THE DIFFERENTIAL EFFECTS OF OMEGA-6 AND OMEGA-3 POLYUNSATURATED FATTY ACIDS ON INTESTINAL MICROBIAL ECOLOGY AND HOST REDOX RESPONSES

by

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Abstract

The gastrointestinal tract harbors complex bacteria which plays an important role in health and disease. Gut microbial antigens, in conjunction with ingested dietary components, are important in intestinal immune homeostasis. High omega-6 polyunsaturated fatty acid (n6 PUFA) can induce oxidative stress and inflammation in the gut. In contrast, omega-3 (n3 PUFAs) supplementation can cure several inflammatory diseases. However, the relationship between dietary PUFAs and the intestinal microbiota remain unknown. Our study was to determine the effect of high fat diets with varying n6 and n3 PUFA on mice microbiota and host responses. We used 20% corn oil (high n6 PUFA), corn + fish oil (19% wt/wt corn oil added to 1% wt/wt fish oil; high n6 PUFA + long chain n3 PUFA), 20% canola oil (low n6 PUFA) as diets keeping 5% wt/wt corn oil as a chow control. After feeding mice the high fat diets for 5 weeks, Quantitative Polymerase Chain Reaction (qPCR) was used to examine the gut microbiota. Immunofluorescence was carried out to examine immune and redox responses. All high fat diets, regardless of composition, significantly reduced *Bacteroides spp*. and increased in intestinal epithelial cell death. Mice fed 20% corn oil had high levels of bacteria from the Clostridium and Enterobacteriaceae; associated with inflammatory bowel disease (IBD). In contrast, mice fed corn oil diets supplemented with fish oil, had enriched beneficial microbe *Lactobacillus* and lower levels of Enterobacteriaceae and Clostridia species. Fish oil also reduced neutrophil infiltration as well suggesting that n3 PUFA is anti-inflammatory. In addition, unexpectedly, fish oil supplementation induced oxidative stress in the colon evident by the increased presence of 4-hydroxy-2-nonenal, a lipid peroxide product, and dual oxidase 2, which generates H_2O_2. In addition, catalase, an antioxidant was also low in the fish oil group. Canola oil, which contains n3, n6 PUFAs and a monounsaturated fat oleic acid, alters the microbiota similar to the corn oil

group. Overall, our research suggests that n6 PUFA alters the microbial composition, enriching it with detrimental microbes. Fish oil supplementation can reverse this effect. However, we also provide evidence of fish oil supplementation increasing oxidative stress.

Preface

The animal experiments were approved by UBC animal care committee committee (Animal care #: A11-0367, Bio-saftey #: B10-0064) and performed in UBC Vancouver by Ben Dai under the supervision of Dr. Deanna Gibson and Dr. Sanjoy Ghosh. Samples were brought to UBC Okanagan for further analysis. I conducted the microbiota analysis and immunofluorescence techniques. Part of this work was presented as a poster presentation at the February, 2012 Canadian Digestive Diseases Week (CDDW) conference. All chapters in this thesis were written by me with advice and suggestions from my supervisor Dr. Deanna Gibson and other committee members. The thesis was professionally edited by Vivian Albrecht MA, TESL (Okanagan College).

Publications

1. Ghosh S, Dai C, Brown K, **Rajendiran E**, Makarenko S, Baker J, Ma C, Halder S, Montero M, Ionescu VA, Klegeris A, Vallance BA, Gibson DL. (2011). "Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status and ion transporter gene expression". Am J Physiol Gastrointest Liver Physiol, 301(1), G39-49.

2. Baker J., Brown, K., Molcan E., Dai C., Makarenko S., **Rajendiran E**., Mahmoud S., and D. L. Gibson. (2012). "Medicinal lavender modulates the enteric microflora to protect against *Citrobacter rodentium*-induced colitis". Am J Physiol Gastrointest Liver Physiol, 303 (7), G825-836.

3. Kirsty Brown, Erin Molcan, **Ethendhar Rajendiran**, Angela Nusrat, Jessica Baker, Stephanie Rucheinsky, Deanna L. Gibson. Book chapter, "Oxidative Stress in Gastro intestinal disease" (Invited; under review).

4. Ghosh S, DeCoffe D, Brown K, **Rajendiran E**, Yip A, Estaki M, Dai C, Gibson DL. Fish oil attenuates ω-6 PUFA-induced) intestinal dysbiosis and murine colitis but enhances systemic inflammation. PLOsONE (Accepted with revisions).

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List of Symbols, Abbreviations

%	Percentage
°C	Degree centigrade
16S rRNA	16S ribosomal ribonucleic acid
5-LOX	Lipooxigenase pathway
bNOS	Bacterial nitric oxide synthase
CFU	Colony forming unit
COX 2	Cyclooxygenase pathways 2
DAPI	4', 6-diamidino-2-phenylindole is a blue florescent stain
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DUOX2	Dual oxidase 2
dUTPs	Deoxyuridine 5´-triphosphate(s)
E.coli	*Escherichia coli*
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
GALT	Gut associated lymphoid tissue
GI	Gasto-intestinal
GPO	Glutathione per oxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HHE	Trans-4-hydoxy-2-hexenal
HNE	Trans-4-hydroxy-2-noneal
HT-29	Human colon adenocarcinoma cell line
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LT	Leukotrienes

MAMPs	Microbial associated molecular patterns
MHC II	Major histocompatibility complex II
Nf κ B	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PPAR-γ	Peroxisome proliferator activated receptor gamma
PRRs	Pattern recognition receptors
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SFB	Segmented Filamentous Bacteria
SOD	Superoxide dismutase
spp.	Species
TdT	Terminal deoxynucletidyl transferase
TGF β	Tumor growth factor beta
Th 17	T helper-17
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TX	Thromboxanes
wt/wt	Weight / Weight

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Dedication

I would like to dedicate this work to my beloved parents and family for their moral support and encouragement.

Chapter 1: Introduction

1.1 Literature review

1.1.1 The intestinal microbiota

The gastrointestinal tract (GI) has the most diverse microbial population co-existing in equilibrium in a defined region. The intestinal tract of a human harbors a complex bacterial ecosystem. Actinobacteria, Bacteroidetes, Proteobacteria, and Firmicutes are the major phyla of the GI tract, with most being facultative aerobic and obligate anaerobes (Macfarlane et al., 2004; Wang et al., 1996). Humans and mice share most of their genes and GI microbiota, and the above mentioned phyla bacteria of human GI tract are similar to those of the mice GI tract (Spor et al., 2011). The gut microbiota population can vary in gut regions. For example, the stomach and duodenum (aerobic environment) of healthy adults contain aerobic bacteria to an extent of 10^{2-3} colony forming units (CFU)/ml. The acidic environment of the stomach and the high bile acid concentration (and relatively low transit time) of the duodenum are responsible for determining bacterial counts in these regions. The jejunum and ileum contain 10^{4-8} CFU of bacteria/ml with continuously increasing counts of facultative aerobes to the distal region of ileum. The colon contains a larger population to the extent of 10^{10-13} CFU of bacteria/ml with most bacteria being strict anaerobes (Cummings et al., 1991; Macfarlane et al., 1998). The gut resident microbes are very important to the host's health. For example, the microbiota are critical for maintaining balanced immune responses, synthesizing anti-microbial peptide, displacing pathogens or colonization resistance, and regulating mucus synthesis (Blaser, 2006).

The intestinal epithelial cells are a physical barrier, and act as protective layer against invading bacteria, as well as other luminal antigens from systemic circulation. These intestinal epithelium barriers control the communication between the lymphoid tissue and gut microbiota.

This communication is crucial to determining the mucosal immune responses. The intact epithelial barrier depends on tight junction proteins which must be tightly packed in the epithelial cells (Yu et al., 2012). In addition, some specialized cells present in the epithelial layer are paneth cells and goblet cells. A paneth cell secretes alpha-defensin anti-microbial peptides (Ouellette et al., 1996) and goblet cells regulate the synthesis of mucus (Deplancke et al., 2001). This mucus layer on the epithelium lineage controls physical and chemical abrasion of the epithelia by the luminal contents.

The intestinal physiology and the host defense may contribute to determining the overall composition and distribution of microbes in the GI tract. Physiological conditions include the digestion process, pH, substrate availability, redox potential, and transit time. Host defenses include antigen recognition, antibody secretion, mode of birth, early infections, and antibiotic exposures (Marques et al., 2010). Although not yet confirmed, various disease conditions altered in the intestinal physiology, and altered in the host defenses, may result in changes in the GI tract microbiota population. It is possible then, that studying the gut microbiota could lead to increased understanding of how to maintain or control the overall health status of the host.

1.1.2 Intestinal microbiota dysbiosis and early colonization

An alteration or imbalance in the intestinal gut microbiota ecology is called dysbiosis. It is becoming increasingly evident that environmental factors play an important role in altering the GI microbiota composition. Intestinal dysbiosis was caused by environmental factors including age (Agans et al., 2011), diet (Turnbaugh et al., 2009), and disease (Seksik et al., 2003). This altered microbiota composition is the prime contributor to determine the early stage of maturation of a child's immune system. Thus, the relationship of environmental factors, resident

microbiota, and mucosal surfaces are of primary importance to the maintenance of gut integrity. During gestation, a fetus develops in a sterile environment. After birth, the infant is exposed to the bacteria-rich environment and intestinal colonization occurs thereafter (Marques et al., 2010). Early colonization during the post-natal period may be a crucial factor that determines the overall immune status of an adult. From 2-4 years of age, a child's microbial imprint resembles that of an adult (Palmer et al., 2007). In addition, recent reports suggest that a wide range of conditions during gestation (gestational age, maternal illness, delivery mode and hospitalization) and early colonization (breast feeding, infection, and antibiotic use) determine an adult's GI tract microbiota composition (Marques et al., 2010). Interestingly, preterm birth also shows dysbiosis of specific gut microbiota. For example, low levels of strict anaerobes (*Bifidobacterium* and *Bacteroides*) and high levels of pathogenic bacteria such as Enterobacteria, *Escherichia coli, Bacteroides spp*., *Clostridia*, *Staphylococci, Enterococci, and Streptococci* were observed in the gut of preterm infants (Jacquot et al., 2011; Mshvildadze et al., 2008). Therefore, the composition of GI microbiota, such as proportion of beneficial and pathobiont microbes, may reveal the health status of the host.

1.1.3 Diseases and microbiota

GI microbiota species from different phyla must be present in the right proportion in order to maintain gut homeostasis. Healthy subjects have been observed to have a different proportion of microbiota than GI diseased patients. An alteration of the microbiota in disease conditions raises the question of whether microbiota dysbiosis is a main cause or a side effect of a disease. Emerging studies highlight various diseases associated with GI microbiota dysbiosis, including autoimmune diseases, such as inflammatory bowel disease (IBD) (Schwiertz et al.,

2010; Seksik et al., 2003) and celiac disease (Collado et al., 2007) other diseases including colon cancer (Scanlan et al., 2008), IBS (Salonen et al., 2010), stress (Cryan et al., 2011), alcoholic liver disease (Abu-Shanab et al., 2010) and metabolic diseases such as obesity (Di Baise et al., 2008; Hildebrandt et al., 2009; Santacruz et al., 2009) and diabetes (Giongo et al., 2011).

Our recent study suggests that transferring fecal microbiota from disease resistant mice to disease susceptible mice reduces the disease susceptibility (Ghosh et al., 2011). This transfer from the resistant to susceptible mice results in a reduction in colonic pathology and reduced systemic spread of infectious colitis induced by an enteric pathogen *Citrobacter rodentium*. These observations suggest that beneficial microbes may be associated with increased host-protection against inflammation. Thus, our study demonstrates the critical role gut microbiota plays during enteric disease susceptibility.

The immune homeostasis is needed to balance the GI immune responses of the host. Thus, host health and GI microbes play a major regulatory role. This regulation can be observed in diseases associated with the GI tract, where intestinal immune homeostasis is deregulated or up-regulated. This altered condition is characterized by the inappropriate activation of the immune cells, resulting in high levels of pro-inflammatory interleukin (IL) and chemotactic mediators. These mediators may contribute to enhancing the inflammation process and to destroying host tissues. The overall gut homeostasis mainly depends on the GI bacterial metabolism. The gut microbiota is known to produce lipopolysaccharides, or endotoxins, which alter the intracellular signal cascades by binding with specific cell receptors. Thus, metabolic activities of gut microbiota can have both beneficial and detrimental effects on the host. Interestingly, a relatively abundant change in gut microbiota and the presence of pro-inflammatory mediators from circulating immune cells can contribute to the etiology of GI tract

diseases. One such example is IBD. During IBD, a high level of pro-inflammatory mediator and tissue destruction are observed (Brandtzaeg et al., 1997). In addition, the proportion of intestinal microbiota also varies with higher levels of the family Enterobacteriaceae and lower levels of the phyla Bacteroidetes, and Firmicutes (Frank et al., 2007). Yet, the relationship between the altered microbiota and inflammatory mediators is quite unclear and still needs to be studied.

