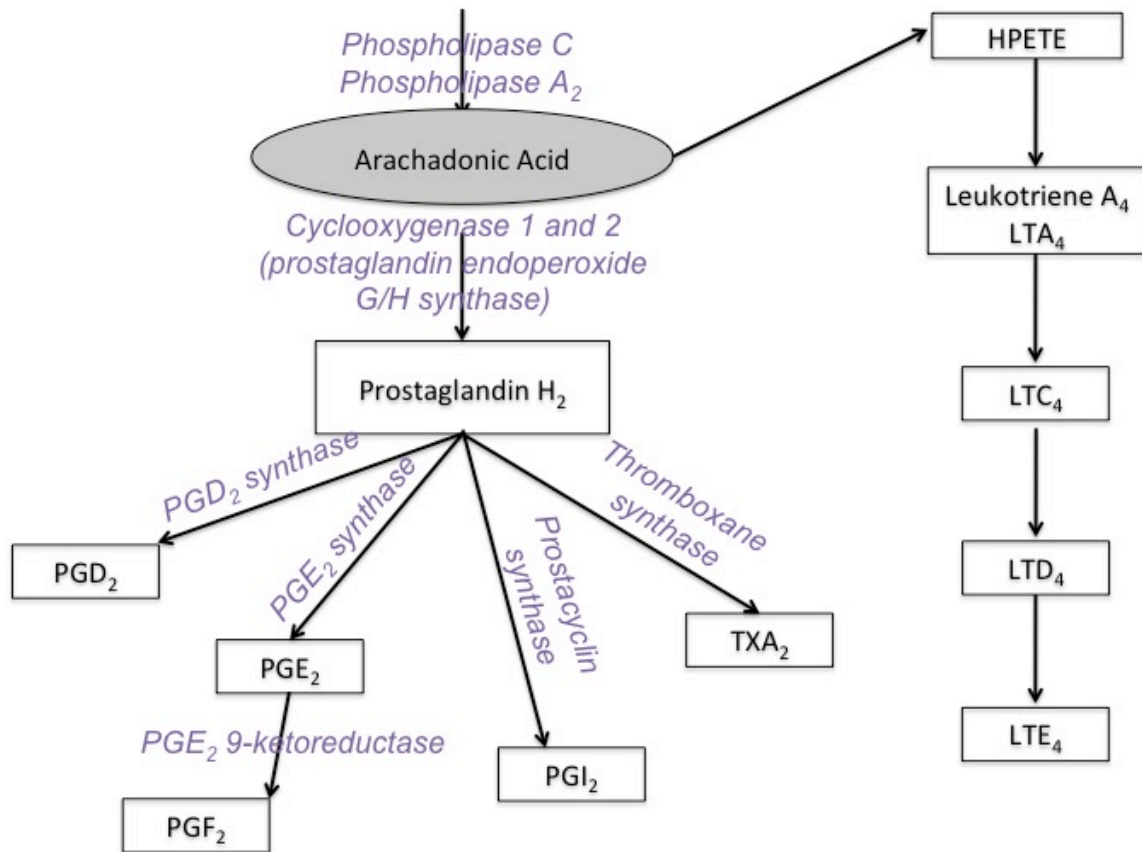
Figure 1.1: Biosynthesis of Prostaglandins and Thromboxanes. Prostaglandins and thromboxanes are synthesized in a step-wise process initiated by the release of AA from membranes.   Prostaglandins are synthesized in response to a number of triggers including, but not limited to, hormones and mechanical 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

PGH_2. 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, PGD_2 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. TXA_2 is the active compound, which is unstable and is non-enzymatically

converted to TXB_2 (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. F_α prostaglandins have two hydroxyl groups on the

cyclopentane ring and formed from PGH_2 by PGF_α reductase (Smyth 2006).

Prostacyclins (PGIs) are formed from PGH_2 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). PGI_2 is an unstable active intermediate, which is non-

enzymatically converted to inactive 6-keto-$PGF1_\alpha$ (Smyth 2006).

The five classes of receptors are named according to the prostanoid letter they have

the highest affinity for (example: PGI_2 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_s, 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 PGH_2. The enzyme is found in two isoforms, termed COX-1

and COX-2. COX is responsible for oxygenation of AA and for reducing PGG_2 to PGH_2.

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-

kB, 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-

hydroxyeicosatetranoic acid (15R-HETE), opposed to the usual PGH_2 (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: PGH_2. The prostaglandins which are synthesized from PGH_2 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).
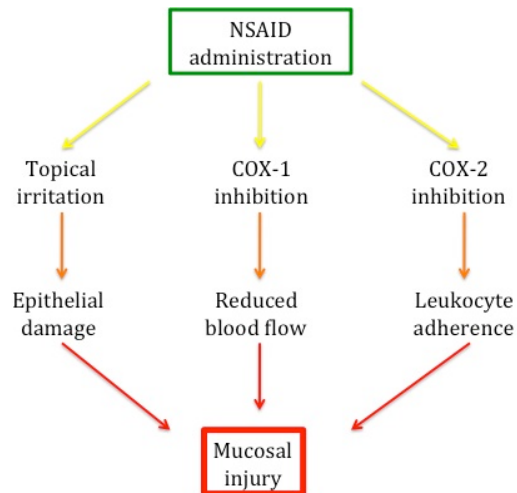
Figure 1.2: Factors contributing to NSAID gastropathy. By disrupting the phospholipids on the mucosal surface of the stomach and duodenum, NSAIDs can result in epithelial damage from acid exposure. Additionally, NSAIDs can result in epithelial cell death by direct contact (see text). Reduction of mucosa 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

asymptomatically (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

Drug	Dose	Administration	Reference
Diclofenac	10 mg/kg	Orally Evaluated 24 hours later	Satoh *et al.* 2013
	8 mg/kg	Intragastrically twice daily Evaluated 7 or 14 days later	Fornai *et al.* 2013
	7.5 mg/kg	Intragastrically twice daily Evaluated 24 hours or 5 days later	Zhang *et al.* 2013
	7.5 mg/kg	Intragastrically single dose Evaluated 24 hours or 5 days later	Chao *et al.* 2012
Indomethacin	5-40 mg/kg	Intragastrically Evaluated 24 hours later	Kent *et al.* 1969
	3.5-15 mg/kg	Orally Evaluated 24 hours later	Sigthorsson *et al.* 2002
	10 mg/kg	Orally Evaluated 24 hours later	Hayashi *et al.* 2014
	18 mg/kg	Orally Evaluated 6 hours later	Shi *et al.* 2014
Naproxen	10 mg/kg	Orally twice daily Evaluated 4.5 days later	Wallace *et al.* 2011
	10 mg/kg	Orally twice daily Evaluated 4.5 days later	Blackler *et al.* 2012
	40 mg/kg	Subcutaneously	Takeuchi *et al.*

Evaluated 24 hours later	2002

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.
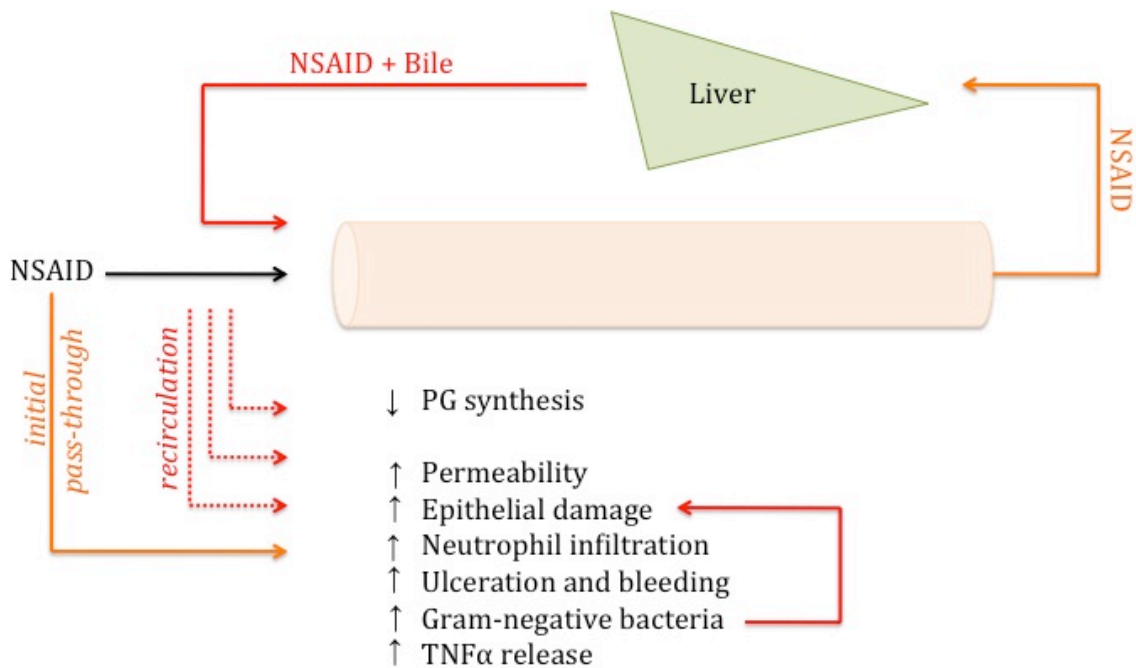
Figure 1.3: Pathogenesis of NSAID enteropathy. NSAIDs produce their effects during their initial pass-through to the small intestine. NSAIDs that are enterohepatically recirculated are absorbed in the distal intestine and glucuronidated in the liver, where they subsequently produce effects as they are 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 it's 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)*.*
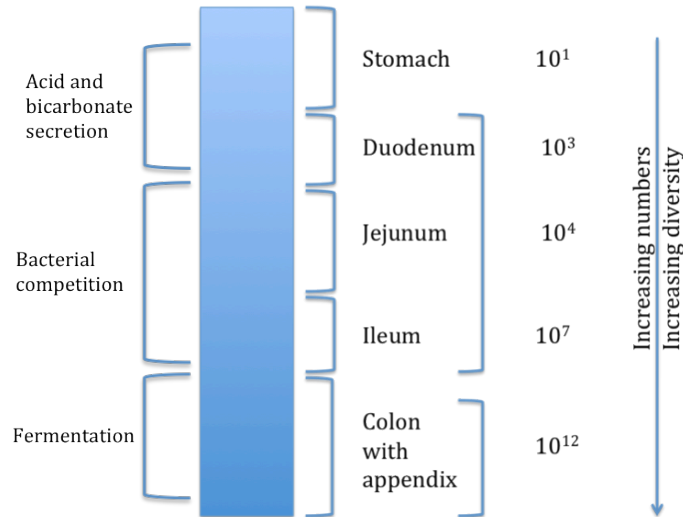
Figure 1.4 Spatial aspects of the intestinal microbiota. The diversity and number
of bacteria increase from proximal to distal GI tract. The numbers and classes of
bacteria that colonize a specific area are due to micro-environmental pressures
related to oxygen content and nutrient availability among 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

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induce intestinal dysbiosis and how this influences the severity of NSAID-induced

small intestinal damage is explored.

– CHAPTER TWO–

THESIS SCOPE AND OBJECTIVES

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 H_2RA).

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 $NaPO_4$ (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 H_2O; Sigma-Aldrich, ON), 50 µL

of mutanolysin (10U/µL; Sigma-Aldrich, ON) and 10 µL of RNase A (10 mg/mL in

H_2O; 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 ddH_2O 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 previously[12] 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 docmented 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 H_2RA; 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 GGG GCA CGG 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 MgCl$_2$, 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.

Target bacterial group/species	Sequence (5'–3')
Actinobacteria phylum	CGCGGCCTATCAGCTTGTTG
	CCGTACTCCCCAGGCGGGG
Bifidobacterium group	CTCCTGGAAACGGGTGG
	GGTGTTCTTCCCGATATCTACA
B longum group	TTCCAGTTGATCGCATGGTC
	TCACGCTTGCTCCCCGAT
B bifidum	CCACATGATCGCATGTGATTG
	CCGAAGGCTTGCTCCCAAA
B breve	CCGGATGCTCCATCACAC
	ACAAAGTGCCTTGCTCCCT
B adolescentis	CTCCAGTTGGATGCATGTC
	TCCAGTTGACCGCATGGT
B catenulatum group	CGGATGCTCCGACTCCT
	CGAAGGCTTGCTCCCGAT
B angulatum	CAGTCCATCGCATGGTGGT
	GAAGGCTTGCTCCCCAAC
B lactis	GTGGAGACACGGTTTCCC
	CACACCACACAATCCAATAC

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 mm,

200 μL of O-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_2O_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 H_2O) 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 PGE_2 assay above. The

concentration of each sample was calculated from the equation obtained from the

standard curve. %B/B_0 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 $FeCl_3$ (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. H_2S concentration

was obtained by using NaHS solutions of varying concentrations as NaHS releases

HS^- when dissolved in water and HS^- forms H_2S with H^+. H_2S 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 H_2RA, 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.

n = 6
per group
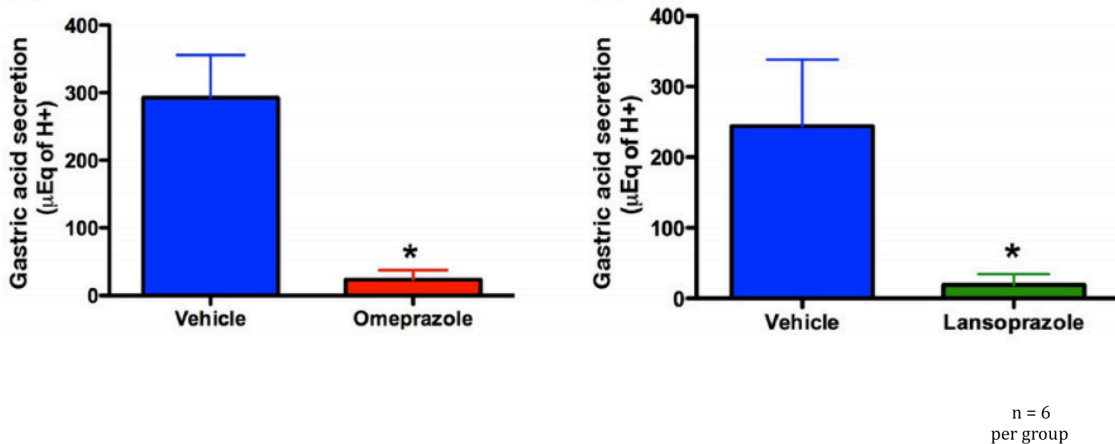
Figure 4.1 PPIs significantly suppress gastric acid secretion. Omeprazole (red
bar) lansoprazole (green bar) at 10 mg/kg twice daily for five days reduced gastric
acid secretion significantly. Bars represent the mean ± standard error of the mean. *
$p<0.05$ vs. the vehicle group; Student's t-test. (Figure 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 PGE_2