1.1.4 Diet and microbiota

From a nutritional standpoint, a diet should contain all essential nutrients in the right proportion in order to ensure normal metabolic function. Recent evidence suggests that high fat diets contribute to various diseases. Several studies have revealed that diet can alter the microbial ecology of the gut, at least transiently. Recently, three different diverse groups of fecal samples were analyzed to identify the gut microbial communities. Three "enterotypes", dominated by *Bacteroides, Prevotella*, and *Ruminococcus* groups, were observed (Arumugam et al., 2011). In addition, a diet rich in protein and animal fats was associated with the *Bacteroides* enterotype, whereas a carbohydrates diet was associated with the prevotella enterotype were observed in fecal samples (Wu et al., 2011). Thus, diversity and dietary habit are associated with specific gut microbiota with corresponding enterotypes. In addition, an altered microbiota was observed in a single day when diet was switched from low fat to high fat, and plant polysaccharide to high-sugar in mice (Turnbaugh et al., 2009). When overweight men were fed a reduced carbohydrate diet, the specific bacterial population in their gut changes (Walker et al., 2011). Another study shows that diet influences the diversity manifested by the significant enrichment in Bacteroidetes and depletion in Firmicutes and Enterobacteriaceae in African rural children compared to European children (De Filippo et al., 2010). In addition, a calorie-restricted diet has an influence

on the gut microbiota in over weight adolescents (Santacruz et al., 2009). Both before and after intervention, levels of bacteria such as *Bacteroides fragilis*, *Clostridium leptum* and *Bifidobacterium cantenulam* counts were significantly higher in the high weight-loss groups; while levels of bacteria such as *Clostridium coccoides*, *Lactobacillus*, *Bifidobacterium, Bfidobacterium breve*, and *Bifidobacterium bifidum* were significantly lower (Santacruz et al., 2009). In general, the gut microbiota is relatively stable in one's life span once it has been colonized in the GI tract. Thus, a once held view that the microbiota was stable throughout one's life appears to be in question and continues to be investigated. While there is evidence that the microbiota can be altered with dietary influence, we still do not know how long these ecological changes last or what type of effect they have on the host's intestinal immune responses and overall GI health.

1.2 The Western diet

The Western lipid diet has shifted from saturated fats to polyunsaturated fatty acids (PUFAs) due to agricultural business, economical, and industrial growth (Simopoulos, 2011). In Canada, consumption of PUFAs has increased by 54% during the past few decades (Canada statistics, 2005). We acquire PUFAs from plant and fish oils (Das, 2006). PUFAs mainly consist of linoleic acid (n6) and α-linolenic acid (n3). This composition varies depending on the source from which PUFAs are obtained. The ratio of n6 and n3 in the present lipid diet is 10:1 to 20-25:1, which deviates significantly from the proportions consumed by our ancestors, who consumed lipids in a nearly in 2:1 ratio (Simopoulos, 2011). In the Western world, a correlation exists between the high lipid diet consumption and disease (Ortega et al., 2012). Infectious diseases have been successfully controlled but new autoimmune, allergic and inflammatory

diseases have been observed in children and adults (Bach, 2002; Blaser, 2006). Emerging evidence suggests that diet has a role in determining the health of the Western world. It is currently unknown if a PUFAs diet has an effect on gut microbiota and this diet may have implications for gut health.

1.3 Polyunsaturated fatty acids in inflammation

Linoleic acid (n6) and α-linolenic acid (n3) are essential components of dietary PUFAs. Humans and mammals must acquire these fatty acids from their diet, because they cannot be synthesized on their own in mammalian cells. In humans, enzymes Δ6 desaturase and Δ5 desaturase convert n6 PUFA and n3 PUFA into their distinctive long-chain metabolites (Das, 2006; Simopoulos, 2006). Human breast milk contains a significant amount of all PUFAs which mainly depends upon the maternal diet. Vegetable oils rich in n6 PUFA come from corn, sunflower, soybean, rapeseed, and flax seed oils, whereas n3 PUFA is found in fish and fish products. The Inuit people have traditionally had a lower incidence of inflammatory disorders than the western population, presumably because of their dietary fats, which mainly come from fish, which are rich in n3 fatty acids including eicosapentaenoic acid (EPA; 20:5 n3) and docosahexaenoic acid (DHA; 22:6 n3), as well as saturated fats.

The two major fatty acids in PUFA, n6 and n3, are important energy sources and cell membrane structural components (Calder, 2009). They are signaling molecules and precursors of potent active metabolite-like eicosanoids, which have a pivotal role in the regulation of inflammation (Calder, 2009). Eicosanoids derived from n6 PUFA contribute to pro-inflammatory functions, whereas eicosanoids from n3 PUFA such as EPA and DHA have anti-inflammatory attributes (Burghardt et al., 2010; Calder, 2003). The proportion of n3 to n6 PUFAs in dietary oil is very important. For example, n3 PUFA has a distinct property that inhibits the inflammatory

eicosanoids generated by the n6 PUFA. Thus, n3 PUFA is known to be beneficial during inflammation conditions.

1.4 Polyunsaturated fatty acids in disease

A Western diet has a higher ratio of n6 PUFAs than n3 PUFAs. The higher ratio of n6 PUFAs and this type of lipid diet have gradually increased in the last few decades. The increased ratio of essential fatty acids (n3 and n3 PUFA) in the western diet has been suggested to be responsible for the increased incidence of many chronic inflammatory diseases and metabolic diseases such as IBD (Mane et al., 2009; Siguel et al., 1996; Tjonneland et al., 2009), diabetes, obesity (Das, 2006), and colon cancer (Singh et al., 1997).

One well known inflammatory disease associated with the GI tract is IBD with unknown etiology. Immunological dysfunction and microbial dysbiosis are believed to be the main causes. IBD has been increasing along with the increased uptake of dietary PUFAs. A recent study shows that excessive consumption of linoleic acid, a n6 PUFA, increases the risk of ulcerative colitis by 30%, whereas consumption of DHA (n3 PUFAs) is associated with a 77% disease reduction (Tjonneland et al., 2009). The association between n6 PUFAs and IBD has been demonstrated experimentally in interleukin-10 (IL) knockout mice that reveal an increased incidence of colitis correlates with an increased intake of n6 PUFAs. A reduced incidence of colitis is associated with a lower intake of a n6 PUFA diet (Mane et al., 2009).

Fish oil is a rich source of n3 PUFAs. In a colitis model, mice fed with n3 PUFAs experienced reduced colonic inflammation when compared to mice fed with a corn oil rich in n6 PUFA (Chapkin et al., 2007). In addition, IBD patients who were treated with fish oil sustained a remission (Belluzzi et al., 1996). In a recent clinical trial, the intake of n3 PUFAs lowered pro-

inflammatory markers (tumor necrosis factor- alpha [TNF-α], C-reactive protein) and increased the level of anti-inflammatory markers (IL-10, transforming growth factors [TGF-β]) in living subjects (Ferrucci et al., 2006). The beneficial action of n3 PUFA is due to production of anti-inflammatory eicosanoids such as EPA and DHA (James et al., 2000). Meanwhile, ingestion of n3 PUFA rich diets may benefit IBD patients since it is known that n3 PUFA reduces the activity of the pro-inflammatory eicosanoids generated by n6 PUFA (Calder, 2009; Wall et al., 2010). Thus, an increased intake of n3 PUFA which increases the membrane content of n3 PUFA can change the eicosanoids production, ultimately affecting T cell reaction and antigen presentation on gut-associated lymphoid tissue. The EPA and the DHA of n3 PUFA potentially contribute to down-regulation of immune responses, which include lymphocyte proliferation, cytokine production, and antigen presentation on antigen presenting cells (Calder, 2003; Chambers et al., 1999). In addition, n3 PUFA alters the production of the cytokine profile. This can be mediated by inflammatory gene expression and transcription factors such as nuclear factor κ B (NF κ B) (Zeyda et al., 2003) and peroxisome proliferator activated receptor (PPAR-γ). NF κ B is known to be a principle transcription factor contributing to the production and regulation of cytokines, adhesion molecules, and cyclooxygenase genes (Sigal, 2006). PPAR-γ is expressed in the colonic tissues and PPAR-γ agonist acts in an anti-inflammatory fashion as shown in the colitis mice model. A low level PPAR-γ expression in mice increases susceptibility to chemical colitis in the PPAR-γ knockout mice model (Desreumaux et al., 2001). Fish oil supplementation is known to be beneficial in inflammatory conditions but in normal base line conditions the role of fish oil is not known. This suggests the importance of studying the effect of fish oil supplemented with n6 PUFA.

1.5 Overview of oxidative stress

Oxidative stress is the imbalance between generation and the removal of reactive molecules in the biological system. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are the natural by products of the normal metabolism; in mild oxidative conditions, tissues often respond by producing more antioxidants enzymes such as glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) and, glutathione peroxidase (GPO) to normalize the free radicals in the biological system. However, severe persistence of oxidative stress conditions depletes antioxidant capacity that leads to lower antioxidant levels in the inflamed tissues. Generation of reactive free radicals such as ROS and RNS are essential to kill invading pathogens by immune cells such as dendritic cells and macrophages (Clark et al., 2001).

1.6 Oxidative stress-mediated tissue damage and sources

Reactive molecules have a very short half-life and are difficult to measure in the biological system. It is possible to measure reactive species via indirect methods. Oxidative lipid products can be measured in tissues and body fluids by thiobarbituric acid reactive substances (TBARS), isoprostanes, diene conjugation, and alkane levels in the breath. Oxidative DNA damage is generally measured by the concentration of 8-hydroxy-2'-deoxyguanosine (8OHdG). Free radicals are generated upon oxidation and attack proteins to produce a 3-nitrotyrosine, 3-chlorotyrosine, and parahydoxy-phenylacetaldehyde. In addition, reactive molecules are usually neutralized by antioxidants in the biological systems (Shah et al., 1999). Further, redox imbalance is known to initiate a biological response signal, such as cell death or cell survival (Miura et al., 2010). Cell death is by apoptosis or necrosis, and survival is through molecular

repair or enhanced defense and repair systems (Miura et al., 2010). Furthermore, NADPH oxidase 1 (Nox1) and dual oxidase 2 (Duox2) are two homologues of the catalytic core of phagocyte NADPH oxidase. This enzyme is highly expressed and identified as a major source of ROS in the GI tract. This superoxide-producing enzyme has a potential role in the immunopathology of the GI tract. Nox1 has the highest level of mRNA in the colon and is often called "colon NADPH oxidase". Duox2 protein is expressed in the colon, duodenum and small intestine and may have a role in inflammation and host defense due to its intrinsic Ca^{2+}-, NADPH- dependent H_2O_2-generating activity (Rokutan et al., 2008). Production of H_2O_2 by Duox2 supports lactoperoxidase-mediated antimicrobial defense mechanisms on the mucosal surface. In addition, dietary PUFAs have a double bond in their structure which is more prone to oxidize and produce a wide variety of oxidative products. Therefore, these products are generally used as a biomarker for protein damage; their measurement indicates the overall redox status of the host.

1.7 Anti-oxidants in the gut

In general, anti-oxidant enzymes can scavenge free radicals. Glutathione peroxidase (GPX) catalyzes the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thus it protects mammalian cells against oxidative damage. SOD destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by CAT and GPX reactions. SOD converts the highly reactive superoxide radical to less reactive H_2O_2. CAT is one of the most efficient enzymes known; even higher concentration H_2O_2 can not saturate CAT. It reacts with H_2O_2 to form water and molecular oxygen to neutralize the oxidative response. Immune cells such as neutrophils and macrophages produce ROS and NO, which increase the levels of

oxidative stress in a localized area. While these processes function to eliminate pathogens or dying cells, this can also be a source of cellular damage if unchecked. Under normal circumstances, host cells have antioxidants (thiols, ascorbate, α-tocopherol) and anti-oxidant enzymes (SOD, CAT, GSH peroxidase) to minimize the damage to host cells in close proximity to the antigen. However, a combination of continuous inappropriate inflammatory activation or a weakened host anti-oxidant response can result in oxidative stress damage to the host cells of the GI tract. Furthermore, dietary anti-oxidants such as vitamins, carotenoids, and polyphenols reduce oxidative damage to the body by depleting or preventing ROS. Vitamin C has been implicated to help reduce oxidative damage and increase total antioxidant capacity (Hermsdorff et al., 2011). Vitamin E prevents lipid peroxidation and small intestinal hypersecretions (Lindley et al., 1994). Carotenoids are plant pigments with anti-oxidant properties, and can be converted into vitamin A. Therefore, host anti-oxidant defense enzymes as well as dietary antioxidants are important in the context of minimized effect of reactive substances (RNS, ROS) in the biological system.

1.8 Polyunsaturated fatty acids and oxidative stress

Double bonds present in PUFAs are more prone to oxidize and produce oxidized fatty acids and hydoxy fatty acids as unstable intermediate compounds known as oxylipin, including both free and esterified forms. Upon oxidation, n6 PUFA produce trans-4-hydroxy-2-nonenal (HNE) and n3 PUFA (DHA and EPA) to produce trans-4-hydoxy-2-hexenal (HHE) intermediates (Spickett et al., 2010). These oxidized products of PUFAs enhance the production of iNOS, RNS, ROS, and induce NF κ B, apoptosis, and generate protein, phospholipid adducts, all of which reduce the level of glutathione (GSH) in the tissues and fluids. We can measure

these products in order to understand the mechanism of dietary PUFA's effects on the biological system (Long et al., 2010).

Research has shown that epithelial cell damage (Rao et al., 1997), inflammation, and mucus deficiency are associated with GI disease due to excessive production of free radicals (Ding et al., 2005; Holmes et al., 1998; Kruidenier et al., 2003). The harmful effects of these reactive radicals may be associated with the initiation and propagation of the disease (Tuzun et al., 2002). Another study shows that fish oil supplement given to ulcerative colitis patients decreases the level of oxidative stress (Barbosa et al., 2003). Nitric oxide (NO) is another free radical produced by three isoforms of nitric oxide synthase (neuronal-nNOS, endothelial-eNOS and inducible-iNOS). Two of these (nNOS and eNOS) are important in the gut because they have a pivotal role in peristalsis and control of mucosal blood flow (Karpuzoglu et al., 2006). The iNOS produces a significant amount of NO which is involved in innate immunity; sustained and excess NO generation is accompanied by IBD (Middleton et al., 1993; Tripathi et al., 2007). In addition, eNOS and iNOS are essential for mucus producing colonic goblet cell functions, and thereby protect against inflammatory stimulation and bacterial translocation (Vallance et al., 2004). Different DNA adducts may be formed from n3 PUFA oxidation and nearly fifteen oxidative products identified from the oxidation of DHA (Marnett, 2002; Nath et al., 1994). Oxygenated α, β-unsaturated aldehydes (O-αβUAs) of n3 PUFA are potentially carcinogenic. DHA supplemented (0.8 and 1.6g/day) for two weeks in a human study caused significant increases in plasma 4-hydroxy-hexenal (4-HHE), a peroxidized product of DHA (Calzada et al., 2008). It was reported that n3 PUFA peroxidation products were related to the risk of chronic hepatitis C and chronic obstructive pulmonary disease (Kitase et al., 2005; Rahman et al., 2002). Furthermore, orally administrated 4-hydroxy-trans-2-nonenal (4-OHE, 3 mg) in mice showed

adducts such as 4-OHE-deoxiguanidine (4-OHE-dG), 4-OHE-deoxycytidine (4-OHE-dC), and 4-OHE-methyl-dC in esophageal, gastric, and intestinal DNA (Kasai et al., 2005). Therefore, a correlation between a rich PUFA diet and oxidative stress can be assumed. However, the role of gut microbiota in oxidative stress is still unclear.

1.9 The gut microbiota and oxidative stress

The gut has a diverse population of bacteria in different regions. Our recent evidence suggests that gut commensal bacteria significantly modulates redox responses in the gut (Ghosh et al., 2011). It is known that attaching/effacing (A/E) luminal enteric pathogens, like enteropathogenic and enterohemorrahgic *E. coli,* induces iNOS. The expression of iNOS is involved in the host defenses in the epithelial cells lining of the intestinal crypts (Vallance et al., 2002). In crypt epithelial cells of C57BL/6 mice*, C. rodentium* induces iNOS expression. This iNOS expression has been linked with oxidative stress and enhances pathogen clearance (Gibson et al., 2008; Vallance et al., 2002). Other *in vitro* studies have found that bacterial LPS of *Salmonella tryphimurium* can induce iNOS and cyclooxigenase-2 (COX-2) expression in the mouse RAW 264.7 macrophages cell line (Shiratori et al., 2005). In addition, the *ex vivo* production of cytokines and NO in the spleen, peritoneum, and peyer's patch leukocytes of mice were analyzed after oral administration of viable lactic acid bacteria. There was no alteration of cytokines or NO in the peyer's patch or spleen cell cultures, but in the peritoneal culture, *L. acidophilus* increased the IFN-γ, IL-6, IL-12, and nitric oxide production whereas *L. helveticus, L. gasseri, L. reuteri* and *Bifidobacterium* impaired the production of IFN-γ, IL-6, and NO (Tejada-Simon et al., 1999). Conclusively, the resident microbiota plays an important role in the redox status of the gut in both infected and non-infected conditions.

While traditionally it is thought that NO promotes protection of host cells against pathogens, NO can also act as a signaling molecule in bacteria to promote their survival as well as modulate host responses, which can be either beneficial or harmful for the host or the microbe (Gusarov et al., 2005). NO derived from bacterial nitric oxide synthase (bNOS) or from host iNOS promotes growth of NO-resistant bacteria (Gusarov et al., 2009). NO generation during oxidative stress has been shown to alter gene expression in *E.coli* (Mukhopadhyay et al., 2004; Nunoshiba et al., 1993) and in *B.subtilis* (Nakano, 2002). As well, *Salmonella*'s DNA base excision repair system is required for resistance against the genotoxic effects mediated by NO *in vivo* (Richardson et al., 2009). The above studies suggest that some bacteria have adapted to utilize the host's protective defenses of NO production for their own advantage and survival.

Bacteria and their cytoplasmic components may contribute to the induction of oxidative stress in host tissues. Commensal products from heat killed *Bifidobacterium* and *Lactobacillus* were shown to induce NO as well as TNF-α and IL-6 in RAW264.7 macrophage cell lines (Tejada-Simon et al., 1999). This suggests that although these commensal strains are normally considered beneficial, if they cross the epithelial barrier they can induce inflammatory and oxidative responses in the underlying immune cells. Other studies reveal the damaging effects of superoxide radicals of bacterial origin on colonic cells. For example, H_2O_2 produced from *Lactobacillus* induces cell death in the human epithelial cell line HT-29 (Strus et al., 2009). This suggests that gut microbiota capable of producing H_2O_2 could be involved in the damage associated with chronic inflammatory diseases, such as inflammatory bowel disease (IBD), by contributing to H_2O_2 production in the inflamed mucosa. In support of this concept, biopsies and stool samples from IBD patients were shown to contain H_2O_2 producing *Enterococci, Streptococci*, and *Lactobacilli*. As well, in the mucosa of IBD patients, the total populations of

aerobic bacteria increased, revealing a higher oxygen tension present in inflamed tissues (Strus, et al., 2009).

Additional studies reveal that some intestinal bacteria can produce extracellular superoxide, hydrogen peroxide, or hydroxyl radicals which arise from O_2. For example, while stool and clinical enterococcal isolates including *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casselijlavus*, and *Enterococcus gallinarum* produce extracellular superoxide, other commensal bacteria like streptococci, staphylococci, and aerobic gram-negative bacilli do not. In addition, production of extracellular superoxide by *E. faecalis* is more common than production by *E. faecium* (Huycke et al., 1996). Among enterococci, *E. faecalis* is most commonly associated with invasive infections (Jett et al., 1994), which indicates that extracellular superoxide is a potential virulence factor. In appropriate *vivo* conditions, extracellular superoxide favors the growth of enterococci in the intestinal tract, thereby facilitating colonization, and possibly, overgrowth (Miller et al., 1995). *E. faecalis* also induces IBD in germ free IL-10 knockout mice, suggesting that *E. faecalis* could induce IBD in genetically susceptible individuals (Balish et al., 2002). Thus, the ability of invasive strains such as *E. faecalis* to produce extracellular superoxide, and then thrive in this microenvironment, suggests that these microbes may not only be associated with chronic inflammatory conditions but could also contribute to disease etiology by damaging host cells.

1.10 Research overview and hypothesis

Linolenic acid (n6 PUFA) is one of the predominant fatty acids in the dietary oils of Western diets and has been linked to IBD (Tjonneland et al., 2009). In addition, it produces the pro-inflammatory eicosanoids which are found in elevated levels during inflammatory

conditions. Furthermore, fish oil (rich in n3 PUFA) supplementation reduces the inflammatory markers during IBD and blocks the generation of pro-inflammatory eicosanoids from n6 PUFA (Calder, 2003; Ferrucci et al., 2006). Therefore, we assigned 20% corn oil as predominant in the n6 PUFA group because of the higher ratio of n6 PUFA compared to n3 PUFA (nearly >10:1). Another group is the fish oil supplementation group (19% corn oil + 1% fish oil). We assumed that fish oil (rich in n3 PUFA) reduces the inflammatory eicosanoids produced by n6 PUFA and reverses the intestinal microbiota of n6 PUFA that could potentially be associated with GI inflammatory disease. The third diet group, 20% canola oil, was included in our study because this diet has a nearly 2:1 ratio of n6 and n3 PUFA. This ratio refers to the diet consumed by our ancestors in ancient days (Simopoulos, 2006, 2011), and considered as a high fat control group in this study. In addition, canola oil has high ratio of a monounsaturated fat, oleic acid, compared to corn and the fish oil supplementation group. We hypothesized that the canola oil group would produce intermediate or beneficial effects compared to 20% corn oil and similar results as the 19% corn oil + 1% fish oil. Finally, 5% corn oil group was considered as a low fat diet (normal chow). Therefore, **we hypothesized that a diet rich in n6 PUFA would cause dysbiosis, which would increase the intestinal immune responses and have an effect on the redox responses. We hypothesized that fish oil supplementation would reverse these effects**.

1.10.1 Objectives

a. Determine the gut microbial ecology of mice fed with different proportions of n6 and n3 PUFA diets (corn oil, corn + fish oil, canola oil, and low fat).
b. Examine aspects of immunity and redox status of GI tract from mice fed different n6 and n3 PUFA diets (corn oil, corn + fish oil, canola oil, and low fat).

Chapter 2: Methodology

2.1 Animal studies and diet experiment

Four weeks old female C57BL/6 mice were randomly sorted into four groups. Each group had 5 to 6 mice. Three groups of mice were fed with three high lipid diets (corn oil, corn + fish oil, canola oil) and one group was fed with a low fat diet (5% wt/wt of corn oil). The high fat diets were isocaloric and isonitrogenous in nature and were prepared by adding 200 grams of oils to 800 grams of basal mix obtained from Harlan Teklad, USA (TD.88232). Appendix B provides additional details. The oils used were 20% w/w canola oil (low n6 PUFA group), 20% w/w corn oil (high n6 group) or 19% corn oil supplemented with 1% w/w fish oil (long chain n3 PUFA group). The fatty acid compositions of the various oils are given in Table 1. The mice were fed the above mentioned diets for five weeks, after which the mice were sacrificed, and luminal content and tissues were collected for microbiota and host inflammatory analysis. The collected samples were stored at -80 °C until processing.

The basal Mix was composed of protein at 21.2% wt/wt (19Kcal % energy) and carbohydrates at 44.4% wt/wt (39Kcal % energy). The 5% corn oil group has 22.6% wt/wt protein (26.4Kcal % energy) and carbohydrates 51.2 % wt/wt (60.1Kcal % energy). Therefore, all 20% wt/wt PUFAs gave 4.53Kcal energy/gm of diet whereas 5% corn oil gave 3.41Kcal energy/gm of diet. For the detailed compositions of Harlan Teklad Basal Mix see Appendices A and B. Respective diets were administered to mice for 5 weeks; food and water were freely accessible for all mice. All of these experiments were performed at the Child and Family Research Institute by Dr. Deanna Gibson, Dr. Sanjoy Ghosh and Ben Dai following the guidelines of Animal Care set out by the University of British Columbia.

2.2 Quantitative polymerase chain reactions (qPCR) for microbiota Analysis

About 60 to 80% of microbes are predicted to be non-cultivatable (Suau et al., 1999). Today, 16S rRNA gene probe hybridization has become widely adopted to detect specific bacterial groups or species in a mixed population. The 16S rRNA sequence contains conserved, variable, and hyper-variable regions which can be used to detect phyla or families at a lower resolution, and genus or species at a higher resolution. A quantitative polymerase chain reaction (qPCR) is another commonly used method to detect the bacteria in a mixed population. In this method, 16S rRNA directed synthetic oligo deoxynucleotide primers are used to amplify the 16S rRNA gene of our bacteria of interest.