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 PGE_2 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).

n = 6
per group
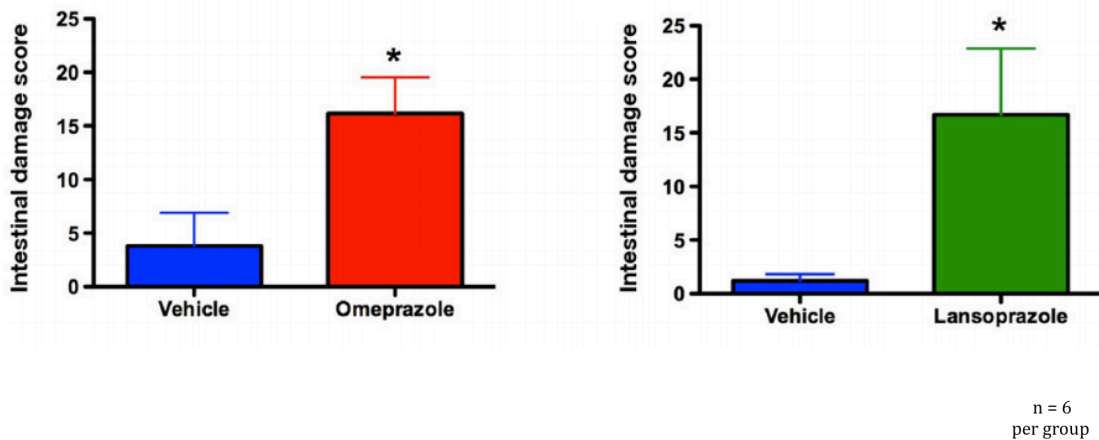
Figure 4.2 PPIs exacerbate small intestinal injury induced by naproxen.
Exacerbation of 10 mg/kg naproxen damage (oral four day dosing) from PPI
administration. Bars represent the mean ± standard error of mean. * $p < 0.05$ vs. the
vehicle group; Student's t-test. (Figure credit: Wallace 2011)

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 PGE_2 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.
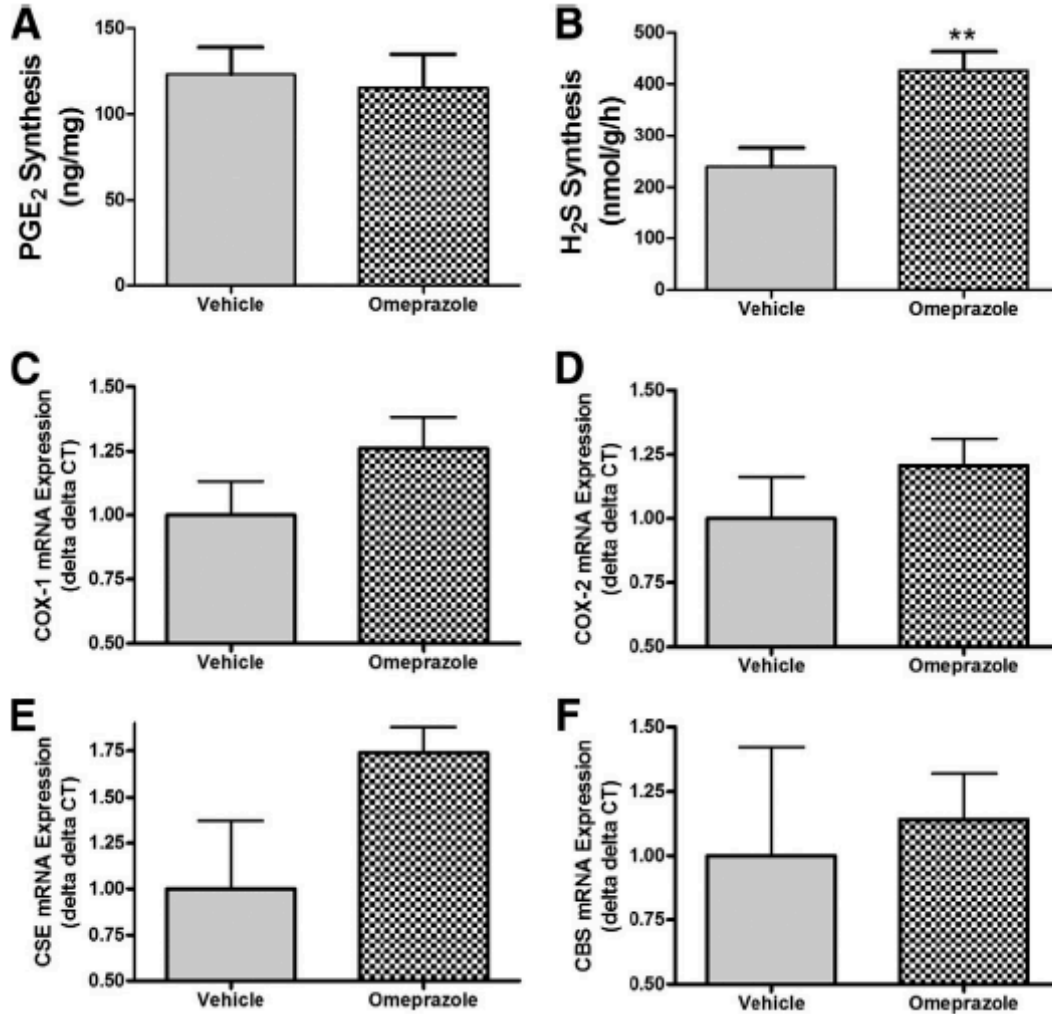
n = 6
per group

Figure 4.3 Treatment with omeprazole significantly increased H$_2$S synthesis but did not alter PG synthesis. Omeprazole treatment did not influence PG synthesis, but did significantly increase H$_2$S 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 H$_2$S 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.
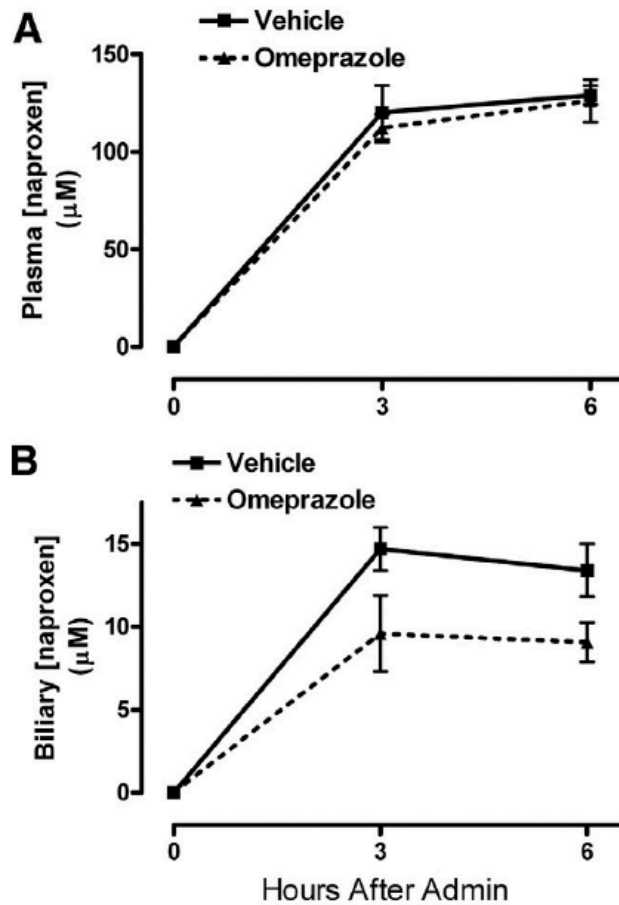
**Figure 4.4 Omeprazole administration does not alter the pharmacokinetics of
naproxen.** Rats were given a single dose of naproxen (10 mg/kg) following five
days of twice-daily omeprazole (10 mg/kg). Rats were anesthetized with isoflurane
3 or 6 hours following naproxen administration. Bile was collected 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.
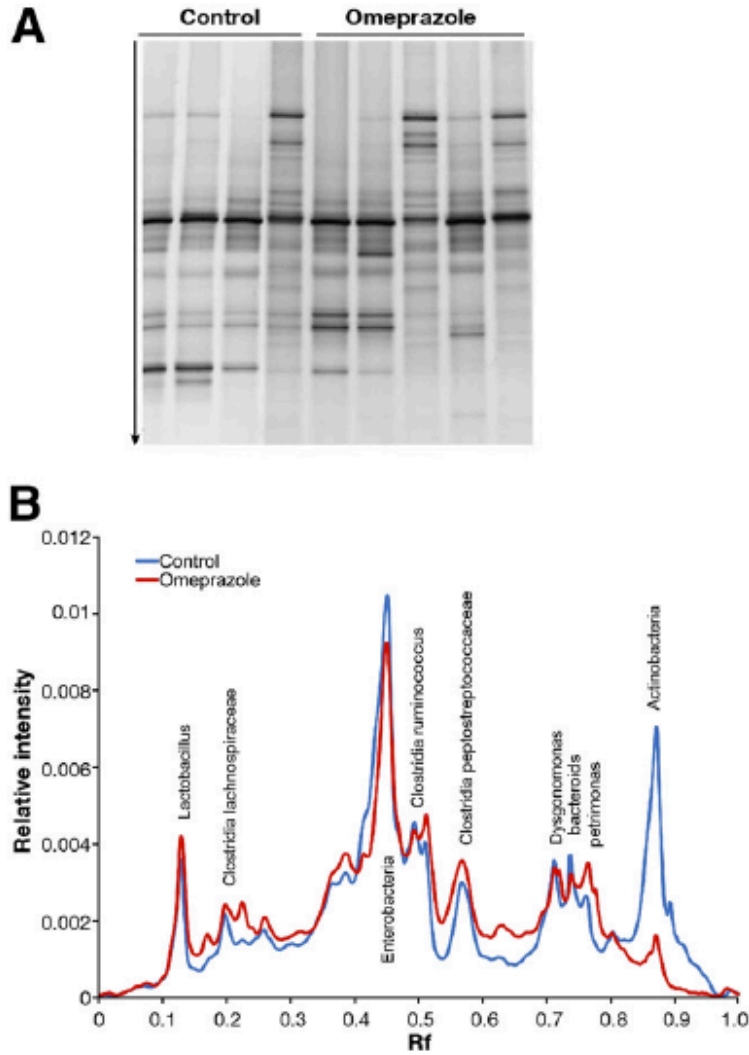
Figure 4.5 Administration of omeprazole resulted in significant intestinal dysbiosis. Rats treated with omeprazole (10 mg/kg) twice daily for 9 days had key changes in their jejunal microbiota compared to vehicle (control) as revealed by PCR-DGGE. Each lane in panel A represents 1 rat. In panel B, the 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 ± 2 and 17 ± 5 respectively). This reduction in damage

score is consistent with the abolition of the decrease in hematocrit observed with

naproxen and omeprazole administration.
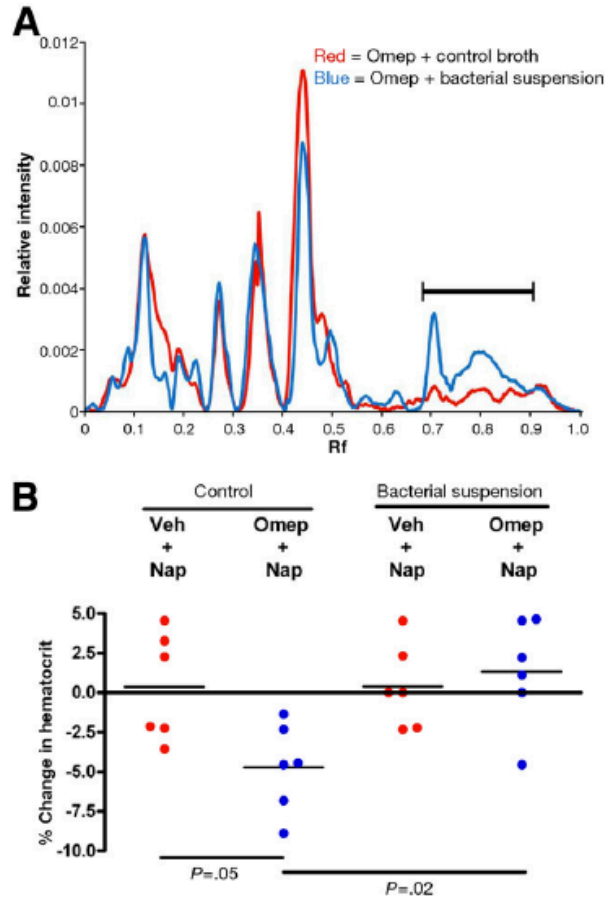
Figure 4.6 PPI-induced dysbiosis and increase in naproxen-induced bleeding were reversed after administration of selected *Bifidobacteria*-enriched commensal bacterial. (A) Jejunal Actinobacteria (black bar) was increased after selected bacterial administration but there was no increase in any other 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.
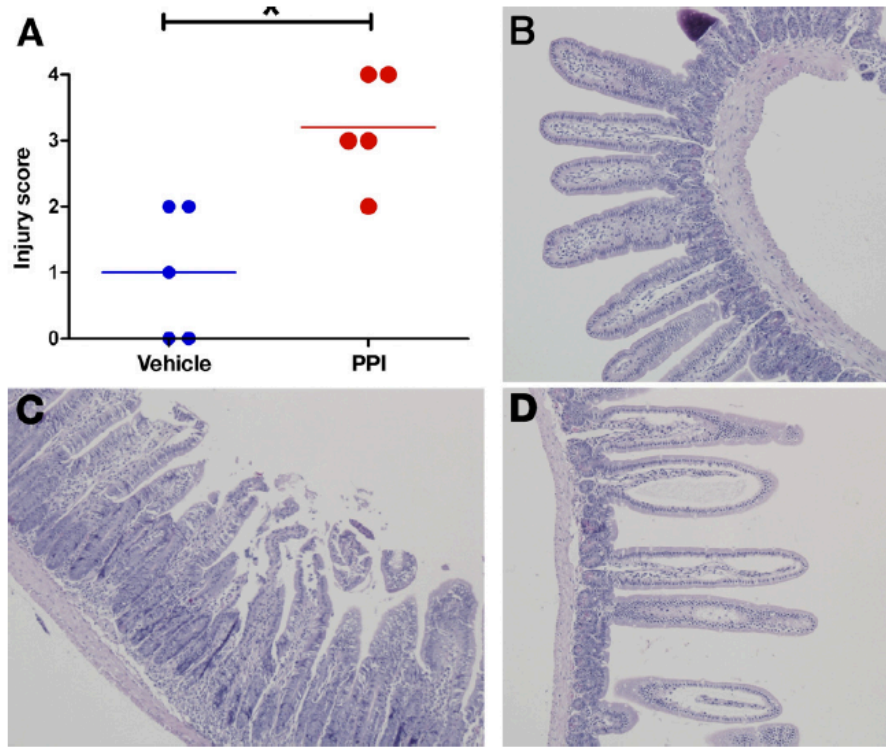
Figure 4.7 PPI-induced exacerbation of naproxen small intestinal damage is transferable via the intestinal microbiota. (A) Germ-free mice administered intestinal bacteria from PPI-treated rats developed more severe naproxen-induced small intestinal damage compared to germ-free mice administered intestinal 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 (10^9 CFU) significantly reduced the extent of intestinal damage induced by naproxen (by ~82%; $p<0.01$; Fig. 1). In contrast, treatment with BL (10^9 CFU) did not significantly affect the severity of small intestinal damage induced by naproxen (Fig. 5.1). When tested at doses of 10^7 CFU and 10^8 CFU, neither strain of *Bifidobacteria* significantly affected the severity of intestinal damage induced by naproxen (data not shown).
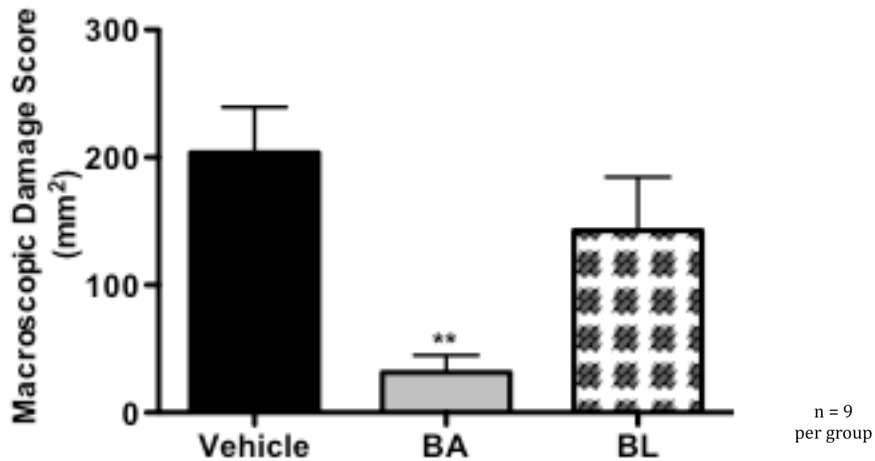
Figure 5.1: Treatment with *Bifidobacteria adolescentis* (BA) reduced naproxen-induced small intestinal damage. When administered to rats once daily for five days prior to initiating twice-daily treatment with naproxen, BA significantly reduced the severity of intestinal damage by ~82%. In contrast, 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).
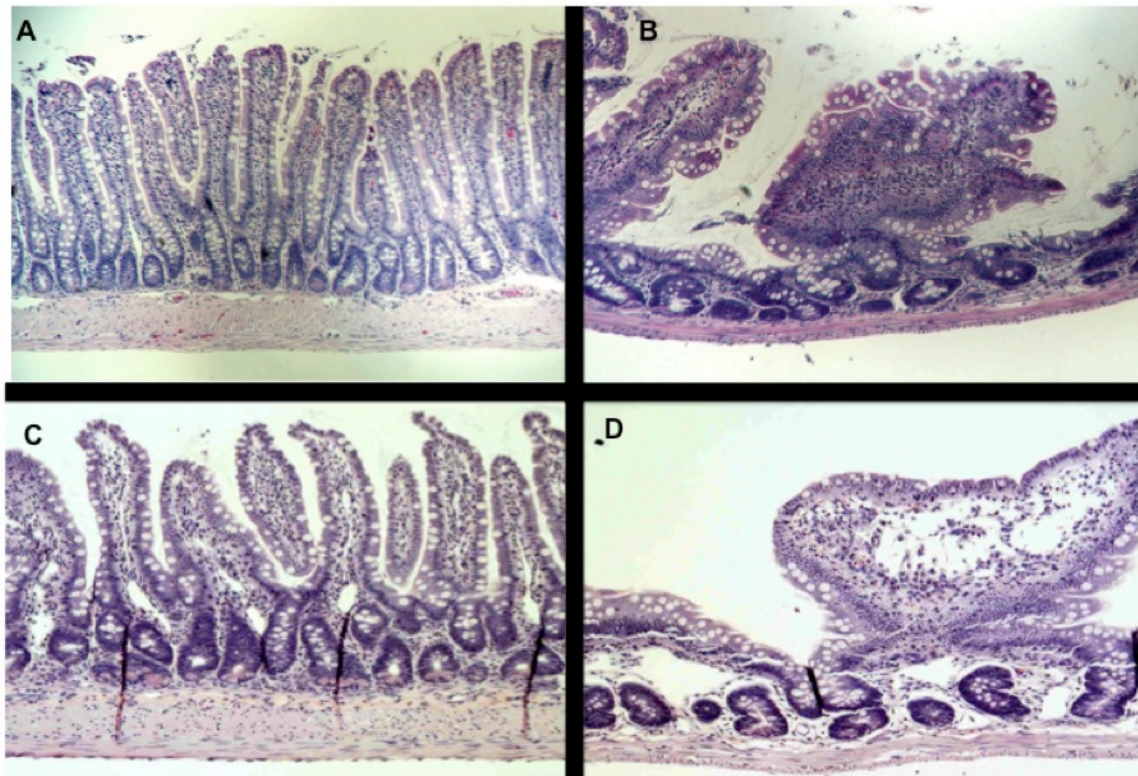
Figure 5.2: Microscopic differences among treatment groups were observed distal to the ligament of Treitz. All panels show samples taken from the mid-jejunum 20 cm distal to the ligament of Treitz at 10X magnification. **A.** Histological sample from a naïve rat. **B.** A sample from a rat that had vehicle 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 mm^2 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.

n = 6
per group
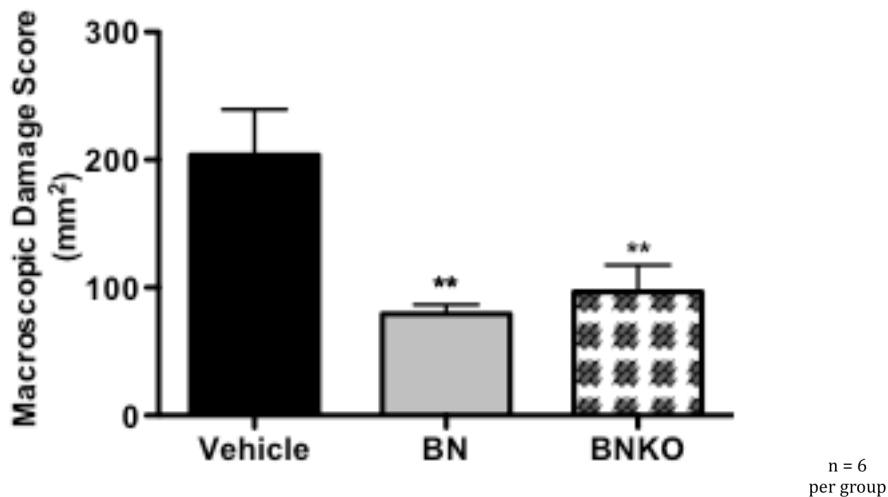
Figure 5.3: *Bifidobacterium longum* **supsp** *longum* **NCC2705 (BN) and its BL0033 gene knockout (BNKO) ameliorate naproxen-induced small intestinal injury.** Similarly to BA treatment, when administered to rats once daily for five days prior to naproxen administration, BN and BNKO significantly reduced 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 FPrau 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 FPrau has been associated with disease activity in Crohn's disease

patients (Sokol 2008; Galecka 2013). These facts were the foundation for the

examination of FPrau administration in our model. Vehicle treatment resulted in an

average macroscopic damage score of 230. We observed that treatment with FPrau

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 FPrau-treated rats as in vehicle-treated rats. There were significantly fewer

ulcerations and erosions in the small intestine of FPrau-treated rats.

Due to the oxygen intolerance of FPrau, the supernatant was tested as a treatment

(FPrau SN). Treatment with the bacteria-free FPrau 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 FPrau SN

treatment did begin more proximately in the small intestine compared to where the

damage initiated in vehicle-treated rat.

n = 6 in FPrau and FPrau SN
n = 4 in control
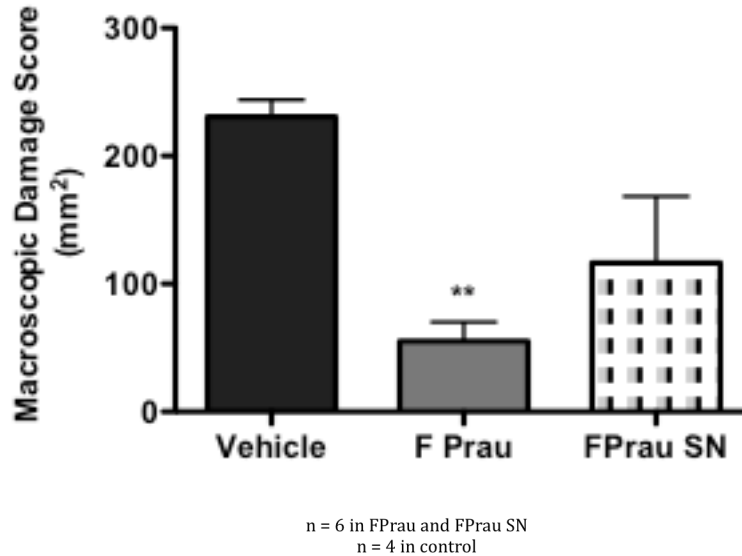
Figure 5.4: Faecalibacterium prausnitzii (FPrau) significantly reduced naproxen-induced intestinal damage. When administered to rats once a day for five days prior to naproxen administration, FPrau significantly decreased macroscopic intestinal damage scores by over 75%. FPrau supernatant was unable 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.
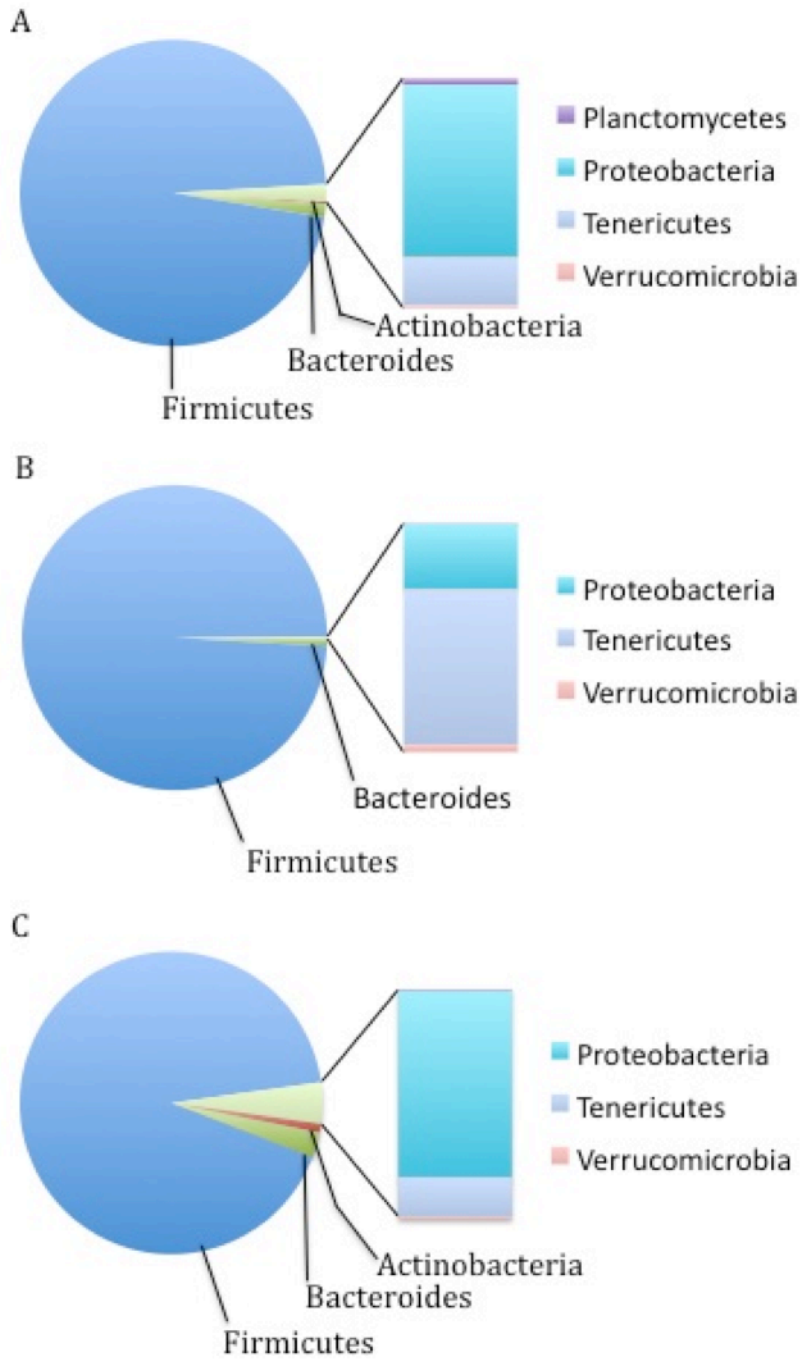
Figure 5.5: Small intestinal bacterial Illumina sequencing data reveals alterations in non-dominant phyla. Tissue and contents were sequenced from small intestinal sections of treated rats. **A.** Vehicle-treated rats had ~96.5 % of the total bacteria assigned to the Firmicutes phylum, ~1.4 % assigned 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 (10^9 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.

n = 7
per group
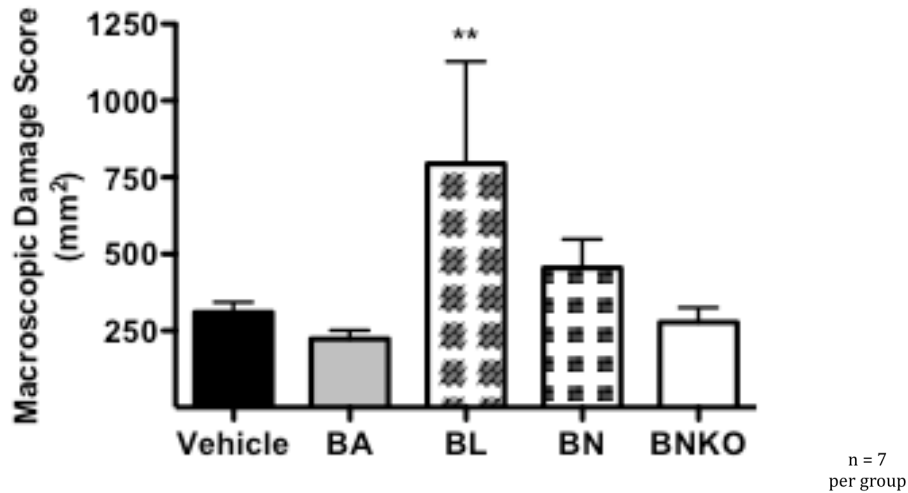
Figure 5.6: *Bifidobacterium longum* **subsp.** *longum* **JCM 1217 (BL) exacerbates diclofenac induced small intestinal damage.** A single oral administration of diclofenac (15 mg/kg, in 1% CMC) was given to rats after the same pretreatment protocol from the naproxen-enteropathy study. Animals were 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 ≥ 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).