2.2.1 Bacterial primer selection

To select the bacterial primers, we conducted a comprehensive literature survey to find primers in published journals. We chose primers that had been successfully used to study the microbiota by qPCR method and were important in the context of diet, GI disease, and inflammation (Table1 and refer Appendix C). We then confirmed the primers specificity using the insillico PCR database (http://insilico.ehu.es/) as well as in the Ribosomal Database Project (http://rdp.cme.msu.edu/). For Segmented Filamentous Bacteria (SFB), I designed our own primer by using the NCBI primer blast tool against *Candidatus Arthomitus spp. SFB-mouse-Japan*, complete genome (GenBank-AP012202.1). The forward primer was used to amplify the region from 186099 - 186118 and the reverse primer was used to amplify the region from 186317-186298 of 16S ribosomal RNA gene of SFB. This SFB primer was also validated using the above mentioned database. The primers were synthesized by Integrated DNA Technologies (IDT), Canada.

2.2.2 Bacterial genomic DNA extraction

The bacterial genomic DNA was extracted from the collected luminal contents of all mice. PBS homogenized luminal content at ~400 mg was used to extract a bacterial genomic DNA by using Qiagen stool kit and each step were performed according to the manufacturer instructions. All extracted DNA concentrations were measured by NanoDrop 2000 (Thermo scientific) and aliquoted at ~50 ng/µl in water (molecular grade water, Fisher scientific, BP2819100) for qPCR analysis.

2.2.3 Primer efficiency check

A temperature gradient PCR was carried out to initially to determine the annealing temperature of each primer set and standardize the initial PCR conditions. The annealing temperature of primers was achieved by performed PCR reactions in different temperatures. Furthermore, the real-time machine not only monitors DNA synthesis during the PCR, it also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double strand depends on its base composition. All PCR products for a particular primer pair should have the same melting temperature - unless there are contamination, mispriming, primer-dimer artifacts. Since SYBR green fluoroscence dye does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature.

Primer efficiencies were then calculated and efficiency was checked according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). We were successful in obtaining efficiencies in an acceptable range (80-120% as shown in Table 3) with the exception of Enterobacteriaceae, *Clostridia spp.*

and *E. feacalis* primers. To determine the primer efficiency, a serial dilution of a template DNA was used to carry out the PCR reaction. The serial dilution Ct value was used to generate a standard curve. The co-efficient correlation (R^2) or Pearson's correlation coefficient from the linear regression line were used to check the primers efficiency. Amplification efficiency **E** is calculated from the slope of the standard curve. For example, efficiency for 10 fold dilution of template DNA is calculated by following formula according to the biorad manual and MIQE guidelines (Bustin et al., 2009)

$$\mathbf{E} = 10^{-1/\text{slope}}$$

$$\% \text{ Efficiency} = (\mathbf{E}-1) \times 100$$

2.2.4 qPCR

Once we standardized the PCR condition, real-time PCR was performed. Bacterial DNA quantification was achieved on a CFX96 real-time PCR machine (Bio-Rad) using Sso Fast Eva Green Supermix (Bio-Rad) under conditions suggested by the supplier, and one reaction volume is 10 µl. All PCR reactions were carried out in duplicate at 10µl volume. High-profile white tubes and ultra clear sealing tapes (Bio-Rad) were used. One reaction volume (10µl) consists of the following materials, which were used under conditions suggested by the supplier.

Sso Fast Supermix	- 5 µl
Forward primer	- 0.3µl (5µM)
Reverse primer	- 0.3 µl (5µM)
Deionized water	- 3.4 µl
Template DNA	- 1 µl (50 ng/ µl)

The following final conditions were used set in the PCR machine to perform the PCR reactions. Initial denaturation at 98°C for 2 minutes followed by 39 cycles of denaturation at 98°C for 30 s, annealing for 30 s (refer to Table 2 for each annealing temperature of each

bacteria) and extension at 72°C for 30 s. Melting curve analyses were carried out from 65 °C to 95°C. Within this temperature range, after every 1°C increase, the plate was read and the temperature was held for 10 s. The CFX manager version2 software was used to analyze our results (Balamurugan et al., 2008). A relative quantification method was used to quantify our bacteria of interest. In this method eubacteria primers were considered as a reference gene for a relative quantification of our microbiota analysis. We have used efficiency values of each bacterial primer (Table 3).

2.3 Immunofluorescence staining

2.3.1 Procedure

Transverse sectioned, 5 μm thickness of Paraffin-embedded colon tissue sections were deparaffinized with xylene and gradually rehydrated with decreasing concentrations of alcohol following standard techniques as previously described (Ghosh et al., 2011; Gibson et al., 2008). Antigen retrieval was performed in rehydrated tissues with a 1 mg trypsin (Sigma) tablet in 1 ml of water for 20-30 minutes at room temperature. The tissues were washed and non-specific sites were blocked with 5% Bovine Serum Albumin (BSA) in PBS (Sigma). The specific primary antibody was diluted in 5% BSA in PBS (we followed the instruction according to the specific antibody supplier). Incubation was performed for 1 ½ hours at room temperature or overnight at 4 °C followed by washing with PBS. The specific secondary antibody in 5% BSA in PBS (1: 150 to 1:200) conjugated with fluorophore was incubated for 1 hour at room temperature. Finally, tissue sections were mounted with Fluoroshield with DAPI (Sigma) and visualized by using an Olympus 1X81 Q-Imaging camera with a built in Meta Morph advanced version 7.7.7.0. This procedure was used to perform the following experiments.

2.3.2 Quantification of macrophages and neutrophils

The procedure mentioned in the section 2.3.2 was followed and tissues section were incubated in a primary antibody made against F4/80 (Cedarlane laboratories anti-rat monoclonal antibody) or myeloperoxidase (Neomarkers, Fremont, CA) used for macrophags and neutrophil analysis respectively. Secondary antibody was incubated with Goat-anti-Rat, labeled with green fluorescent Fluor 488 for macrophage and Goat-anti-Rabbit IgG, Hilyte Fluor 594 labeled-Red for neutrophil. We the instructions suggested by the supplier. Positively stained macrophage and neutrophil cells were counted manually at 400X time magnification under the microscope, on entire colon tissue section of each mouse (5 to 6 mice in the group) and subsequently cells counts of each mouse were averaged to make the graphs.

2.3.3 Apoptosis - TUNEL assay

We examined apoptosis since it is also a marker for oxidative damage of tissues and cells (Sweeney et al., 2005) and followed the procedure mentioned in the section 2.3.1. We measured the apoptotic cells using the Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL). This method, commonly used to detect apoptosis, results in DNA fragmentation where the presence of fragmented DNA-3'OH end can be identified by terminal deoxynucletidyl transferase (TdT). TdT is an enzyme that helps incorporate the deoxythymidine analog deoxyuridine 5´-triphosphate (dUTPs) that is tagged with a fluorophore marker which gives a signal to detect apoptotic cells. We used a FragEL DNA Fragmentation Detection Kit from Calbiochem (product # QIA39-EA) to visualize apoptotic cells. Positively stained apoptotic cells were counted manually at 400X time magnification under the microscope, on 5 to 6 regions of

10 intact crypts of each mouse tissue section in the group (5 to 6 mice in the group) and then averaged values of each mouse were used to make the graphs (Gibson et al., 2008).

2.3.4 HNE staining

The procedure mentioned in the section 2.3.1 was followed. For the primary antibody for HNE staining, we used 1 µl of polyclonal Goat anti- Hydroxynoneal (1µg/µl, abm-Applied Biological Materials, Inc) in 50 µl 5% BSA in PBS that specifically binds to HNE modified protein or detects HNE modified proteins in tissue sections. One µl of secondary antibody conjugated with red fluorophore (Goat-anti-Rabbit IgG, Hilyte Fluor 594 labeled-Red) in 150 µl of 5% BSA in PBS was used to detect the HNE signal in the colon section under the microscope.

2.3.5 DUOX2 staining

We followed the basic procedure mentioned in the section 2.3.1 and for specific DUOX2 staining we used 1 µl of purified rabbit polyclonal antibody raised against DUOX2 of mouse origin (200 µg/1 ml, sc-134442, Santa Cruz Biotechnology, Inc) in 50 µl of 5% BSA in PBS was used and 1 µl of secondary antibody conjugated with red flurophore (Goat-anti-Rabbit IgG, Hilyte Fluor 594 labeled Red) in 150 µl of 5% BSA in PBS was used to observe the DUOX2 expression in colon of the mice.

2.3.6 Catalase staining

The procedure mentioned in the section 2.3.1 was followed. For specific catalase staining we used 1µl of purified rabbit polyclonal antibody raised against catalase of human origin (200µg/1ml, sc-50508, Santa Cruz Biotechnology, Inc) in 50 µl of 5% BSA in PBS was used and 1µl of the secondary antibody conjugated with red fluorophore (Goat-anti-Rabbit IgG,

Hilyte Fluor 594 labeled Red) in 150 µl of 5% BSA in PBS was used to find the catalase expression in colon of the mice.

2.3.7 Glutathione peroxidase-2 (GPX2)

We followed the procedure mentioned in the section 2.3.1. For specific GPX2 staining we used 1 µl of primary purified goat polyclonal antibody raised against GPX2 of human origin (200µg/1ml, sc-54604, Santa Cruz Biotechnology, Inc) in 50 µl of 5% BSA in PBS was used and 1 µl of secondary antibody conjugated with red fluorophore (Rabbit-anti Goat IgG, Hilyte Fluor 594 labeled Red) in 150 µl of 5% BSA in PBS was used to observe the expression of GPX2 in the colon of the mice.

2.4 Statistical analysis

GrapPad Prism4 software was used to performed one-way analysis of variance (ANOVA) with tukey's multiple comparison test to compare the diet treatment group means; and p values less than 0.001(***<p), 0.01 (**<p) and 0.05(*<p) were considered to be significant. Each diet treatment group had 5 to 6 mice.

Table 1: Major fatty acids of dietary oils used in this study (Lipids gms/100 gms of chow)

Fatty acids	Corn oil 20% wt/wt	Canola oil 20% wt/wt	19% Corn + 1% Fish oil (wt/wt)	Corn oil 5% wt/wt (Normal Chow)
Saturated FA	2.56	1.38	2.75	0.64
Linoleic acid (n6)	11.46	3.88	10.9	2.87
Arachidonic acid	0	0	0.025	0
α-Linolenic acid (n3)	0.24	1.5	0.231	0.06
Oleic acid	5.36	12.92	5.31	1.34
DHA/EPA	0	0	0.34	0

All 20% wt/wt high PUFAs diets were isocaloric. The diets were composed of: 20% wt /wt corn oil (high in n6 PUFA), 20% wt/wt canola oil (2.5: 1 of n6 and n3 PUFA), 19% wt /wt of corn oil + 1% wt/wt of fish oil (high n6 PUFA supplemented with n3 PUFA) and a low fat normal chow control (5% wt/wt corn oil).

Table 2: List of bacterial primers used in this study

	Bacterial Name	Forward primer	Reverse primer	Annealing Temp (°C)	Reference
1.	*Bacillus spp.*	GCGGCGTGCCTAATACATGC	CTTCATCACTCACGCGGCGT	60	(Petnicki et al., 2009)
2.	*Lactobacillus spp.*	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	60	(Walter et al., 2001)
3.	*Enterococcus faecalis*	CCCTTATTGTTAGTTGCCATC	ACTCGTTGTACTTCCCATTG	60	(Rinttila et al., 2004)
4.	*Clostridium coccoides*	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCG	60	(Matsuki et al., 2004)
5.	*Eubacterium rectale*	ACTCCTACGGGAGGCAGC	GCTTCTTAGTCAGGTACCGT	60	(Barman et al., 2008)
6.	*Clostridia spp.*	GCTGCTAATACCGCATGATA	CAGACGCGAGTCCATCTCAG	60	(Deloris et al., 2006)
7.	SFB	CGGAGCATGTGGTTTAATTC	GCTGTCTTCGCTAAAGTGCT	55	This study
8.	*Bacteroides spp.*	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG	60	(Petnicki et al., 2009)
9.	*Bacteroides fragilis*	AYAGCCTTTCGAAAGRAAG	CCAGTATCAACTGCAATTTT	60	(Matsuki et al., 2004)
10.	Enterobacteriaceae	GTGCCAGCMGCCGCGGTAA	GCCTCAAGGGCACAACCTCC	60	(Barman et al., 2008)
11.	*Bifidobaacterium spp.*	CTCCTGGAAACGGGTGG	GGTGTTCTTCCCGATATCTA	60	(Matsuki et al., 2002)
12.	*Enterococcus faecium*	CCACCGGAGATTGCTCCACC	CCGTCAAGGGATGAACAGTT	53.4	(Kang et al., 2010)
13.	Eubacteria	CGGTGAATACGTTCCCGG	TACGACTACCTTGTTACGAC	60	(Sokol et al., 2009)

SFB – *Segmented Filamentous Bacteria*

The list of bacterial primers used in this study. These primers were retrieved from other microbiota studies using qPCR in published journals and were important in the context of diet, GI disease and inflammation (see appendix C).