n = 7
per group
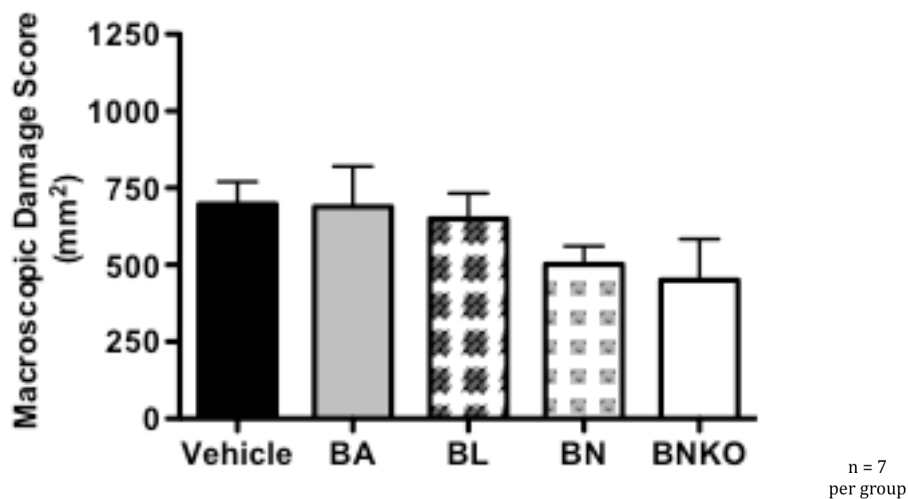
Figure 5.7: None of the bacterial pretreatments influenced small intestinal damage induced by a single administration of indomethacin. After the bacterial pretreatment protocol, 7.5 mg/kg of indomethacin was orally administered and rats were euthanized 24 hours later. No bacterial pretreatment altered 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 ddH$_2$O 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).

aybe

The antibiotic treatment administered in drinking water was made up of four antibiotics: ampillicin 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.
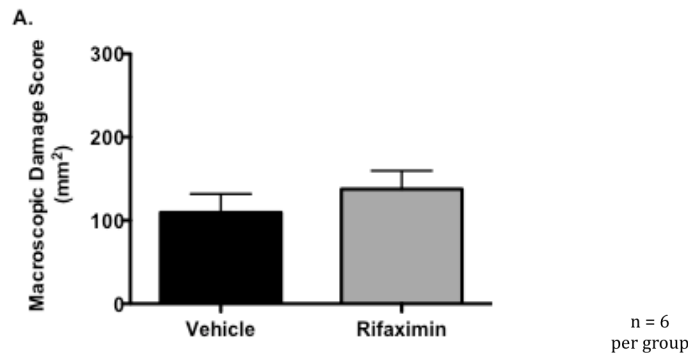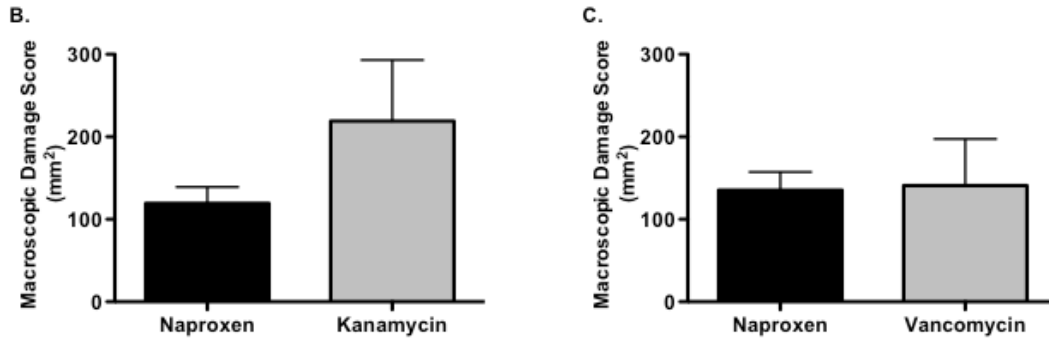
Figure 6.1: Antibiotic treatment did not significantly alter the severity of naproxen-induced small intestinal damage. A. When administered to rats once daily for five days prior to initiating a twice-daily naproxen treatment, rifaximin did not significantly alter the severity of intestinal damage. B. 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.
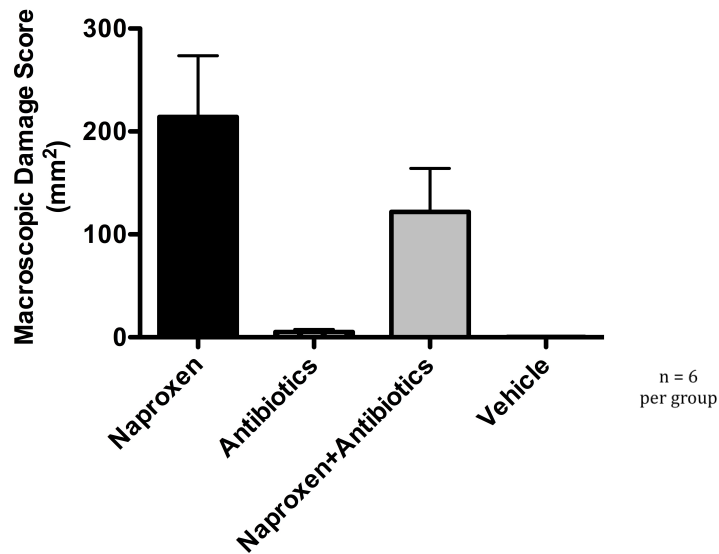
Figure 6.2: An oral antibiotic cocktail administration failed to protect against naproxen-induced small intestinal damage. Rats pretreated orally with an antibiotic cocktail of neomycin, streptomycin and bacitracin were not protected from naproxen-induced enteropathy. Antibiotic treatment alone did not 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 ampilicin (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)
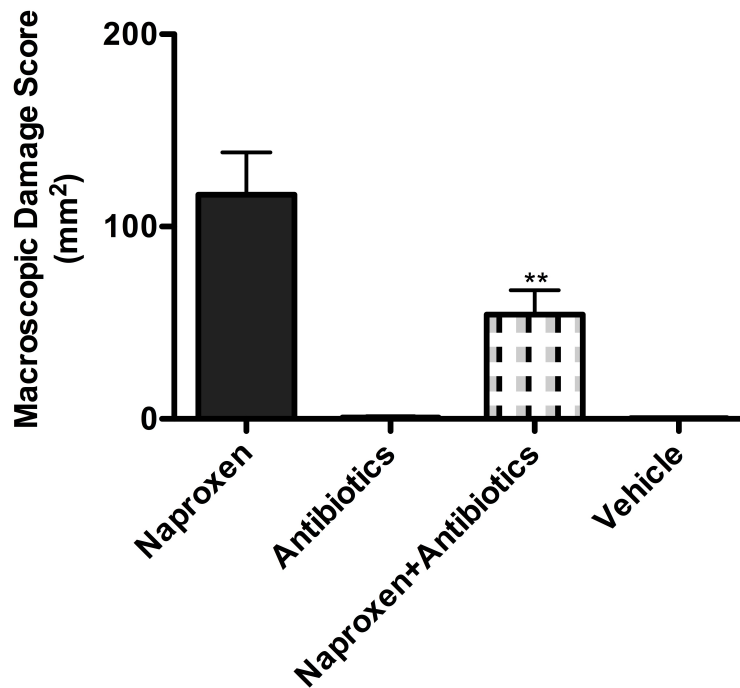
Figure 6.3: Antibiotic treatment in drinking water ameliorated naproxen-induced small intestinal damage. An antibiotic cocktail administered to rats via drinking water significantly decreased the small intestinal macroscopic damage induced by naproxen. Bars represent the mean ± standard error of the mean of 6 rats per group. ** $p<0.01$ vs. the naproxen group.
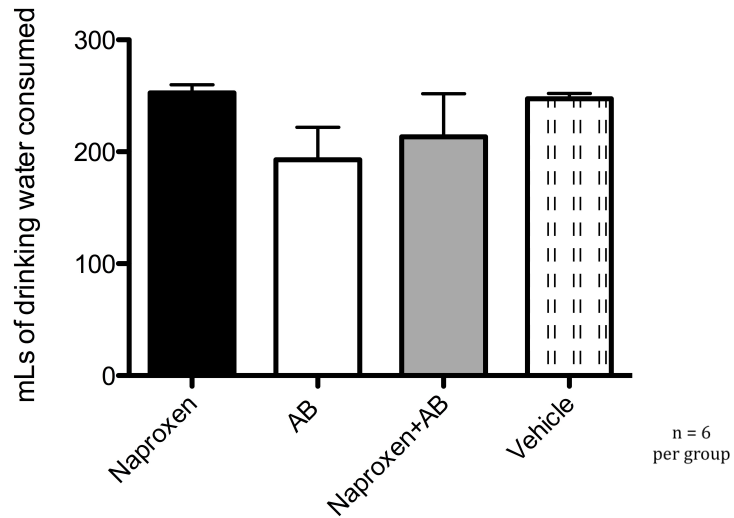
Figure 6.4. Water consumption by rats in antibiotic drinking water study.
Regardless of their treatment groups, rats consumed equal amounts of drinking
water throughout the duration of the study. Bars represent the mean ± standard
error of the mean. p=0.2827; one-way ANOVA.

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).
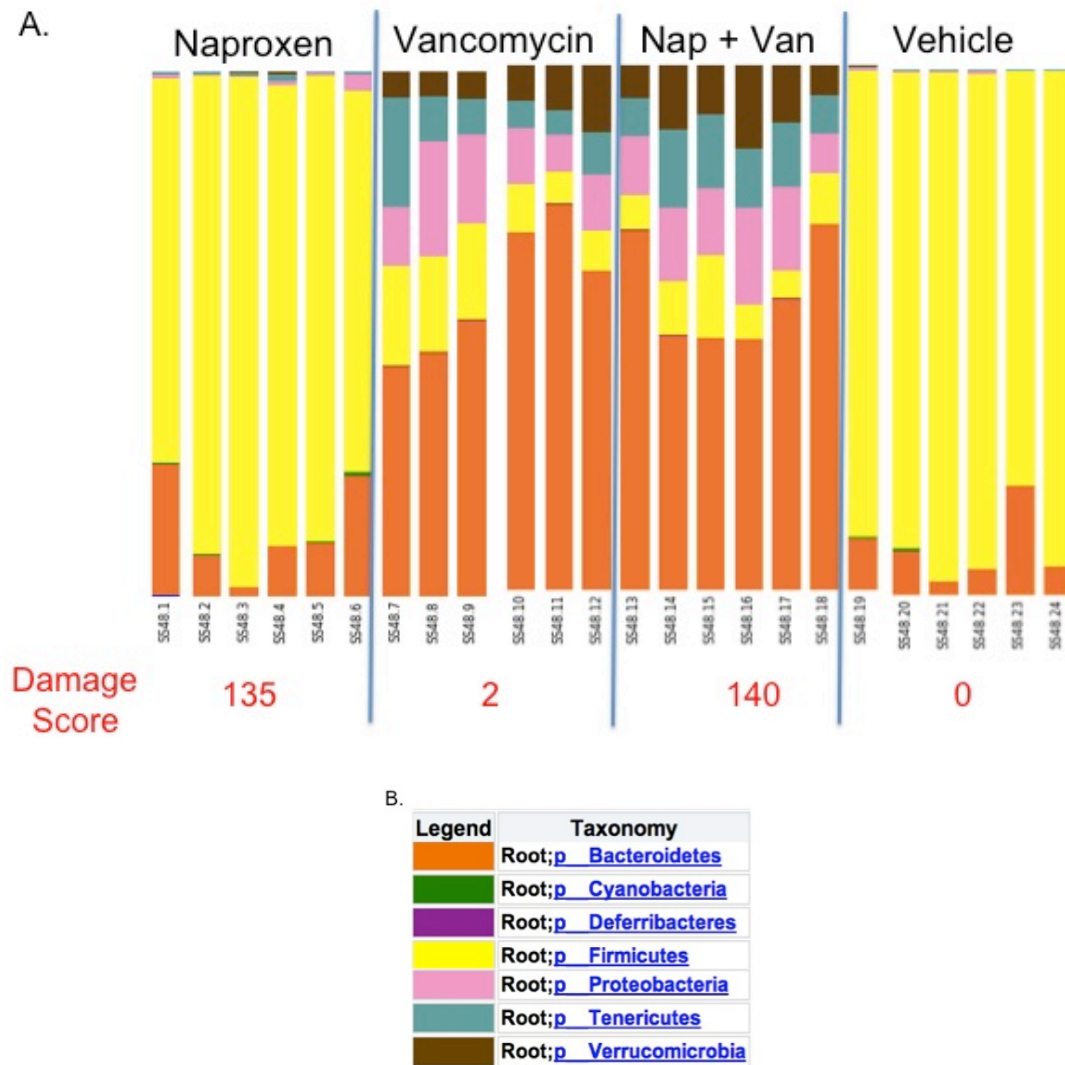
**Figure 6.5: Taxa summary of cecal microbiota contents from rats treated with
vancomycin**. A. Taxa summaries from cecal samples of naproxen-treated rats are
similar to those from vehicle-treated rats. They are categorized by a large
proportion of Firmicutes bacteria. Their levels of Firmictues are 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% 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 Firmictues 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 Firmictues 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 mm^2).
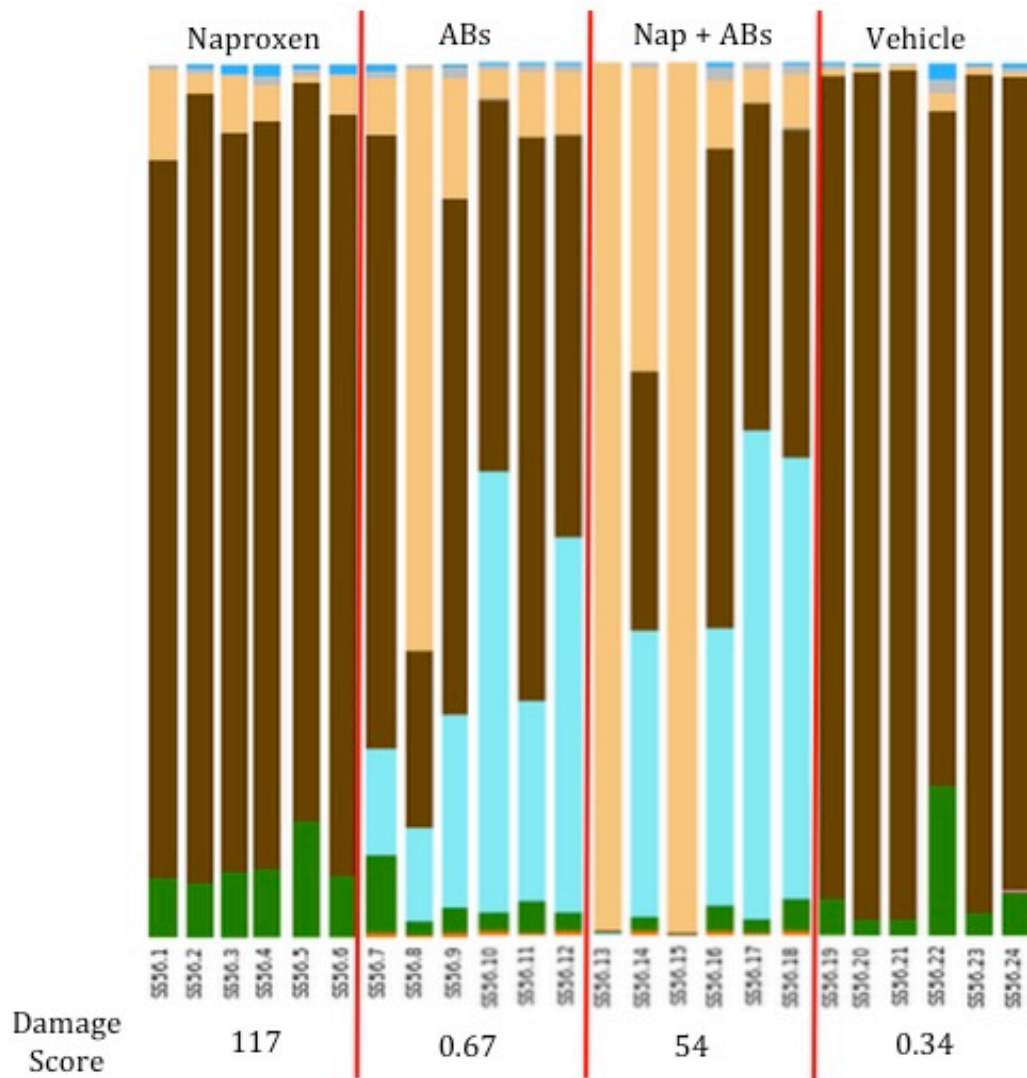
B.

Legend	Taxonomy	
🟦	Root;p__Acidobacteria	
🟧	Root;p__Actinobacteria	
🟩	Root;p__Bacteroidetes	
🟦	Root;p__Cyanobacteria	
🟫	Root;p__Firmicutes	
🟧	Root;p__Proteobacteria	
⬜	Root;p__Tenericutes	
🟦	Root;p__Verrucomicrobia	

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.
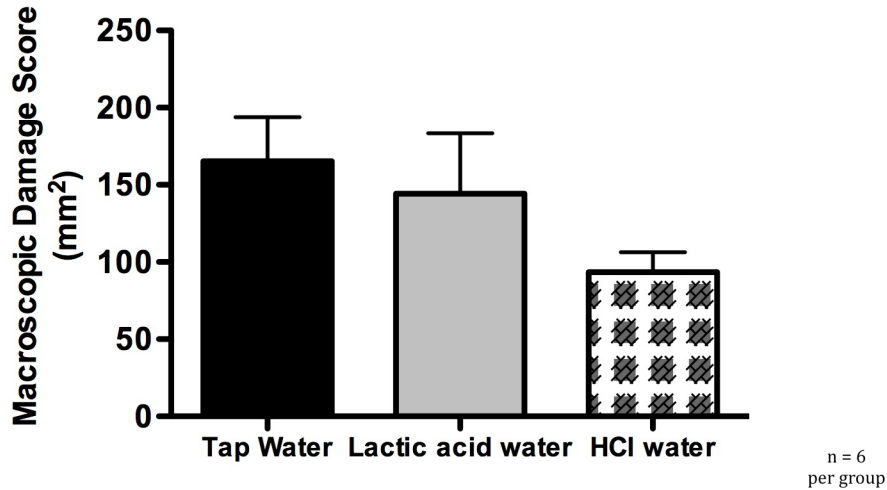
Figure 7.1: Lactic acid-supplemented water did not influence naproxen-induced small intestinal damage.
Rats given water supplemented with lactic acid did not significantly change the naproxen-induced small intestinal damage severity. Similarly, rats given HCl-supplemented water did not affect the severity 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

2005; Goldstein 2005; Maiden 2005; Fijimora 2010). 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_2RA. 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.
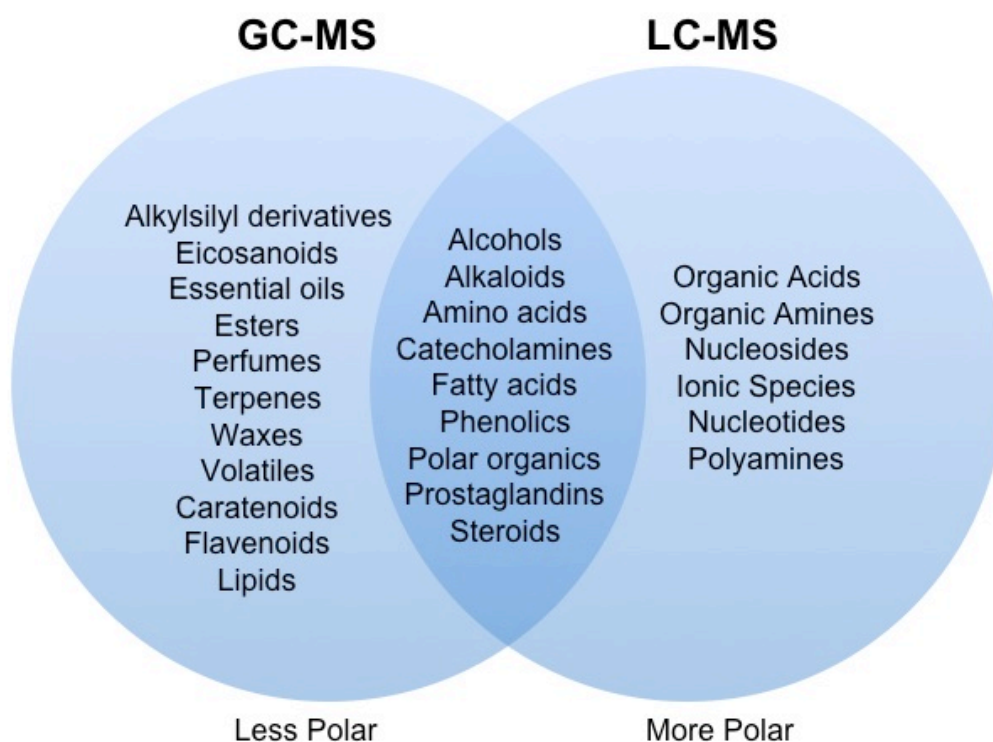
Figure 8.1: Classes of chemicals and the analytical techniques with which they are most compatible. Although the two analytical techniques do have cross over with respect to chemical classes they detect, there are distinct metabolites which are better suited for one of the two analyses.

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 *in vivo* as compared to *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 *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

(Hold 2003; Oikonomou 2013; Castillo 2007; Nava 2011; Foglesong 1984). 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 1.4×10^{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 *Bifidobact1eria* 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 is it 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 *in vitro* and *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 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 NFκB 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.

Its 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 prophylatic monosodium glutamade 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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Conflicts of interest: None

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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).
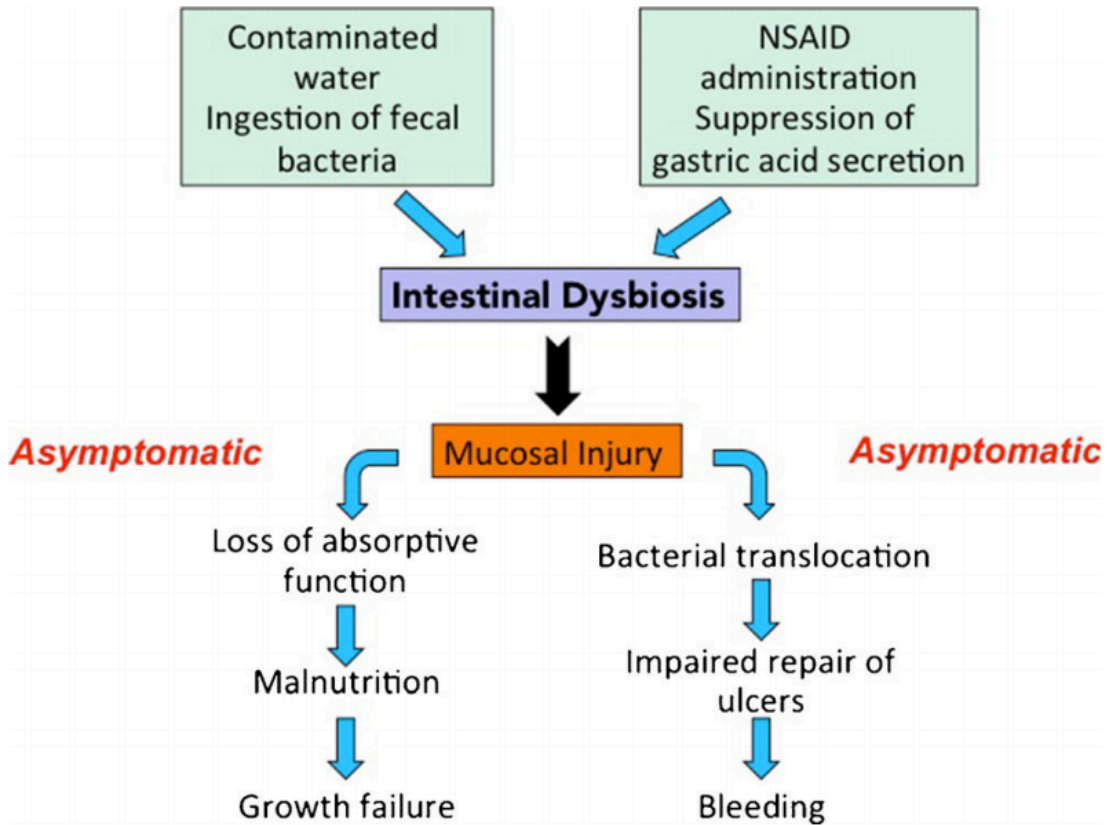
Figure A1: Common elements of pathogenesis of environmental and NSAID-induced enteropathy. Intestinal dysbiosis is a common feature that may drive the mucosal inflammation and injury in both disorders. In the case of environmental enteropathy, mucosal injury primarily results in malabsorption and growth 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).

Treatment	Species	Effect on Enteropathy	References
PPI	Rat	Worsens	Wallace 2011 Satoh 2012
H2RA	Rat	Worsens	Satoh 2012
Misoprostol	Human	Inconsistent	Bjarnason 1989 Morris 1994 Fujimori 2009
Metronidazole	Rat	Improves	Yamada 1993
Metronidazole	Human	Improves*	Bjarnason 1992
Rebamipide	Rat	Improves	Mizoguchi 2001
Rebamipide	Human	Inconsistent	Niwa 2008 Fujimori 2011
Probiotic: *L. acidophilus*	Rat	Improves	Kinouchi 1998
Probiotic: *B. adolescentis*	Rat	Improves	Kinouchi 1998
Probiotic: *B. adolescentis*	Rat	Improves	Syer 2012
Probiotic: VSL#3	Human	Improves	Montalto 2010
Probiotic: *L. casei*	Rat	Improves	Watanabe 2009
Probiotic: *L. casei*	Human	Improves	Endo 2011

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.

A.8 References

Andreasson K., Scheja A., Saxne T., *et al.* (2011) Faecal calprotectin: a biomarker of gastrointestinal disease in systemic sclerosis. *J Intern Med.* 270, 50-57.

Bartram J. & Cairncross S. (2010). Hygiene, sanitation, and water: Forgotten foundations of health. *PLoS Medicine, 7* (11), e1000367.

Bhowmik S.K, An J.H., Lee S.H., *et al.* (2012). vMAlteration of Bile Acid Metabolism in Pseudo Germ-Free Rats. Arch Pharm Res, 25 (11), 1969-1977.

Bjarnason I., Hayllar J., Smethurst P., *et al.* (1992) Metronidazole reduces intestinal inflammation and blood loss in non-steroidal anti-inflammatory drug induced enteropathy. *Gut* 33, 1204-1208

Bjarnason I., Price A.B., Zanelli G., *et al.* (1988) Clinicopathological features of nonsteroidal antiinflammatory drug-induced small intestinal strictures. *Gastroenterology* 94, 1070-1074.

Bjarnason I., Smethurst P., Fenn C.G., *et al.* (1989) Misoprostol reduces indomethacin-induced changes in human small intestinal permeability. *Dig Dis Sci* 34, 407-411.

Brune K., Dietzel K., Nurnberg B., *et al.* (1987) Recent insight into the mechanism of gastrointestinal tract ulceration. *Eur J Rheumatol Inflamm* 9, 8-14.

Calder P.C., Krauss-Etschmann S., de Jong E.C., *et al.* (2006). Early nutrition and immunity - progress and perspectives. *The British Journal of Nutrition, 96*(4), 774-790.

Chen P., Soares A.M., Lima A.A., *et al.* (2003). Association of vitamin A and zinc status with altered intestinal permeability: Analyses of cohort data from northeastern Brazil. *Journal of Health, Population, and Nutrition, 21*(4), 309-315.

Endo H., Higurashi T., Hosono K., *et al.* (2011) Efficacy of Lactobacillus casei treatment on small bowel injury in chronic low-dose aspirin users: a pilot randomized controlled study. *J Gastroenterol* 46, 894-905

Endo H., Sakai E., Higurashi T., *et al.* (2012) Differences in the severity of small bowel mucosal injury based on the type of aspirin as evaluated by capsule endoscopy. *Dig Liver Dis* 44, 833-838

Fujimori S., Seo T., Gudis K., *et al.* (2009) Prevention of nonsteroidal anti-inflammatory drug-induced small-intestinal injury by prostaglandin: a pilot

randomized controlled trial evaluated by capsule endoscopy. *Gastrointest Endosc* 69, 1339-1346.