Table 3: Bacterial primer's efficiencies and their phylum

	Bacterial Primer	Efficiency Value at 60°C	Phylum
1	*Bacillus spp.*	114.0 %	Firmicutes
2	*Lactobacillus spp.*	113.9 %	"
3	*Enterococcus faecium*	118.1 %	"
4	*Enterococcus faecalis*	137.6 %	"
5	*Clostridium coccoides gp.*	105.9 %	"
6	*Eubacterium rectale*	99.4 %	"
7	*Clostridia spp.*	123.1 %	"
8	SFB	92.2 %	"
9	*Bacteroides spp.*	100.1 %	Bacteroidetes
10	*Bacteroids fragilis*	104.7 %	"
11	*Enterobacteriaceae*	140.7 %	Proteobacteria
12	*Bifidobacterium spp.*	96.6 %	Actinobacteria
13	Eubacteria	111.5 %	-

The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines suggests that primer's efficiency should lie between 80-120%.

Chapter 3: Results

3.1 Dietary corn oil increases *Clostridia spp*. in the gut

To determine the effects of the high fat diets fed to mice we examined microbial dysbiosis using specific primers to 16S RNA and qPCR. We examined *Clostridium coccoides gp*., *Clostridia spp*. and *Eubacterium rectale* of clostridium cluster XIVa (Firmicutes phylum) because these species are opportunistic pathogens or pathobionts (Lozupone et al., 2012) associated with IBD (Duck et al., 2007) and IBS (Jeffery et al., 2011; Schoepfer et al., 2008) and can cause systemic infection (Decousser et al., 2007; Elsayed et al., 2004; Finegold et al., 2005). In our study, we found that the ileum of mice fed with dietary corn oil group, rich in n6 PUFAs, was enriched with the *Clostridium coccoides gp*. (ANOVA, df=3, F=4.393, *p< 0.0195; Tukey's post-hoc *p< 0.05; Figure 1B, panel b1) and *Clostridia spp.* (ANOVA, df=3, F=5.298,**p< 0.01; Tukey's post-hoc *p< 0.05; Figure 1B, panel b2) when compared to ileum of mice fed with corn + fish oil diets and 5% corn oil. All high fat diets promoted the *Clostridia spp.* in the colon when compared to 5% corn oil but not statistically significant (ANOVA, df=3, F=3.42, p> 0.0492; Tukey's post-hoc, p> 0.05; Figure 1A, panel a2). Furthemore, only 20% corn oil rich diets enhanced this species in the ileum compared to mice fed with corn + fish oil and 5% corn oil (ANOVA, df=3, F=5.298,**p< 0.01; Tukey's post-hoc *p< 0.05; Figure 1B, panel b2). There was no difference in *Eubacterium rectale* in the colon (ANOVA, df=3, F=0.4953, p> 0.6901; Tukey's post-hoc, p> 0.05; Figure 2A, panel a3), in the ileum of high and low fat fed mice (ANOVA, df=3, F=2.598, p> 0.0882; Tukey's post-hoc, p> 0.05; Figure 2B, panel a3). Therefore, high fat diets composed of corn oil increase the opportunistic pathogens such as *Clostridium coccoides gp*., *Clostridia spp.* in the ileum of gut but fish oil supplementation with corn oil reduces these bacterial species.

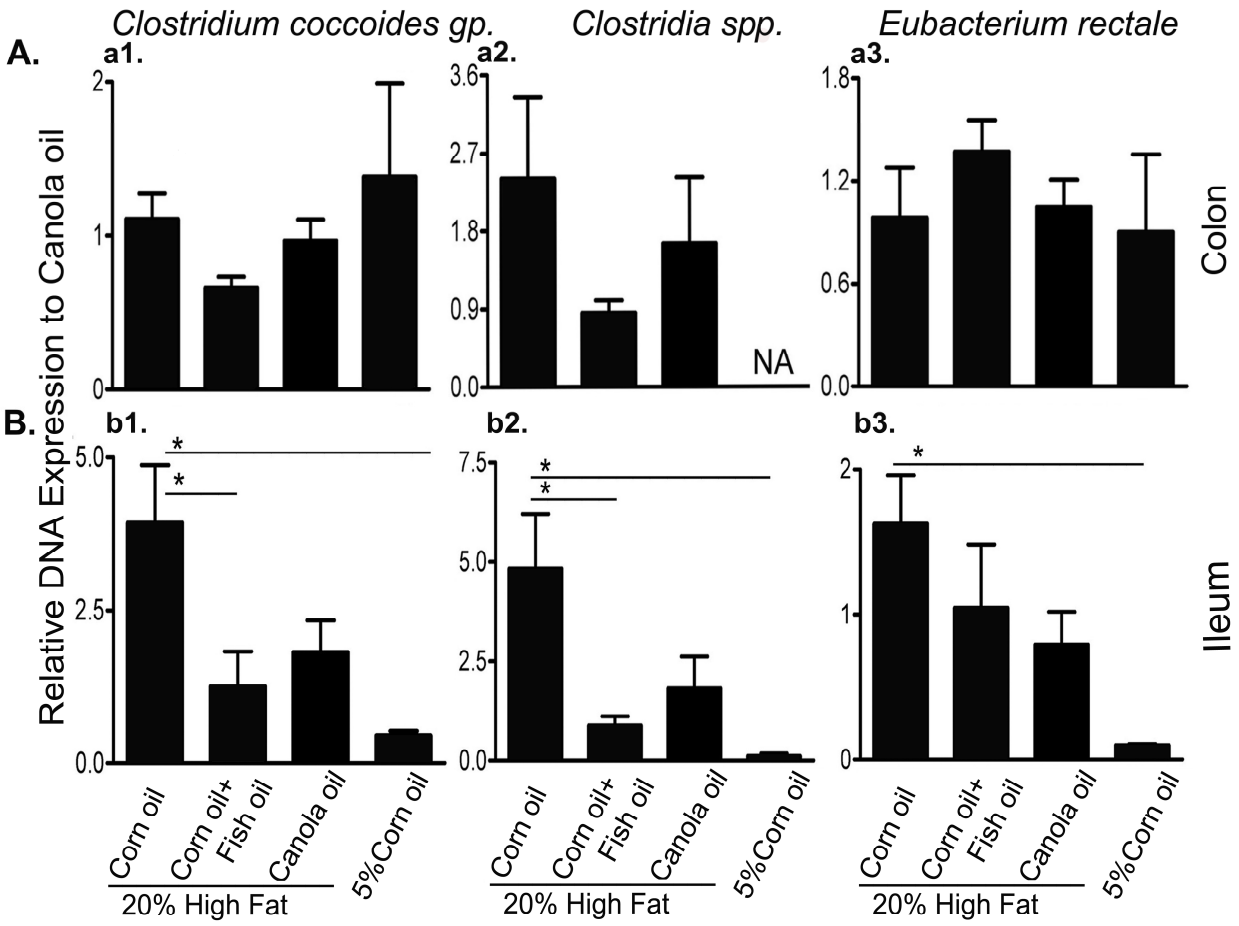

Figure 1: Dietary corn oil enriches *Clostridium coccoides gp.* and *Clostridia spp.* in the ileum of the gut.

A. Colons of mice fed with high fat diets (20% of corn oil, canola oil and 19 % corn + 1% fish oil) show a high level of *Clostridia spp.* when compared to 5% corn oil (panel a2). There was no amplification in 5% corn oil fed mice. There was no correlation between dietary differences in PUFA intake with the occurrence of *Eubacterium rectale* (panel a3). ND= not detected **B**. *Clostridium coccoides gp*. and *Clostridia spp.* were significantly higher in the ileum of mice fed diets rich in corn oil when compared to corn + fish oil and 5% corn oil [panel b1 & b2 (*p< 0.05)], and *Eubacterium rectale* was not significantly higher in mice fed high fat diets and low fat diet (panel b3). Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice.

3.2 Fish oil supplementation increases *Lactobacillus spp.* in the colon.

We analyzed several important immune modulating microbes from the Firmicutes phyla. Segmented Filamentous Bacteria (SFB) is gram positive and non-culturable bacteria of the gut microbiota. SFB is important in T helper 17 cell maturation (Ivanov et al., 2008; Umesaki et al., 1999) and high levels of Th17 cells are infiltrated in IBD patient's intestines (Monteleone et al., 2012). Therefore, we have studied how different dietary PUFAs affect SFB levels in the gut. Normally, SFB are abundant in the terminal ileum of GI tract (Ivanov et al., 2009). Our results indicated that mice fed a diet rich in corn oil appear to have increased SFB in the colon when compared to mice fed a diet of 5% corn oil (low fat or normal chow, Figure 2A, panel a1), Although the difference was not significant (ANOVA, df=3, F=1.593, p> 0.2242; Tukey's post-hoc, p> 0.05; Figure 2A, panel a1). Fish oil supplementation to corn oil diets was able to reverse this trend showing similar levels of SFB as compared to normal chow (Figure 2A, panel a1]. While there was no difference in SFB in the ileum of mice fed with high fat diets and low fat diet (ANOVA, df=3, F=1.021, p> 0.4096; Tukey's post-hoc, p> 0.05; Figure 2B, panel b1). The *Bacillus spp*. is a gram positive, spore producing, and facultative anaerobe or obligate aerobe of gut microbiota. Anti-microbial peptides from the *Bacillus spp.* have a probiotic activity against GALT maturation (Abriouel et al., 2011; Hamdache et al., 2011). Due to the known beneficial properties of *Bacillus spp.,* we examined the effect of the high fat diets on changing their quantity in the gut. There was no significant impact of *Bacillus spp.* in the colon (ANOVA, df=3, F=2.587, p> 0.0832; Tukey's post-hoc, p> 0.05; Figure 2A, panel a2) or in the ileum of high fat and low fat fed mice (ANOVA, df=3, F=2.293, p> 0.117; Tukey's post-hoc, p> 0.05; Figure 2B, panel b2). The *Lactobacillus spp.* is gram positive and a facultative anaerobe, and has many beneficial probiotic properties (Foye et al., 2012; Ohashi et al., 2009), like improving the

mucosal barrier function (Hamer et al., 2008; Ivanov et al., 2009; Schlee et al., 2008). In our results, *Lactobacillus spp.* was found to be enriched in the colons of mice fed with corn + fish oil compared to the colons of mice fed with corn oil (ANOVA, df=3, F=6.095,**p< 0.0044; Tukey's post-hoc, *p< 0.05; Figure 2A, panel a3), canola oil (ANOVA, df=3, F=6.095,**p< 0.0044; Tukey's post-hoc, *p< 0.05; Figure 2A, panel a3], and 5% corn oil (ANOVA, df=3, F=6.095,**p< 0.0044; Tukey's post-hoc, *p< 0.05; Figure 2A, panel a3]. There was no significant change of *Lactobacillus* in the ileum. These results suggest that fish oil supplementation may modulates the populations of several important immune-modulating microbes by depleting TH17 inducing microbes known to promote colitis (SFB) and enriching protective microbes (*Lactobacillus*) in the colon. This suggests that fish oil supplementation may have an effect on the inflammatory status of the host through the microbes.