Fujimori S., Takahashi Y., Gudis K., *et al.* (2011) Rebamipide has the potential to reduce the intensity of NSAID-induced small intestinal injury: a double-blind, randomized, controlled trial evaluated by capsule endoscopy. *J Gastroenterol* 46, 57-64

Galpin L., Manary M.J., Fleming K., *et al.*(2005). Effect of lactobacillus GG on intestinal integrity in Malawian children at risk of tropical enteropathy. *The American Journal of Clinical Nutrition, 82*(5), 1040-1045.

Graham D.Y., Opekun A.R., Willingham F.F., *et al*. (2005) Visible small-intestinal mucosal injury in chronic NSAID users. *Clin Gastroenterol Hepatol* 3, 55-59

Hagiwara M., Kataoka K., Arimochi H., *et al.* (2004) Role of unbalanced growth of gram -negative bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug. *J Med Invest* 51: 43-51

Humphrey J.H. (2009). Child undernutrition, tropical enteropathy, toilets, and handwashing. *Lancet, 374*(9694), 1032-1035.

Kent T.H., Cardelli R.M., Stamler F.W. (1969) Small intestinal ulcers and intestinal flora in rats given indomethacin. *Am J Pathol* 54, 237-249

Kinouchi T., Kataoka K., Bing S.R., *et al.* (1998) Culture supernatants of Lactobacillus acidophilus and Bifidobacterium adolescentis repress ileal ulcer formation in rats treated with a nonsteroidal antiinflammatory drug by suppressing unbalanced growth of aerobic bacteria and lipid peroxidation. *Microbiol Immunol* 42, 347-355

Lanas A., García-Rodríguez L.A., Polo-Tomás M., *et al.* (2009) Time trends and impact of upper and lower gastrointestinal bleeding and perforation in clinical practice. Am J Gastroenterol; 104: 1633-1641.

Lin A., Arnold B.F., Afreen S., *et al.* (2013). Household environmental conditions are associated with enteropathy and impaired growth in rural Bangladesh. *The American Journal of Tropical Medicine and Hygiene, 89*(1), 130-137.

Lindenbaum J. (1968). Small intestine dysfunction in Pakistanis and Americans resident in Pakistan. *The American Journal of Clinical Nutrition, 21*(9), 1023-1029.

Lombardo L., Foti M., Ruggia O., *et al.* (2010) Increased incidence of small intestinal bacterial overgrowth during pro- ton pump inhibitor therapy.

Clin Gastroenterol Hepatol 8, 504-508

Maiden L., Thjodleifsson B., Theodors A., *et al.* (2005) A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. *Gastroenterology* 128, 1172-1178

McKay S., Gaudier E., Campbell D.I, *et al.* (2010). Environmental enteropathy: New targets for nutritional interventions. *International Health, 2*(3), 172-180.

Mizoguchi H., Ogawa Y., Kanatsu K., *et al.* (2001) Protective effect of rebamipide on indomethacin-induced intestinal damage in rats. *J Gastroenterol Hepatol* 16, 1112-1119

Montalto M., Gallo A., Curigliano V., *et al.* (2010) Clinical trial: the effects of a probiotic mixture on non-steroidal anti-inflammatory drug enteropathy - a randomized, double- blind, cross-over, placebo-controlled study. *Aliment Pharmacol Ther* 32, 209-214.

Morris A.J., Murray L., Sturrock R.D., *et al.* (1994) Short report: the effect of misoprostol on the anaemia of NSAID enteropathy. *Aliment Pharmacol Ther* 8, 343-346.

Niwa Y., Nakamura M., Ohmiya N., *et al.* (2008) Efficacy of rebamipide for diclofenac- induced small-intestinal mucosal injuries in healthy subjects: a prospective, randomized, double-blinded, placebo- controlled, cross-over study. *J Gastroenterol* 43, 270-276

Poullis A., Foster R., Mendall M.A., *et al.* (2003) Proton pump inhibitors are associated with elevation of faecal calprotectin and may affect specificity. *Eur J Gastroenterol Hepatol.* 15, 573-574.

Reuter B.K., Davies N.M., Wallace J.L. (1997) Nonsteroidal anti- inflammatory drug enteropathy in rats: role of permeability, bacteria, and enterohepatic circulation. *Gastroenterology* 112, 109-117.

Sartor RB. (2005) Probiotic therapy of intestinal inflammation and infections. *Curr Opin Gastroenterol* 21, 44.

Satoh H., Amagase K., Takeuchi K. (2012) Exacerbation of non- steroidal anti-inflammatory drug-induced small intestinal lesions by antisecretory drugs in rats: the role of intestinal motility. *J Pharmacol Exp Ther* 343, 270-277.

Syer S.D., McKnight W., Aucouturier A., *et al.* (2012) Bifidobacteria exert a protective effect against NSAID-induced enteropathy that is dependent on lactate production. *Gastroenterology* 142, S-489.
Ranakrishan

Thurnham D.I., Northrop-Clewes C.A., McCullough F.S., *et al.* (2000). Innate immunity, gut integrity, and vitamin A in Gambian and Indian infants. *The Journal of Infectious Diseases, 182 Suppl 1*, S23-8.

Trehan I., Shulman R.J., Ou C.N., *et al.* A randomized, double-blind, placebo-controlled trial of rifaximin, a nonabsorbable antibiotic, in the treatment of tropical enteropathy. Am J Gastroenterol. 2009; 104:2326–33.

Uejima M., Kinouchi T., Kataoka K., Hiraoka I., Ohnishi Y. (1996) Role of intestinal bacteria in ileal ulcer formation in rats treated with a nonsteroidal antiinflammatory drug. *Microbiol Immunol* 40, 553-560.

Wallace J.L., Syer S., Denou E., de Palma G., Vong L., McKnight W., *et al.* (2011) Proton pump inhibitors exacerbate NSAID-induced small intestinal injury by inducing dysbiosis. *Gastroenterology* 141, 1314-1322

Wallace J.L. (2012) NSAID gastropathy and enteropathy: distinct pathogenesis likely necessitates distinct prevention strategies. *Br J Pharmacol* 2012; 165, 67-74.

Wallace J.L. (2013) Mechanisms, prevention and clinical implications of nonsteroidal anti-inflammatory drug-enteropathy. *World J Gastroenterol* 19, 1861-1876

Watanabe T., Nishio H., Tanigawa T., Yamagami H., Okazaki H., Watanabe K., *et al.* (2009) Probiotic Lactobacillus casei strain Shirota prevents indomethacin-induced small intestinal injury: involvement of lactic acid. *Am J Physiol Gastrointest Liver Physiol* 297, G506-G513.

Williams C., McColl K.E. (2006) Review article: proton pump inhibitors and bacterial overgrowth. *Aliment Pharmacol Ther* 23, 3-10

Williams E.A., Elia M., Lunn P.G., (2007) A double-blind, placebo-controlled, glutamine-supplementation trial in growth-faltering Gambian infants. *Am J Clin Nutr* 86, 421–7.

Yamada T., Deitch E., Specian R.D., Perry M.A., Sartor R.B., Grisham M.B. (1993) Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. *Inflammation* 17, 641-662

- APPENDIX II -
REFERENCES

Ashwell M. (2002). Concepts of Functional Foods (ILSI Europe Concise Monograph Series Ed. Walker, R). Available at: http://www.ilsina.org/file/ILSIFuncFoods.pdf.

Adachi J.A., DuPont H.L. (2006). Rifaximin: a novel nonabsorbed rifamycin for gastrointestinal disorders. Clin Infect Dis, 42(4):541-547

Adams R.B., Planchon S.M., Roche J.K. (1993) IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. J Immunol;150(6):2356-63.

Alakomi H.L., Skytta E., Saarela M., *et al.* (2000). Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. Appl Environ Microbiol, 66(5):2001-2005.

Anderson D., Ghandi T., Caspi, A., (2010). Vimovo (naproxen/esomeprazole magnesium). P T. 35(9 Section 2): 2-4.

Andreasson K., Scheja A., Saxne T., *et al.* (2011). Faecal calprotectin: a biomarker of gastrointestinal disease in systemic sclerosis. J Intern Med, 270:50-57.

Antoon J.S., Perry M.A., (1997). Role of neutrophils and mast cells in acute indomethacin induced small bowel injury in the rat. J Gastroenterol, 32, 747-757.

Appleyard C.B., McCafferty D.M., Tigley A.W., *et al.* (1996). Tumor necrosis factor mediation of NSAID-induced gastric damage: role of leukocyte adherence. Am J Physiol, 270, G42-G48.

Arndt H., Palitzsch K.D., Grisham M.B., et al. (1994). Metronidazole inhibits leukocyte-endothelial cell adhesion in rat mesenteric venules. Gastroenterology, 106(5):1271-1276.

Bailey M.T., Dowd S.E., Galley J.D., *et al.* (2001). Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. Brain Behav Immun 25(3), 397-407.

Banerjee A.K., Peters T.J., (1990). Experimental nonsteroidal anti-inflammtory drug-induced enteropathy in the rat: similarities to inflammatory bowel disease and effect of thromboxane synthase inhibitors. Gut, 31: 1358-1364

Benson A., Pifer R., Behrendt C.L., *et al.* (2009). Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. Cell Host Microbe 6, 187-196.

Bergheim I., Weber S., Vos M., *et al.* (2008). Antibiotics protect against fructose-

induced hepatic lipid accumulation in mice: Role of endotoxin. J Hepatol 48(6), 983-929

Biedermann L., Zeitz J., Mwinyi J., *et al.* (2013). Smoking Cessation Induces Profound Changes in the Intestinal Microbiota in Humans. PLoS One, 8(3):e39260.

Bjarnason I., Williams P., Smethurst P., *et al.* (1986). Effect of nonsteroidal antiinflammatory drugs and prostaglandins on the permeability of the human small intestine. Gut, 27, 1292-1297.

Bjarnason I., (1990). Experimental evidence of the benefit of misoprostol beyond the stomachs in humans. J Rheumatol, 17, 38-41.

Bjarnason I., Hayllar J., MacPherson A.I., *et al.* (1993). Side effects of nonsteroidal antiinflammatory drugs on the small and large intestine in humans. Gastroenterology, 104, 1832-1847.

Blackler R., Syer S., Bolla M., *et al.* (2012) Gastrointestinal-sparing effets of novel NSAIDs in rats with compromised mucosal defence. PloS One, 7(4)e35196

Bloom B.S. (1988). Direct medical costs of Disese and Gastrointestinal Side Effects During Treatment for Arthritis. Am J Med, 84 (Suppl 2A): 20-4

Boelsterli U.A., Ramiraz-Alcantara V. (2011). NSAID acyl glucuronides and enteropathy. Curr Drug Metab, 12(3): 245-252

Brufani M., Cellai L., Cerrini S., *et al.* (1984). X-ray crystal structure of 4-deoxy-3'-bromopyrido[1'2'-1,2]imidazo[5,4-c]rifamycin SV. J Antibiot (Tokyo) 37:1623-1627.

Burke A., Smyth E., Fitzgerald G.A., (2006). Analgesic-antipyretic and anti-inflammatory agents; pharmacotherapy of gout. In: Brunton LL, Lazo JS, Parker KL, eds. Goodman & Gilman's the Pharmacological Basis of Therapeutics. 11th ed. NY, USA: McGraw Hill Companies, 671-715.

Boyle, R. J., Robins-Browne R. M., Tang, M. L. (2006). Probiotic use in clinical practice: What are the risks? Am. J. Clin. Nutr, 83(6), 1256-64.

Caplan M.S., Miller-Catchpole R., Kaup S., *et al.* (1999) Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology;117(3):577-83.

Cani P.D., Bibiloni R., Knauf C., *et al.* (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes;57(6):1470-81.

Cani P.D., Amar J., Iglesias M.A., *et al.* (2007a) Metabolic endotoxemia initiates

obesity and insulin resistance. Diabetes;56(7):1761-72.

Cani P.D., Neyrinck A.M., Fava F., *et al.* (2007b) Selective increases of *bifidobacteria* in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia;50(11):2374-83.

Camp J.G., Kanther M., Semova I., *et al.* (2009). Patterns and scales in gastrointestinal microbial ecology. Gastroenterology, 136(6): 1989-2002.

Castillo M., Skene G., Roca M., *et al.* (2007). Application of 16S rRNA gene-targetted fluorescence in situ hybridization and restriction fragment length polymorphism to study porcine microbiota along the gastrointestinal trat in response to different sources of dietary fibre. FEMS Microbiol Ecol, 59:138-146.

Cellai L., Cerrini S., Segre A., *et al.* (1985). A study of structure-activity relationships in 4-deoxypyrido[1',2'-1,2]imidazo[5,4-c]rifamycin SV derivates by electron spectroscopy for chemical analysis and ^{1}HNMR. Mol Pharmacol 27:103-108
Chao G., Zhang S., (2012). Therapeutic effects of muscovite to non-steroidal anti-inflammatory drugs-induced small intestinal disease. Int J Pharm, 436(1-2):154-160

Chmaisse H.M., Antoon J.S., Kvietys P.R., *et al.*(1994). Role of leukocytes in indomethacin induced small bowel injury in the rat. Am J Phys; 266:G239-46

Claesson M.J., Jeffery I.B., Conde S., *et al*. (2012). Gut microbiota composition correlates with diet and health in the elderly. Nature 488, 178-184.

Compare D., Pica L., Rocco A., *et al.* (2011). Effects of long-term PPI treatment on producing bowel symptoms and SIBO. Eur J Clin Invest 41:380-386.

Coyne C.B., Vanhook M.K., Gambling T.M., *et al.* (2002) Regulation of airway tight junctions by proinflammatory cytokines. Mol Biol Cell;13(9):3218-34.

Crofford L.J. (1997). COX-1 and COX-2 tissue expression: implications and predictions. J Rheumatol Suppl, 24, 15-19.

Dalby A.B., Frank D.N., St. Amand A.L., *et al.* (2006). Culture-independent analysis of indomethacin-induced alterations in the rat gastrointestinal microbiota. Appl Environ Microbiol 72:6707-6715.

Davies P., Bailey P.J., Goldenberg M.M., (1984). The role of arachidonic acid oxygenation products in pain and inflammation. Annu. Rev. Immunol. 2: 335-357.

De Bandt, J. P., Waligora-Dupriet, A. J., Butel, M. J., (2011). Intestinal microbiota in inflammation and insulin resistance: Relevance to humans. Curr. Opin. Clin. Nutr. Metab. Care 14(4), 334-340.

Dharmani P., Srivastava V., Kisson-Singh V., *et al.* (2009). Role of Intestinal Mucins in Innate Host Defence Mechanisms against Pathogens. J Innate Immun 1:123-135.

Douthwaite A.H., Lintott S., (1938). Gastroscopic observation of the effect of aspirinand certain other substances on the stomach. Lancet, 2, 1222–1225.

Duncan S.H., Hold G.L., Harmsen H.J.M., *et al.* (2002). Growth requirements and fermentation products of *Fisobacterium prausnitzii,* and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. Int J Syst Evol Microbiol 52(Pt 6):2141-2146.

DuPont H.L. (2003). Community-acequired diarrheal disease in western countries: Applications of nonabsorbable oral antibiotic therapy. Adv Stud Med 3(supplA): S945-S950.

Estes L.L., Fuhs D.W., Heaton A.H., *et al.* (1993). Gastric ulcer perforation, associated with the use of injectable ketorolac. Ann Pharmacother, 27, 42-43.

Faith J.J., Guruge J.L., Charbonneau M., *et al.* (2013). Long-term Stability of the Human Gut Microbiota. Science, 341(6141);1237439.

Fedorak, R. N., Madsen, K. L., (2004) Probiotics and prebiotics in gastrointestinal disorders. Curr Opin Gastroen, 20(2), 146-155.

Fiorucci S, Antonelli E., Distrutti E., *et al.* (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology, 129;1210-1224

Flower R.J., Vane J.R. (1972). Inhibition of prostaglandin synthetase in Brain explains the Anti-pyretic Activity of Paracetamol (4-Acetamidophenol). Nature, 240; 410-411.

Freitas, M., Tavan, E., Cayuela C., *et al.* (2003) Host-pathogens cross-talk. Indigenous bacteria and probiotics also play the game. Biol Cell 95(8), 503-506.

Foglesong M.A., Cruden D.L., Markovetz A.J. (1984). Pleomorphism of fusobacteria isolated from the cockroach hindgut. J Bacteriol 158:474-480.

Fornai M., Antonioli L., Colucci R., *et al*. (2013). NSAID-induced enteropathy: Are the currently available selective COX-2 inhibitors all the same? J Pharmacol Exp Ther 348(1), 86-95.

Frank D.N., *et al.* (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Aca Sci USA 104, 13780-13785.

Fujimori, S., St. Amand A.L., Feldman R.A., *et al.* (2010) Distribution of small intestinal mucosal injuries as a result of NSAID administration. Eur J Clin Invest 40(6), 504-510.

Fujimoto T., Imaeda H., Takahashi K. *et al.* (2013) Decreased abundance of faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J. Gastroen Hepatol, 28(4), 613-619.

Fukiya S., Suzuki T., Kano Y. *et al.* (2011) Current Status of *Bifidobacterium* Gene Manipulation Technologies. In: Sonomoto, K. (Ed.) Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research. Hokkaido, Japan: Caister Associated Press, pp 33-34.

Fukuda S., Toh H., Hase K.. *et al.* (2011)Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469(7331), 543-547.

Fuller R. (1992). History and Development of Probiotics. In: Fuller, R (Ed.). Probiotics, the scientific basis. London, UK: Chapman and Hall, pp1-8.

Funk C.D., (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. Science, 294, 1871-1875.

Ganrot-Norlin K., Stalhandske T., Karlstrom R. (1981). Lack of anti-inflammatory activity of metronidazole. Acta Pharmacol Toxicol (Copenh) 49:130-133
Ghosh S., Karin M. (2002). Missing pieces of the NFkappaB puzzle. Cell, 109;Suppl: S81-S96.

Gionchetti P., Rizzello F., Morselli C., *et al.*(2005), Inflammatory bowel disease: Does rifaximin offer any promise? Chemotherapy 51 (suppl 1): 110-114

Giraud M.N., Motta C., Romero J.J., *et al.* (1999). Interaction of indomethacin and naproxen with gastric surface-active phospholipids: a possible mechanism for the gastric toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs). Biochem Pharmacol, 57, 247-254.

Goldstein J.L., Eisen G.M., Lewis B., *et al.* (2005). Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, andplacebo. Clin Gastroenterol Hepatol, 3, 131-141.

Graham D.Y., Smith J.L., Holmes G.I., *et al.* (1985). Nonsteroidal anti-inflammatory effectof sulindac sulfoxide and sulfide on gastric mucosa. Clin Pharmacol Ther, 38, 65-70.

Graham D.Y., Opekun A.R., Willingham F.F., *et al.* (2005). Visible small-intestinal mucosal injury in chronic NSAID users. Clin Gastroenterol Hepatol, 3, 55–59.

Green F.W., Kaplan M.M., Curtis L.E., *et al.* (1978). Effect of acid and pepsin on blood coagulation and platelet aggregation. A possible contributor prolonged gastroduodenal mucosal hemorrhage. Gastroenterology, 74, 38-43.

Greenblum S., Turnbaugh P.J, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. PNAS, 109(2): 594-599.

Griffin M.R., (1998). Epidemiology of nonsteroidal anti-inflammatory drug-associated gastrointestinal injury. Am J Med, 104: 23S-9S.

Guarner, F., Malagelada J. R., (2003) Gut flora in health and disease. Lancet, 361(9356), 512-519.

Hagiwara M., Kataoka K., Arimochi H., *et al.* (2004). Role of unbalanced growth of gram-negative bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug. J Med Invest; 51:43-51

Hammes W.P, Neuhaus F.C. (1974). On the Mechanism of Action of vancomycin: Inhibition of Peptidoglycan Synthesis *in Gaffkya homari*. Antimicrob Agents Chemother. 6(6): 722–728

Hayashi S., Kurata N., Yamaguchi A. *et al.* (2014). Lubiprostone prevents nonsteroidal anti-inflammatory drug-induced small intestinal damage by suppressing the expression of inflammatory mediators via EP4 receptors. J Pharmacol Exp Ther 349(3):470-470.

Hemler M., Lands W.E.M., Smith W.L., (1976). Purification of the cyclooxygenase that forms prostaglandins: demonstration of two forms of iron in the holoenzyme. J Biol Chem, 251, 5575-5579.

Hijova E., Chmelarova A., (2007).Short chain fatty acids and colonic health. Bratislavske Lekarske Listy 108(8), 354-358.

Hold G.L., Schwiertz A., Aminov R.I., *et al.* (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Appl Environ Microbiol, 69:4320-4324.

Hooper, L.V., Midtvedt, T., Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22, 283-307.

Hunt R.H., Lanas A., Stichtenoth D.O., *et al.* (2009). Myths and facts in the use of anti-inflammatory drugs. Ann Med 8:1-16

Husnik F., Chrudimsky T., Hypsa V. (2011). Multiple origins of endosymbiosis within the Enterobacteriaceae (gamme-proteobacteria): convergence of complex phylogenetic approaches. BMC Biol. 28,9:87.

Illumina. *Illumina Genome Network.* California: Illumina Inc, 2014. Print.

Insel P.A., (1990). Analgesic, antipyretics and antiinflammatory agents; drugs employed in the treatment of rheumatoid arthritis and gout. In: Gilman AG, Rall TW, Nies AS, Taylor P, eds. Goodman and Gilman's the Pharmacological Basis of Therapeutics. 8th ed.Tarrytown, NY: Pergamon, 638–681.

Jeffery I.B., O'Toole P.W., Ohman L., *et al.* (2011) Irritable bowel syndrome subtype define by species-specific alterations in faecal microbiota. Gut 61(7), 997-1006.

Kajanne R., Leppä S., Luukkainen P., *et al.* (2007). Hydrocortisone and indomethacin negatively modulate EGF-R signaling in human fetal intestine. Pediatr Res, 62, 570-575.

Kassinen A., Krogius-Kurikka L., Makivuokko H., *et al.* (2007). The fecal microbiota of ittitable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology 133, 24-23

Kato S., Ito Y., Nishio H., *et al.* (2007). Increased susceptibility of small intestine to NSAID-provoked ulceration in rats with adjuvant-induced arthritis: involvement of enhanced expression of TLR4. Life Sci; 81(16):1309-1316.

Kelly D., Campbell J.I., King T.P., *et al.* (2003). Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamme and RelA. Nat Immunol. 5(1):104-112.

Kent T.H., Cardelli R.M., Stamler F.W., (1969). Small intestinal ulcers and intestinal flora in rats given indomethacin. Am J Patho, 54, 237-245.

Keszthelyl D., Jansen S.V., Schouten G.A., *et al.* (2010). Proton pump inhibitor use is associated with an increased risk for microscopic colitis: a case-control study. Ailment Pharmacol Ther 23:1124-1128.

Kleessen B, Hartmann L, Blaut M. (2003) Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. Br J Nutr;89(5):597-606.

Koga H., Aoyagi K., Matsumoto T., *et al.* (1999). Experimental enteropathy in athymic and euthymic rats: synergistic role of lipopolysaccharide and indomethacin. Am J Physiol., 276:G576-G582

Konaka A., Kato S., Tanaka A., *et al.* (1999). Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. Pharmacol Res, 40, 517-524.

Kujubu D.A., Fletcher B.S., Varnum B.C., *et al.* (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem, 266, 12866-12872.

Kump P.K., Grochenig H.P., Lackner S., *et al.* (2013). Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis. Inflamm Bowel Dis. 19(10): 2155-2165.

Kunin C.M., (1966). Absorption, distribution, excretion and fate of kanamycin. Ann N.Y. Acad. Sci. 132:811-818

Laine L., (2001). Approaches to nonsteroidal anti-inflammatory drug use in the high-risk patient. Gastroenterology, 120, 594-606.

Laine L., Maller E.S., Yu C., *et al.* (2003). Ulcer formation with low-dose enteric-coated aspirin and the effect of COX-2-selective inhibition: a double-blind trial. Gastroenterology, 127, 395-402.

Lanas A., Baron J.A., Sandler R.S., *et al.* (2007). Peptic ulcer and bleeding events associated with rofecoxib in a 3-year colorectal adenoma chemoprevention trial. Gastroenterology,132, 490-497.

Lanas A., Garcia-Rodriguez L.A., Polo-Tomas M., *et al.* (2009). Time trends and impact of upper and lower gastrointestinal bleeding and perforation in clinical practice. Am J of Gastroenterol, 104, 1633-1641.

Langenbach R., Morham S.G., Tiano H.F., *et al.* (1995). Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin induced gastric ulceration. Cell, 83, 483-492.

Lecomte M., Laneuville O., Ji C., DeWitt D.L., Smith W.L., (1994). Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. J Biol Chem, 269, 13207-13215.

Leite A.Z., Sipahi A.M., Damiao A.O. *et al.* (2001). Protective effect of metronidazole on uncoupling mitrochondrial oxidative phosphorylation induced by NSAID: a new mechanism. Gut 48; 163-176

Ley R.E. Turnbaugh P.J., Klein S., *et al.* (2006). Microbial ecology: human gut microbes associated with obesity. Nature 444, 1022-1023.

Lichtenberger L.M., Zhou Y., Dial E.J., *et al.* (2006). NSAID injury to the

gastrointestinal tract: evidence that NSAIDs interact with phospholipids to weaken the hydrophobic surface barrier and induce the formation of unstable pores in membranes. J Pharm Pharmacol, 58, 1421-1428.

Lupp C., Robertson M.L., Wickham M.E., *et al.* (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of enterobacteria. Cell Host Microbe, 16,2(2): 119-129.

Lyra A., Saarinen M., Putaala H., *et al.* (2012). Bifidobacterium nimalis ssp. lactis 420 protects against indomethacin-induced gastric permeability in rats. Gastroenterol Res Pract 2012:615051

Ma L., del Soldato P., Wallace J.L.. (2002). Divergent effects of new cyclooxygenase inhibitors on gastric ulcer healing: shifting the angiogenic balance. PNAS, 99, 13243-13247.

MacNaughton W.K., Cirino G., Wallace J.L., (1989). Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. Life Sci, 45, 1869-1876.

Maiden L., Thjodleifsson B., Theodors A., *et al.* (2005). A quantitative analysis of NSAID-induced small bowel pathology by capsule endoscopy. Gastroenterology, 128, 1172-1178.

Mani S., Boelsterli U. A., Redinbo M. R., (2013) Understanding and modulating mammalian-microbial communication for improved human health. Annu. Rev. Pharmacol. Toxicol., 54, 559-580.

Manichanh C., Borruel N., Casellas F., *et al.* (2012). The gut microbiota in IBD. Nat Rev Gastroenterol Hepatol. 9(10):599-608.

Makivuokko H., Tiihonen K., Tynkkynen S., *et al.* (2010) The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. Br J Nutr 103:227-234.

Marchi E., Montecchi L., Venturini A.P. *et al* (1985)*. 4-Deoxypyrido[1',2':1,2]imidazo[5,4-c]rifamycin SV derivatives. A new series of semisynthetic rifamycins with high antibacterial activity and low gastroenteric absorption. J Med Chem; 28:960-963.

Maslowski, K. M., Vieira, A. T., Ng, A., *et al.* (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature, 461(7268), 1282-1286.

McCarthy D.M., (1990). NSAID-induced gastrointestinal damage – a critical review of prophylaxis and therapy. J Clin Gastroenterol, 12(Suppl. 2), S13-S20.

McCarthy D.M., (2009). GI bleeding: problems that persist. Gastrointest Endosc, 70:225-228.

Medzhitov R., Preston-Hurlburt P., Kopp E., *et al.* (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell, 2:253-258

Meier, R., Steuerwald, M., (2005) Place of probiotics. Curr Opin Crit Care, 11(4), 318-325.

Miquel S., Martin R., Rossi O., *et al.* (2013) Faecalibacterium prausnitzii and human intestinal health. Curr. Opin. Microbiol, 16(3), 255-261

Miura S., Suematsu M., Tanaka S., *et al.* (1991). Microcirculatory disturbance in indomethacin-induced intestinal ulcer. Am J of Physiol, 261, G213-G219.

Neish A.S.. (2009) Microbes in gastrointestinal health and disease. Gastroenterology, 136(1): 65-80.

Nusrat A, Turner JR, Madara JL. (2000) Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am J Physiol Gastrointest Liver Physiol;279(5):G851-G857.

Nava G.M., Stappenbeck T.S. (2011). Diversity of the autochthonous colonic microbiota. Gut Microbes 2:99-104.

Nikaido H., Outer membrane. In: Neidhardt F.C., *et al.* editors. *Escherichia coli* and *Salmonella:* cellular and molecular biology. 2nd ed. Vol 1. Washington, D.C.: American Society for Microbiology; 1996. pp29-47.

Oikonomou G., Teixeira A.G., Foditsch C., *et al.* (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. PLoS ONE 8:363157.

Ong C. K., Lirk P., Tan C. H., Seymour R. A., (2007) An evidence-based update on nonsteroidal anti-inflammatory drugs. J. Clin. Med. Res., 5(1), 19-34.

Ott S.J., Musfeldt M., Wenderoth D.F., *et al.* (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut, 53: 685-693.

Pai R., Szabo I.L., Giap A.Q., *et al.* (2001). Nonsteroidal anti-inflammatory drugs inhibit reepithelialization of wounded gastric monolayers by interfering with actin, Src, FAK, tensin signaling. Life Sci, 69, 3055-3071.