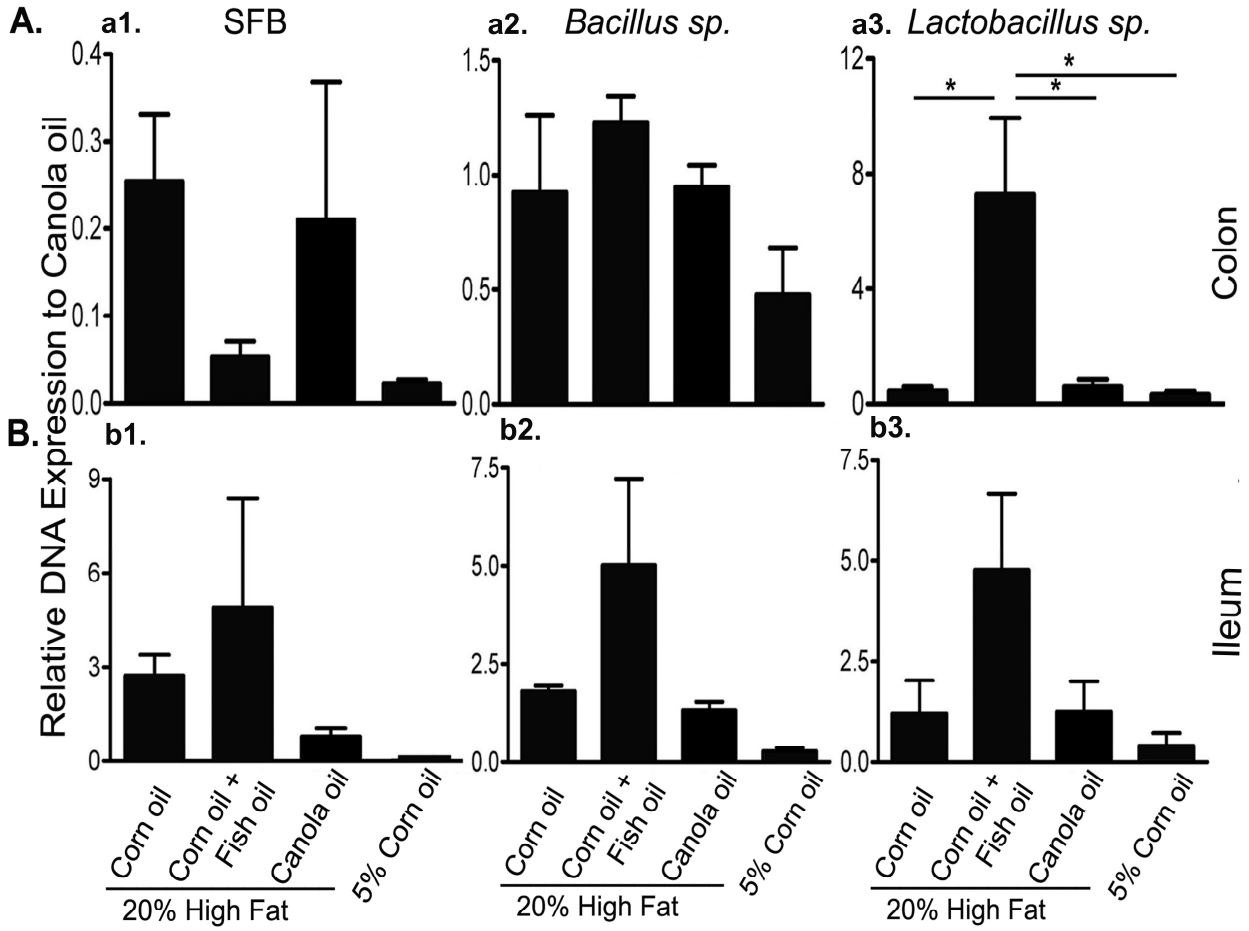

Figure 2: Immune-modulating Firmicutes are altered in the gut as a result of fish oil supplementation to corn oil diets fed to mice.

A.SFB levels trend was higher in the colons of corn oil rich diet fed mice compared to corn + fish oil fed mice (panel a1) as well as compared to the colons of 5% corn oil fed mice (panel a1) There was no significant impact of *Bacillus spp.* high fat fed mice and low fat mice colon (panel a2). Mice fed with corn oil+ fish oil show higher levels of *Lactobacillus spp.* proportions [panel a3 (*p< 0.05)] when compared to corn oil, canola oil and 5% corn oil fed mice. **B.** SFB levels were not significantly higher in the ileum of mice fed with high fat diets rich and low fat diet (panel b1). *Bacillus spp.* levels trends similar like SFB and were not significant among high fat and low fat. For *Lactobacillus* species trend in the ileum is similar to colon but not significant (panel b2). Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice.

3.3 High fat diets, regardless of type, decrease *Bacteroides* species

Bacteroidetes species are the second dominating phylum of gut microbiota and in general are considered beneficial. These species are gram negative anaerobes and a reduction of Bacteroidetes species has been observed in IBD patients (Frank et al., 2007). All high fat diets (corn oil, corn oil + fish oil and canola oil) decreased the appearance of *Bacteroides spp.* in the gut. This was expected because high fat diets and conditions like obesity have been linked to reduction in this group of bacteria (Hildebrandt et al., 2009; Mozes et al., 2008). All of our mice were obese with the most significant weight gain in the n6 PUFA group (data not shown). Interestingly, trends of *Bacteroides spp*. was the same in the ileum and in the colon. The colons of mice fed with all high fat show significantly low levels of *Bacteroides spp*. when compared to 5% corn oil fed mice (ANOVA, df=3, F=22.79,***p< 0.0001; Tukey's post-hoc ***p< 0.001; Figure 3A, panel a1], as well as in the ileum (ANOVA, df=3, F=15.53,**p< 0.0001; Tukey's post-hoc ***p< 0.001; Figure 3B, panel b1]. In addition, *Bacteroides fragilis,* another species from the Bacteroidetes phylum associated with colon cancer (Sears, 2009; Sinkovics, 2012). Our microbiota results showed that *Bacteroides fragilis* were not significant impact of all high fat diets in the colon (ANOVA, df=3, F=0.4548, p> 0.07184; Tukey's post-hoc, p> 0.05; Figure 3A, panel a2 and only detected in two mice of 5% corn oil group). In addition, in the ileum of all high fat fed mice shown similar trend of *Bacteroides fragilis* (ANOVA, df=3, F=0.7392, p> 0.5450; Tukey's post-hoc, p> 0.05; Figure 3B, panel b2) as same as colon but in the 5% corn oil group, where none was amplified (ND= not detected). Therefore, while all high fat diets promoted the growth of this microbe, the composition of the fat had no effects.

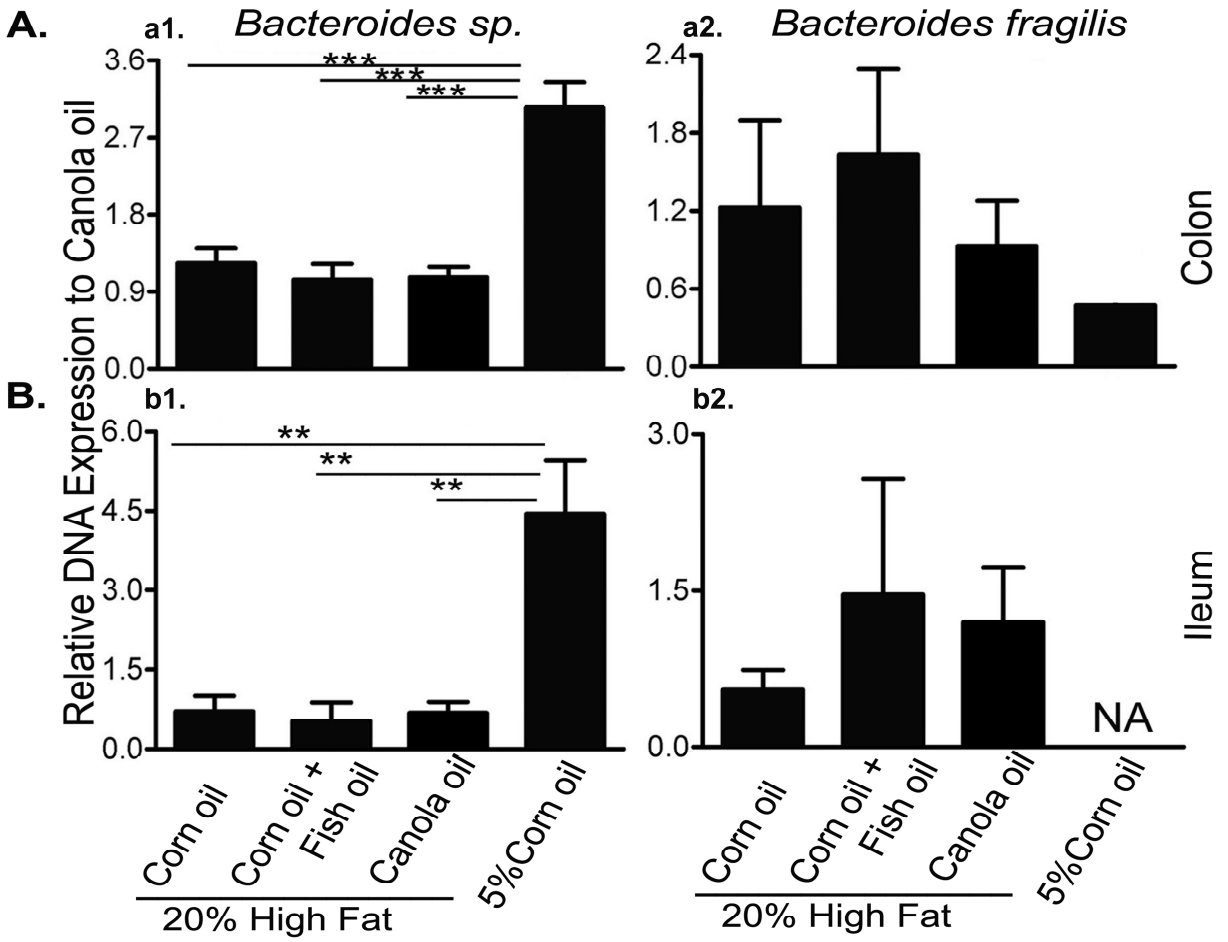

Figure 3: All high fat diets, regardless of type, decrease *Bacteroides spp.* in the gut.
A. *Bacteroides spp*. in the colons of mice fed an all 20 % high fat diet (corn oil, corn +fish oil, canola oil) were significantly lower when compared to the colons of mice fed 5% corn oil [panel a1 (***$p < 0.001$)]. Similarly, *Bacteroides fragilis* were not significant impact in the ileum of all high 20% fat diet fed mice but this species was only detected in two mice of the 5% corn oil group [panel a2] **B.** Similar to the colon, the ileum of mice fed an all 20% high fat diet (corn oil, corn +fish oil, canola oil) were significantly lower the *Bacteroides spp*. compared to 5% corn oil [panel b1 (*** $p < 0.001$)] fed mice. In addition, differences in *Bacteroides fragilis* were observed in mice fed high fat diet group, compared to mice fed a 5% corn oil diet [panel b2]. Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice. ND = not detected.

3.4 Dietary corn oil increases Enterobacteriaceae and fish oil reverses this trend

Most of the gram negative pathogenic bacteria belong to the Enterobacteriaceae family (Zheng et al., 2008). This species is found in elevated levels in mucosa lesions of GI inflammatory disease (Lupp et al., 2007; Seksik et al., 2003). Therefore, we investigated the Enterobacteriaceae in our microbiota analysis and showed that corn oil (rich in n6 PUFA) fed mice colons had significantly higher levels of this bacteria compared to corn + fish oil fed mice (ANOVA, df=3, F=5.028,**p< 0.0105; Tukey's post-hoc,*p< 0.05; Figure 4A] and compared to 5% corn oil fed group (ANOVA, df=3, F=5.028,**p< 0.0105; Tukey's post-hoc,*p< 0.05; Figure 4A]. The addition of fish oil to the corn oil diet reverses this effect. However, Enterobacteriaceae was not abundant in the ileum of all high fat as well as 5% corn oil fed mice (Figure 4B). Our microbiota analysis shows that dietary corn oil (n6 PUFA) enriches Enterobacteriaceae in the colon.

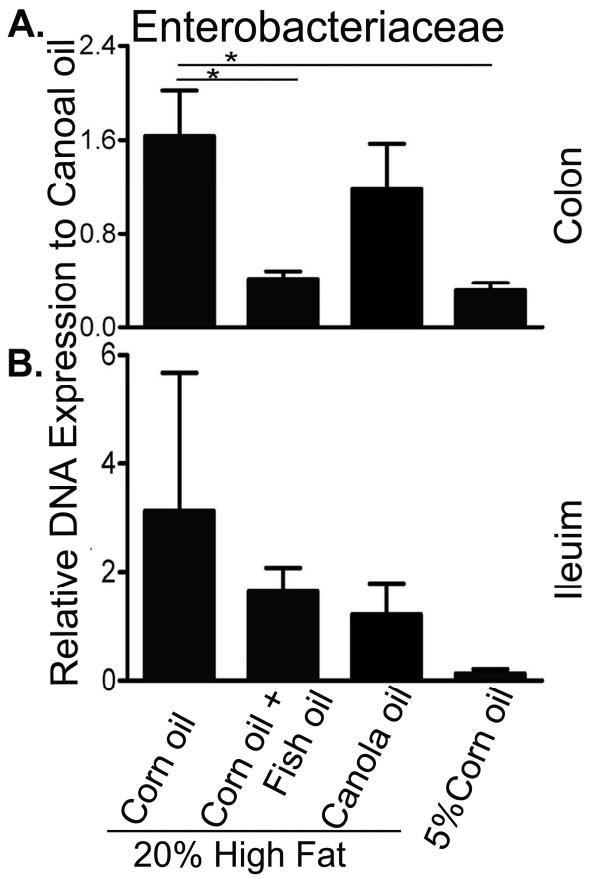

Figure 4: Dietary corn oil increases Enterobacteriaceae in the colon.
Elevated levels of Enterobacteriaceae were observed in the colon of rich corn oil diet fed mice when compared to corn + fish oil fed mice (*p<0.05; panel A), as well as mice fed with 5% corn oil (*p< 0.05; panel A)**.** All high fat diets and low fat diets did not alter the Enterobacteriaceae in the ileum (panel B). Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice.