Palmer M. (Ed.). (2012). Notes to Biochemical Pharmacology; Chapter 9 Eicosanoids related drugs. New York, NY: Wiley & Sons

Pardi D.S., Kelly C.P. (2011). Microscopic Colitis. Gastroenterology, 141:1155-1165.

Petruzzelli M., Vacca M., Moschetta A., *et al.* (2007). Intestinal mucosal damage caused by non-steroidal anti-inflammatory drugs: role of bile salts. Clin Biochem, 40, 503-510.

Polisson R., (1996). Nonsteroidal anti-inflammatory drugs: Practical and theoretical considerations in their selection. Am J Med, 100(Suppl. 2A): 31S-6S

Poullis A., Foster R., Mendall M.A., (2003). Proton pump inhibitors are associated with elevation of faecal calprotectin and may affect specificity. Eur J Gastroenterol Hepatol., 15, 573-574.

Qin J. Li R., Raes J., *et al.* (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59-65.

Quigley E. M., Quera R., (2060) Small intestinal bacterial overgrowth: Roles of antibiotics, prebiotics, and probiotics. Gastroenterology, 130(2 Suppl 1), S78-90.

Rainsford K.D., Willis C., (1982). Relationship of gastric mucosal damage induced in pigs by anti-inflammatory drugs to their effects on prostaglandin production. Dig Dis Sci, 27, 624-635.

Ramirez-Farias C., Slezak K., Fuller Z., *et al.* (2009). Effects of inulin on the human gut microbiota: stimulation of *Bifidobacteria adolesentis* and *Faecalibacterium prausnitzii*. Br J Nutr, 101, 533-542.

Rath H.C., Schultz M., Freitag R., *et al.* (2001). Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. Infect Immun, 69(4):2277-2285.

Ray B. (2005). Factors Influencing Microbial Growth in Food. In: Fundamental Food Microbiology, Third Edition. CRC Press Boca Raton, Florida, pp 74-75.

Reuter B.K., Davies N.M., Wallace J.L., (1997). Nonsteroidal anti-inflammatory drug enteropathy in rats: role of permeability, bacteria, and enterohepatic circulation. Gastroenterology, 112, 109-117.

Ricciotti E., Fitzgerald G.A., (2011). Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol., 31, 986-1000.

Riordan S.M., McIver C.J., Thomas D.H. *et al.* (1997). Luminal bacteria and small-intestinal permeability. Scand J gastroenterol 32:556-563.

Robert A., Asano T., (1977). Resistance of germ free rats to indomethacin-induced lesions. Prostaglandins, 14, 331-341.

Rossi M., Corradini C., Amaretti A., *et al.* (2005) Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl Environ Microbiol 71, 6150–6158.

Round, J.L., Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Na Rev Immunol 9, 313-323.

Ruoff G., (1998). Management of pain in patients with multiple health problems: a management of chronic pain. Am J Med, 105: 45S-52S.

Ruseler-van Embden JG, van Lieshout LM, Gosselink MJ *et al.* (1995) Inability of *Lactobacillus casei* strain GG, L. acidophilus, and *Bifidobacterium bifidum* to degrade intestinal mucus glycoproteins. Scand J Gastroenterol;30(7):675-80.

Salminen S., Deighton M.A., Benno Y., *et al.* (1998). Lactic acid bacteria in health and disease. In: Salminen S., von Wright A., (Eds.) Lactic acid bacteria: microbiology and functional aspects. New York, N.Y.: Marcel Dekker, Inc.; pp221-253.

Sartor R.B., (2005) Probiotic therapy of intestinal inflammation and infections. Curr Opin Gastroen, 21(1), 44-50.

Satoh H., Amagase K., Takeuchi K. (2014). Musocal protective agents prevent exacerbation of NSAID-induced small intestinal lesions caused by antisecretory drugs in rats. J Pharmacol Exp Ther, 348(2):227-235.

Scarpignato C., Hunt R.H., (2010). Nonsteroidal anti-inflammatory drug-related injury to the gastrointestinal tract: clinical picture, pathogenesis, and prevention. Gastroenterol Clin North Am., 39, 433-464.

Scheiman J.M., Yeomans N.D., Talley N.J., *et al.* (2006). Prevention of ulcers by esomeprazole in at-risk patients using non-selective NSAIDs and COX-2 inhibitors. Am J Gastrol, 101, 701-710.

Serhan C.N., Oliw E., (2001). Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. J Clin Invest., 107, 1481-1489.

Shaheen N.J., Hansen R.A., Morgan D.R., *et al.* (2006) The burden of gastrointestinal and liver diseases, 2006. Am. J. Gastroenterol., 101(9), 2128-2138.

Shi S., Wang H., Gao H. *et al.* (2014). Increased gap density predicts weakness of the

epithelial barrier in vivo by confocal laser endomicroscopy in indomethacin-induced enteropathy. Dig Dis Sci 58(7): 1398-1405

Shimotoyodome A., Meguro S, Hase T., *et al.* (2000). Short-chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comp Biochem Physiol A Mol integr Physiol; 125:525-531.

Sigthorsson G., Simpson R.J., Walley M. *et al.* (2002). COX-1 and 2, intestinal integrity and pathogenesis of nonsteroidal anti-inflammatory drug enteropathy in mice. Gastroenterology 122(7): 1913-1923.

Silverstein FE, Faich G, Goldstein JL, *et al.* (2000). Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteroarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. JAMA, 284, 1247-1255.

Smalley W.E., Griffin M.R., Fought R.L., *et al.* (1996). Excess costs from gastrointestinal diease associated with nonsteroidal anti-inflammatory drugs. J Gen Intern Med, 11:461-469

Smith, K., McCoy, K.D., Macpherson, A.J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol 19, 59-69.

Smith WL. (1989). The eicosanoids and their biochemical mechanism of action. Biochem. J. 259; 315-324.

Smith W.L., Garavito R.M., DeWitt D.L., (1996). Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem., 271, 33157-33160.

Smith W.L., DeWitt D.L., Garavito R.M., (2000). Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem., 69, 145-182.

Smyth E.M., Burke A., Fitzgerald G.A., (2006). Lipid-derived autacoids: eicosanoids and platelet-activating factor. In: Brunton LL, Lazo JS, Parker KL, eds. Goodman & Gilman's the Pharmacological Basis of Therapeutics. 11th ed. NY, USA: McGraw Hill Companies, 653-668.

Sokol, H., Pigneur, B., Watterlot, L. *et al.* (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. PNAS 105(43), 16731-16736.

Somasundaram S., Hayllar H., Rafi S., *et al.* (1995). The biochemical basis of nonsteroidal anti-inflammatory drug-induced damage to the gastrointestinal tract: a review and a hypothesis. Scand J Gastroenterol., 30, 289-299.

Somasundaram S., Rafi S., Hayllar H., *et al.* (1997). Intestinal inflammation: Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine. Gut 1997;41:3 344-353

Somasundaram S., Sigthorsson G., Simpson R.J., *et al.* (2000). Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat. Aliment Pharmacol Ther., 14, 639-650.

Sostres C., Gargallo C.J., Arroyo M.T., *et al.* (2010). Adverse effects of non-steroidal antiinflammatory drugs (NSAIDs, aspirin, and coxibs) on upper gastrointestinal tract. Best Pract Res Cl Ga, 24, 121-132.

Stadnyk A.W., Dollard C., Izzekutz T.B., *et al.* (2002) Neutrophil migration into indomethacin induced rat small intestinal injury is CD11a/CD18 and CD11b/CD18 codependent. Gut, 50:629-635.

Stecher, B., Hardt, W.D. (2008). The role of microbiota in infectious disease. Trends Microbiol, 16, 107-114.

Stone E. (1763). An account of the success of the bark of the willow in the cure of agues. Phil Trans R Soc B, 53, 195-200.

Swidsinski A., Loening-Baucke V., Verstraelen H., *et al.* (2008). Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. Gastroenterology 135:568-579.

Tanaka A., Hase S., Miyazawa T., *et al.* (2002). Up-regulation of cyclooxygenase-2 by inhibition of cyclooxygenase-1: a key to nonsteroidal anti-inflammatory drug-induced intestinal damage. J. Pharmacol. Exp. Ther., 300, 754-761.

Takeuchi K., Miyazawa T., Tanaka A., *et al.* (2002). Pathogenic importance of intestinal hypermotility in NSAID-induced small intestinal dmage in rats. Digestion 66(1):30-41.

Tarnawski A., Brzozowski T., Sarfeh I.J., *et al.* (1988). Prostaglandin protection of human isolated gastric glands against indomethacin and ethanol injury. Evidence for direct cellular action of prostaglandin. J. Clin. Invest., 81, 1081-1089.

Turnbaugh P., Ridaura V., Faith J., *et al.* (2009). The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Sci Transl Med, 1(6);6ra14

Uchida A., Yamada T., Hayakawa T., *et al.* (1997). Taurochenodeoxycholic acid ameliorates and ursodeoxycholic acid exacerbates small intestinal inflammation. Am J Physiol, 272; G1249-G1257.

Uejima M., Kinouchi T., Kataoka K., *et al.* (1996). Role of intestinal bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug. Microbiol Immunol, 40, 553-560.

Umezawa H., Mizuno S., Yamazaki H., *et al.* (1968), Inhibition of DNA-dependent RNA synthesis by rifamycins. J Antibiot (Tokyo); 21:234-236

Vaananen P.M., Meddings J.B., Wallace J.L., (1991). Role of oxygen-derived free radicals in indomethacin-induced gastric injury. Am J Physiol, 261, G470-G475.

Vane J.R., (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature, 231, 232-235.

Vane J.R., Flower R.J., Botting R.M., (1990). History of aspirin and its mechanism of action. Stroke, 21(Suppl IV), 12-23.

Vane J.R., Bakhle Y.S., Botting R.M., (1998). Cyclooxygenases 1 and 2. Annu. Rev. Pharmacol. Toxicol., 38, 97-120.

Videla S., Vilaseca J., Guarner F., *et al.* (1994). Role of intestinal microflora in chronic inflammation and ulceration of the rat colon. Gut, 35: 1090-1097.

Videla S., Vilaseca J., Antoin M., *et al.* (2001). Dietary Inulin Improves Distal Colitis Induced by Dextran Sodium Sulfate in the Rat. Am J Gastroenterol, 96: 1486-1493.

Wallace J.L., Keenan C.M., Granger D.N., (1990). Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. Am. J. Physiol. Gastrointest. Liver Physiol., 259, G462-G467.

Wallace J.L., (1992). Prostaglandins, NSAIDs, and cytoprotection. Gastroenterol Clin North Am, 21, 631-641.

Wallace J.L., McKnight W., Miyasaka M., *et al.* (1993). Role of endothehial adhesion molecules in NSAID-induced gastric mucosal injury. Am. J. Physiol., Cell Physiol. , 265, C993-C998.

Wallace J.L., Reuter B., Cicala C., *et al.* (1994). Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcergenic properties in the rat. Gastroenterology, 107, 173-179.

Wallace J.L., Bak A., McKnight W., *et al.* (1998). Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology, 115, 101-109.

Wallace J.L., (1999). Selective COX-2 inhibitors: is the water becoming muddy?. Trends Pharmacol Sci., 20, 4-6.

Wallace J.L., McKnight W., Reuter B.K., Vergnolle N., (2000). NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology, 119, 706-714.

Wallace J.L., Dicay M., McKnight W., *et al.* (2007), Hydrogen sulfide enhances ulcer healing in rats. FASEB J, 21;4070-4076.

Wallace J.L., Vong L., McKnight W., *et al.* (2009). Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology, 137; 569-578.

Wallace J.L., Syer S., Denou E., *et al.* (2011). Proton pump inhibitors exacerbate NSAID-induced small intestinal injury by inducing dysbiosis. Gastroenterology, 141, 1314-1322.

Wallace J.L., (2012). NSAID gastropathy and enteropathy: distinct pathogenesis likely
necessitates distinct prevention strategies. Br J Pharmaco, 165, 67-74.

Watanabe T., Higuchi K., Kobata A., *et al.* (2008). Non-steroidal anti-inflammatory drug-induced small intestinal damage is toll-like receptor 4 dependent. Gut, 57, 181–187.

Watanabe T., Sugimori S., Kameda N., *et al.* (2008). Small bowel injury by low-dose enteric-coated aspirin and treatment with misoprostol: a pilot study. Clin. Gastroenterol. Hepatol., 6, 1279-1282.

Watanabe T., Nishio H., Tanigawa T., *et al.* (2009). Probiotic Lactobacillus casei strain Shirota prevents indomethacin-induced small intestinal injury: involvement of lactic acid. Am J Physiol Gastrointest Liver Physiol 297(3):g506-513.

Wei X., Guo Z., Zhurina D., *et al.* (2012). Fructose uptake in Bifidobacterium longum NCC2705 is mediated by an ATP-binding cassette transporter. J Biol Chem 287(1):357-367.

Whittle B.J., (1981). Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and the gastrointestinal damage induced by indomethacin in the rat. Gastroenterology, 80, 94-98.

Wick M.J., Madara J.L., Fields B.N. *et al.* (1991). Molecular cross talk between epithelial cells and pathogenic microorganisms. Cell, 67(4): 651-659.

Wilcox G.M., Mattia A.R. (2009). Microscopic colitis associated with omeprazole and

esomeprazole exposure. J Clin Gastroenterol 43:551-535.

Willemsen L.E.M., Koetsier M.A., van Deventer S.J.H., *et al.* (2003). Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E1 and E2 production by intestinal myofibroblasts. Gut, 51(10): 1442-1447.

Xie W., Chipman J.G., Simmons D.L., *et al.* (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. PNAS, 88, 2692-2696.

Yamada T., Deitch E., Specian R.D*., et al.* (1993). Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. Inflammation, 17, 641-662.

Yang R, Han X, Uchiyama T *et al*. (2003) IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice. Am J Physiol Gastrointest Liver Physiol;285(3):G621-G629

Yeomans N.D., Tulassay Z., Juhasz L. *et al.* (1998). A comparison of omeprazole with ranitidine for ulcers associated with nonsteroidal anti-inflammatory drugs. Acid Suppression Trial: Ranitidine versus Omeprazole for NSAID-associated Ulcer Treatment (ASTRONAUT) Study Group. N Engl J Med, 338:719-726

Zanardo R.C., Brancaleone V., Distrutti E., *et al.* (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J, 20(12); 2118-2120.

Zhang S., Chao G.Q., Lu B. (2013). Proton pump inhibitors are not the key for therapying non-steroidal anti-inflammatory drugs-induced small intestinal injury. Rheumatol Int, 33(10):2513-2521

Zhou Y., Dial E.J., Doyen R., *et al.* (2010). Effect of indomethacin on bile acidphospholipid interactions: implication for small intestinal injury induced by nonsteroidal anti- inflammatory drugs. Am. J. Physiol. Gastrointest. Liver Physiol., 298, G722-G731.