3.5 All high fat rich diets fed to mice enriches beneficial *Bifidobacterium* species in the gut

The *Bifidobacterium* species is a gram positive anaerobe. There is overwhelming evidence in literature that *Bifidobacterium* is beneficial (Ivanov et al., 2006; Jeon et al., 2012; Philippe et al., 2011; Veiga et al., 2010). Hence, we have examined the *Bifidobacterium* species in our microbiota analysis and we observed that high levels of this species were found in the colon (ANOVA, df=3, F=2.771, p< 0.0715; Tukey's post-hoc, p> 0.05; Figure 5A) as well as in the ileum of high fat fed mice compared to low fat fed mice (ANOVA, df=3, F=3.9058,*p< 0.0461; Tukey's post-hoc, p> 0.05; Figure 5B). Interestingly, this species was not detected in any of the mice in the 5% corn oil group (ND = not detected).

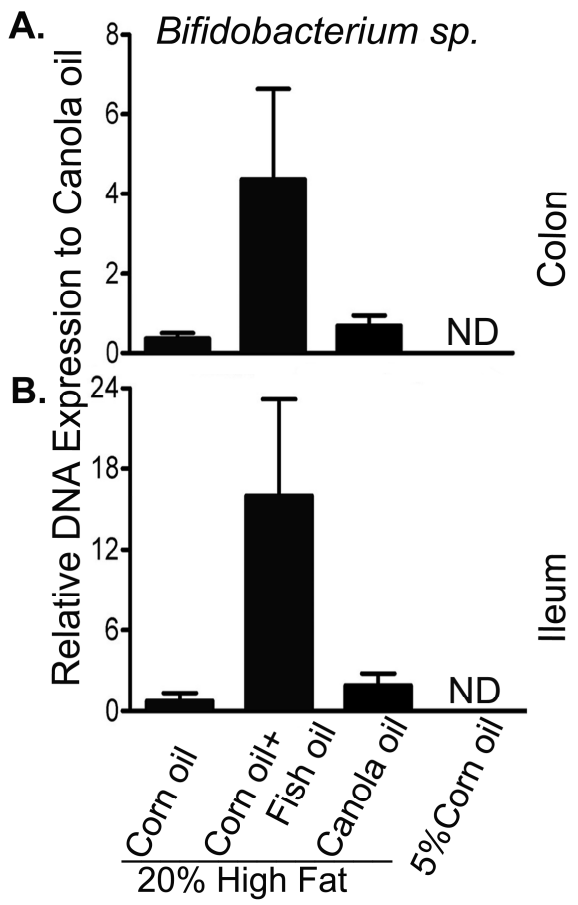

Figure 5: All high fat diets enriches the beneficial *Bifidobacterium spp*. in the gut.
All high fat (20% of corn oil, corn + fish oil and canola oil) fed mice showed higher level of *Bifidobacterium spp*. in the colons (panel A) as well as in the ileum (panel B) when compared to the colon, the ileum of 5% corn oil fed mice. This species was not detected in 5% corn oil fed mice. Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice. ND = not detected.

3.6 High fat diets do not significantly alter the levels of *Enterococcus* species in the gut

We analyzed *Enterococcus faecium* and *Enterococcus faecalis* of *Enterococcus* species are opportunistic pathogens, and associated with a variety of human infections (Jett et al., 1994). Our microbiota analysis of *Enterococcus faecium* (ANOVA, df=3, F=1.991, p> 0.1495; Tukey's post-hoc, p> 0.05; Figure 6A, panel a1) and *Enterococcus faecalis* (ANOVA, df=3, F=1.108, p> 0.3705; Tukey's post-hoc, p> 0.05; Figure 6A, panel a2) were not significant impact in the colon as well as in the ileum of all high fat fed mice (*Enterococcus faecium*-ANOVA, df=3, F=0.4073, p> 0.7498; Tukey's post-hoc, p> 0.05; *Enterococcus faecalis*- ANOVA, df=3, F=0.5703, p> 0.6426; Tukey's post-hoc, p> 0.05: Figure 6B, panel b1 & b2). Interestingly, *Enterococcus faecalis* levels are shown to be similar across the colons of all high fat fed mice (Figure 6A, panel a2). Thus, all high fat diets (corn oil, corn + fish oil, and canola oil) and their composition are not a major influence on *Enterococcus* species in the gut.

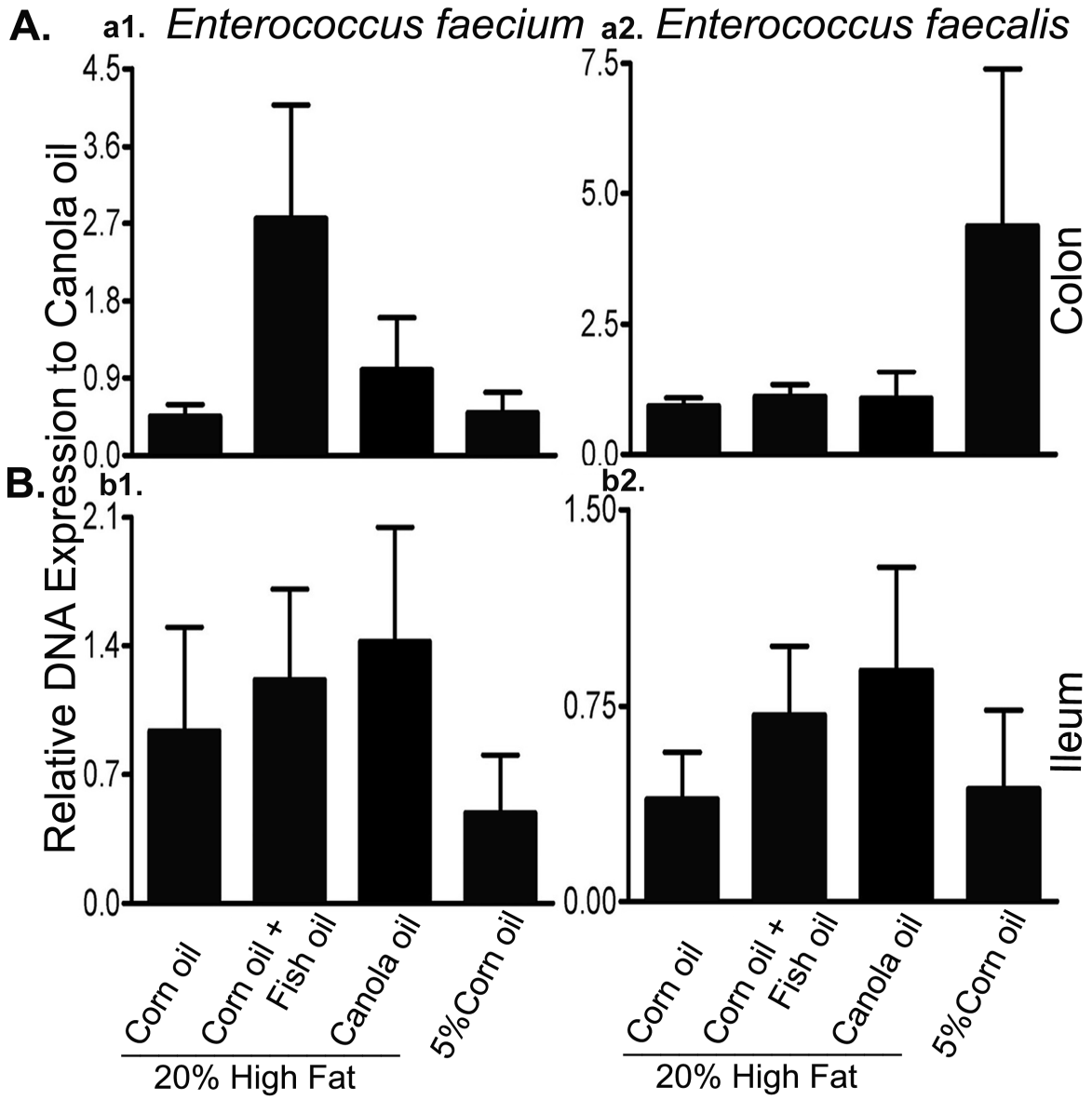

Figure 6: High fat diets have no impact on the *Enterococcus* species in the gut.

A. Colons of all 20% high fat diet (corn oil, corn + fish oil and canola oil) fed mice show no significant changes of *Enterococcus faecium* and *Enterococcus faecalis*. **B.** No significant changes of *Enterococcus* species were observed in the ileum of mice fed with 20% high fat diets (corn oil, corn + fish oil and canola oil). Tukey's multiple comparison test was performed to test the significance and each group had 5 to 6 mice.

3.7 Dietary fish oil normalize the macrophages similar to low fat in the colon

We decided to focus on the host inflammation responses in the colon because our lab research is mainly focusing on IBD. We using a mouse model of colitis which induces inflammation and oxidative stress in the colons of mice when they are infected with the bacterial pathogen, *C. rodentium*. Additionally, we examined the redox status in the colon part of the GI tract because colon is abundant in anaerobic microbial populations.

Macrophages and neutrophils are first line immune cells and the study of their presence in the submucosa and lamina propria of the colon may reveal the immune status of the gut. Our immunofluorescence results suggested that dietary corn oil reduced levels of tissue resident macrophages in the colon while fish oil supplementation can normalize this effect similar to normal chow (ANOVA, df=3, F=4.342, *p< 0.0341; Tukey's post-hoc, *p< 0.05; Figure 7, A&B). In contrast, while corn and canola oil diets did not alter neutrophil recruitment, compared to the normal chow group, but fish oil supplementation appears to have reduced level of neutrophil infiltration into the colon but not statistically significant (ANOVA, df=3, F=2.027, p> 0.1483; Tukey's post-hoc, p> 0.05; Figure 8, A&B). Our results suggested that dietary fish oil supplementation into corn oil normalize the tissue resident macrophages similar to 5% corn oil but corn oil alone reversed this effect.

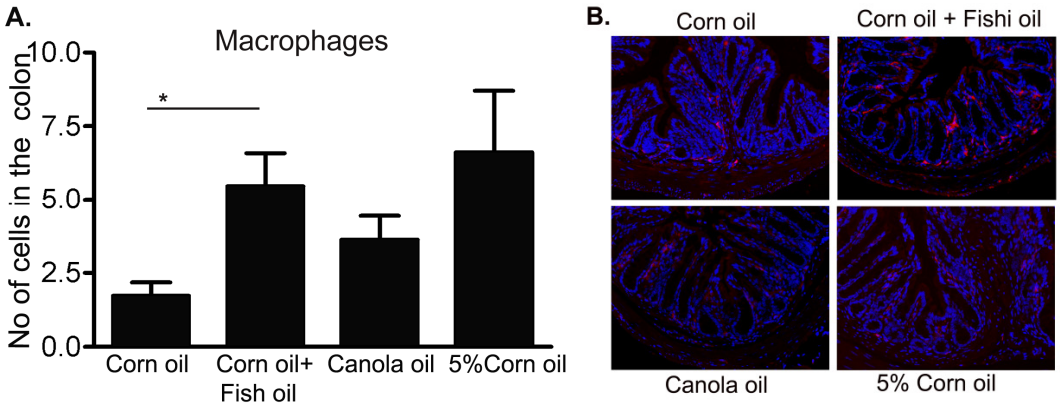

Figure 7: Dietary fish oil normalizes the macrophages similar to 5% corn oil in the colon. All high fat diets lowered the level of macrophages in the colon compared to 5% corn oil. A rich in corn oil fed mice colon macrophages were substantially less in number compared to corn + fish oil (*p<0.05) which had similar level of macrophages compared to 5% corn oil. Panel A, Macrophage counts were made by counting F4/80 positive cells in the submucosa and lamina propria regions of the colon for each tissue section and Panel B, Represents immunofluorescence images of respective diets group. Positively stained macrophages cells were counted manually at 400X time magnification under the microsecope, on entire region of each colon tissue section of each mouse (each group had 5 to 6 mice) and then averaged the cells count values of each mouse were used to make the graphs. Tukey's multiple comparison test was performed to test the significance.

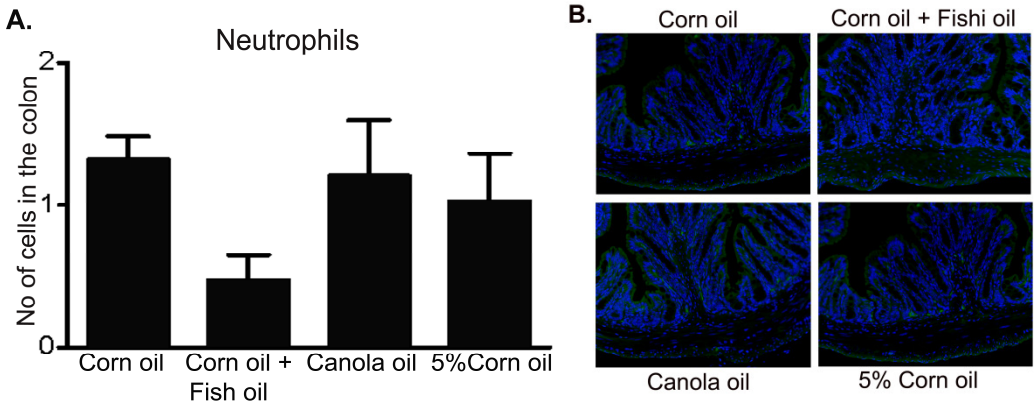

Figure 8: Corn, canola and 5% corn oil recruit similar level of neutrophils in the colon. Colon of mice fed with fish oil added to corn oil diet appear to have reduced the number of neutrophils compared to diet rich in corn oil and canola oil fed mice colon which had similar levels of netrophils as in 5% corn oil fed mice (panel A), Neutrophils counts were made by counting MPO positive cells in the submucosal region of the colon for each tissue section and Panel B, Represents immunofluorescence images of respective diets group. Positively stained macrophages cells were counted manually on 5-7 region of each colon tissue section of each mouse (each group had 5 to 6 mice) and then averaged the cells numbers of each section. These averaged values of each mouse were used to make the graphs.

3.8 All high fat diets induce epithelial cell death

Measurements of mucosal cell death may demonstrate the influence of high fat diets in mucosal homeostasis in two distinctive aspects, such as epithelial integrity and oxidative stress. We measured cell death in the crypts region of the colon (Gibson et al., 2008) by using Terminal deoxynucleotidyl transferase dUTP nick end labeling assay TUNEL staining and our results revealed that all high fat diets significantly induced epithelial cell death compared to 5% corn oil (ANOVA, df=3, F=9.071, **p< 0.0014; corn oil, corn + fish oil vs. 5% corn oil, *p< 0.05-tukey's post-hoc; Figure 9) and canola oil vs 5% corn oil, tukey's post-hoc *p<0.05; Figure 9). Therefore, each high fat diet (corn oil, corn + fish oil and canola oil) induced apoptosis in the epithelium.

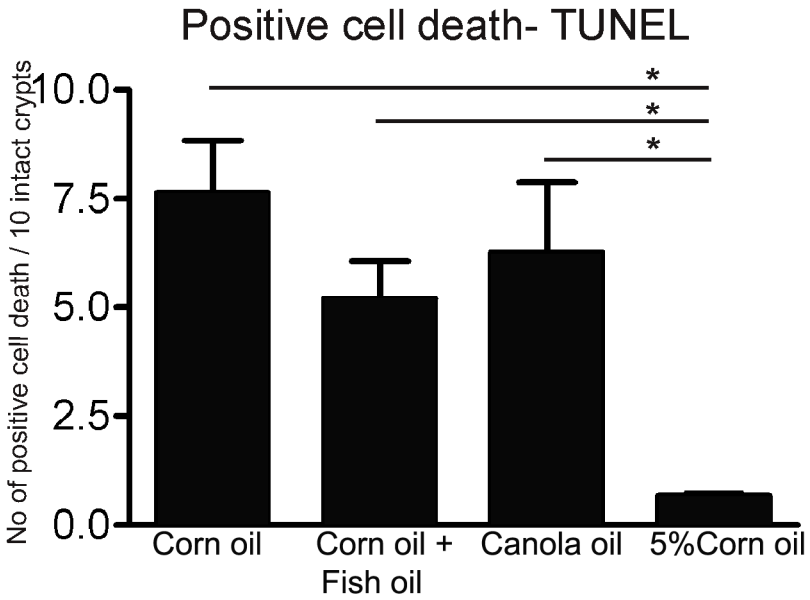

Figure 9: All high fats induce epithelial cell death of the colon epithelium.
Colon of all 20 % high fat diets fed mice (corn oil, corn + fish oil, and canola oil) substantially induced epithelial cell death compared to the 5% corn oil fed mice (corn oil, corn + fish oil vs. 5% corn oil,*p<0.05; canola oil vs. 5% corn oil, *p<0.05). Positively stained apoptotic cells were counted manually in 10 intact crypts of each mouse tissue section (each group had 5 to 6 mice) and then averaged the positive cells number values of each mouse were used to make the graphs (Gibson et al., 2008). Tukey's multiple comparison test was performed to test the significance.

3.9 Supplementation with fish oil to corn oil rich diets increases HNE production, as an oxidative stress marker

HNE is the lipid oxidation product of n6 PUFA (Long et al., 2010). We examined this because HNE is an oxidative marker and our high fat diets were composed of varied proportions of n6 PUFA and n3 PUFA (Table 1). HNE adducts react with proteins and peptides to produce a stable compound. Measuring stable oxidized adducts is the best method to determine an oxidative status in tissues (Long et al., 2010; Spickett et al., 2010). Higher proportions of n3 PUFA were present in two diet groups; canola oil and corn + fish oil diet has 1.5 % wt/wt, 0.57 % wt/wt of n3 PUFAs respectively (Table 1). Using immunofluorescence we analyzed the entire region of each colon tissue section of each mouse (each group had 5 to 6 mice) and found that the canola oil and fish oil fed groups produced high levels of HNE in the sub mucosa region whereas corn oil and 5% corn oil had lower levels of HNE staining (Figure 10). This suggests that n3 PUFA increases oxidative responses in the colon.

3.10 Supplementation with fish oil to corn oil rich diets increases dual NADPH oxidase in the blood vessels in the submucosa

Dual NADPH oxidase is involved in generation of H_2O_2 (Rada et al., 2008; Rokutan et al., 2008), this enzyme staining was qualitatively observed with immunofluorescence technique in the entire region of each colon tissue section of each mouse (each group had 5 to 6 mice). In our results, fish oil added to corn oil showed the highest levels of DUOX2 expression in blood vessel present in the submocosa compared to (Figure 11) corn oil, canola oil and 5% corn oil fed mice colons which have lower level staining of DUOX2 in the sub mucosa regions. This suggests that fish oil may have the role of increase H_2O_2 synthesis through NADPH oxidase activity. Thus n3 PUFA increases oxidative responses in the colon.

3.11 Supplementation of fish oil to corn oil rich diets reduces catalase expression in the colon

Catalase is an enzyme involved in the conversion of harmful H_2O_2 into water (Gaetani et al., 1996; Mueller et al., 1997). This enzyme expression was measured with immunofluorescence staining in the colon and we found decreased level of staining in the two diet groups that have n3 PUFA; canola oil and corn + fish oil diet has 1.5 % wt/wt, 0.57 % wt/wt of n3 PUFAs respectively (Table 1). we observed qualitatively in the entire colon tissue section of each mouse (each group had 5 to 6 mice) and found that the fish oil added to corn oil and canola oil diet resulted in the lowest levels of catalase immunostaining in the muscularis mucosa and circular muscle of the colon (Figure 12). High levels of positive catalase staining were observed in the colons of mice fed with rich corn oil diets and 5% corn oil. This suggests that diets rich in n3 PUFA (corn + fish oil) diminish the expression level of the enzyme catalase and decreases responses that protect against oxidative responses in the colon.

3.12 Expression of an antioxidant glutathione peroxidase (GPX2) in the epithelium was unaffected by the diet

GPX2 is an antioxidant enzyme involved in the conversion of harmful H_2O_2 into water as well as scavenging free radicals (Chu et al., 1995; Toppo et al., 2009) . This enzyme is unique to the GI tract, where it has been reported to be highly expressed in the epithelium of the small intestine (Chu et al., 1995). In our results, we found no differences in the colon of mice fed the high fat diet and 5% corn oil (Figure 13). This suggests that diets rich in n3 PUFA or n6 PUFA do not alter the expression level of the GPX2 enzyme in the GI tracts.

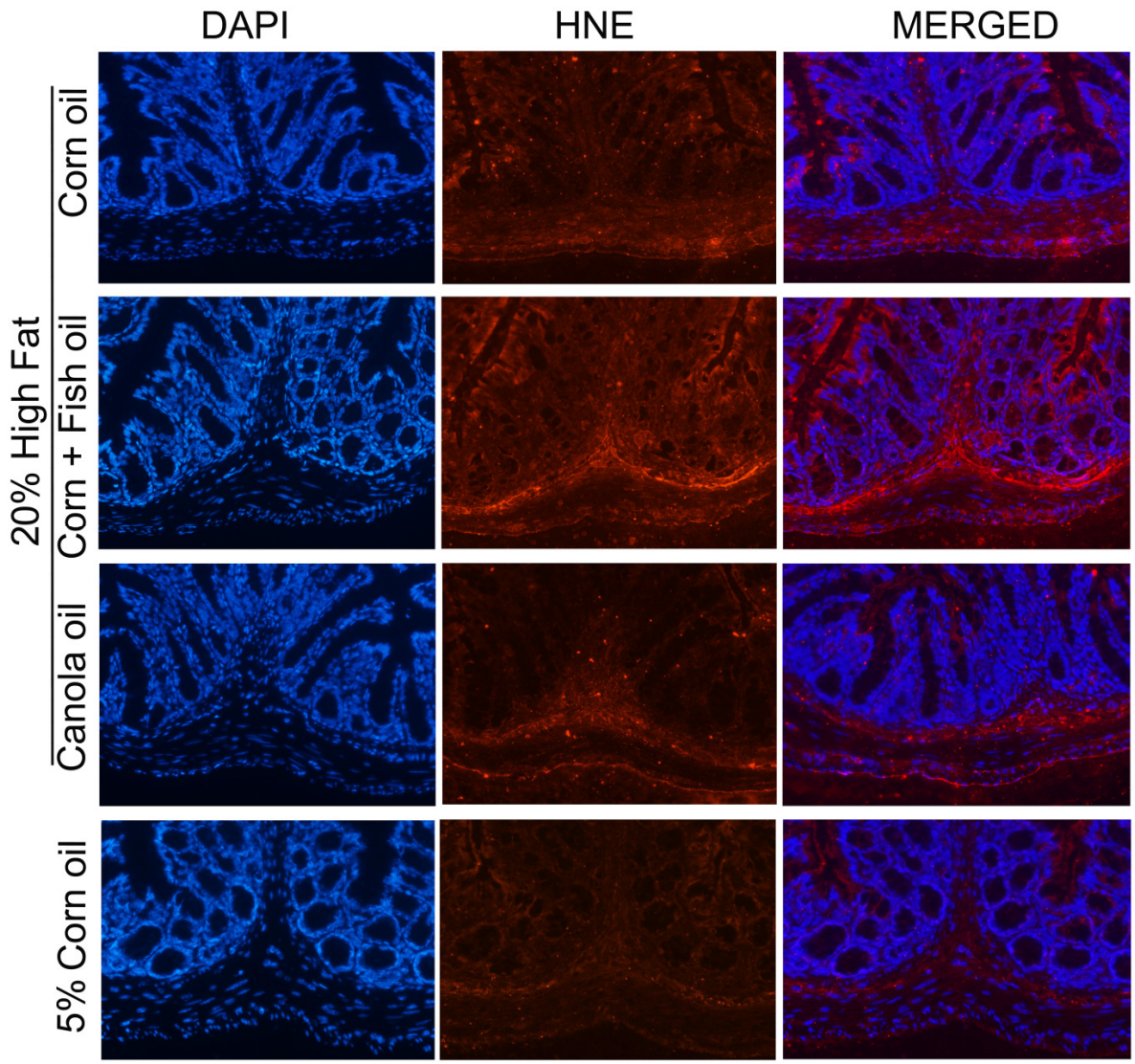

Figure 10: Canola oil and fish oil increase the generation of HNE in muscularis mucosa of the colon. Our HNE immunofluorescence results showed fish oil added in to corn oil (2nd row) and canola oil (3rd row) fed mice produced a high level of HNE in muscularis mucosa as well as in submucosa regions of the colon compared to corn oil (1st row). In addition, 5% corn oil mice showed low levels of HNE compared to all high PUFAs (4th row). We observed qualitatively HNE expression in the entire colon tissues section of each mouse (each group had 5 to 6 mice). Above immunofluorescence images in each row illustrates typical staining patterns in each treatment group. All above pictures were taken at 200x magnification.

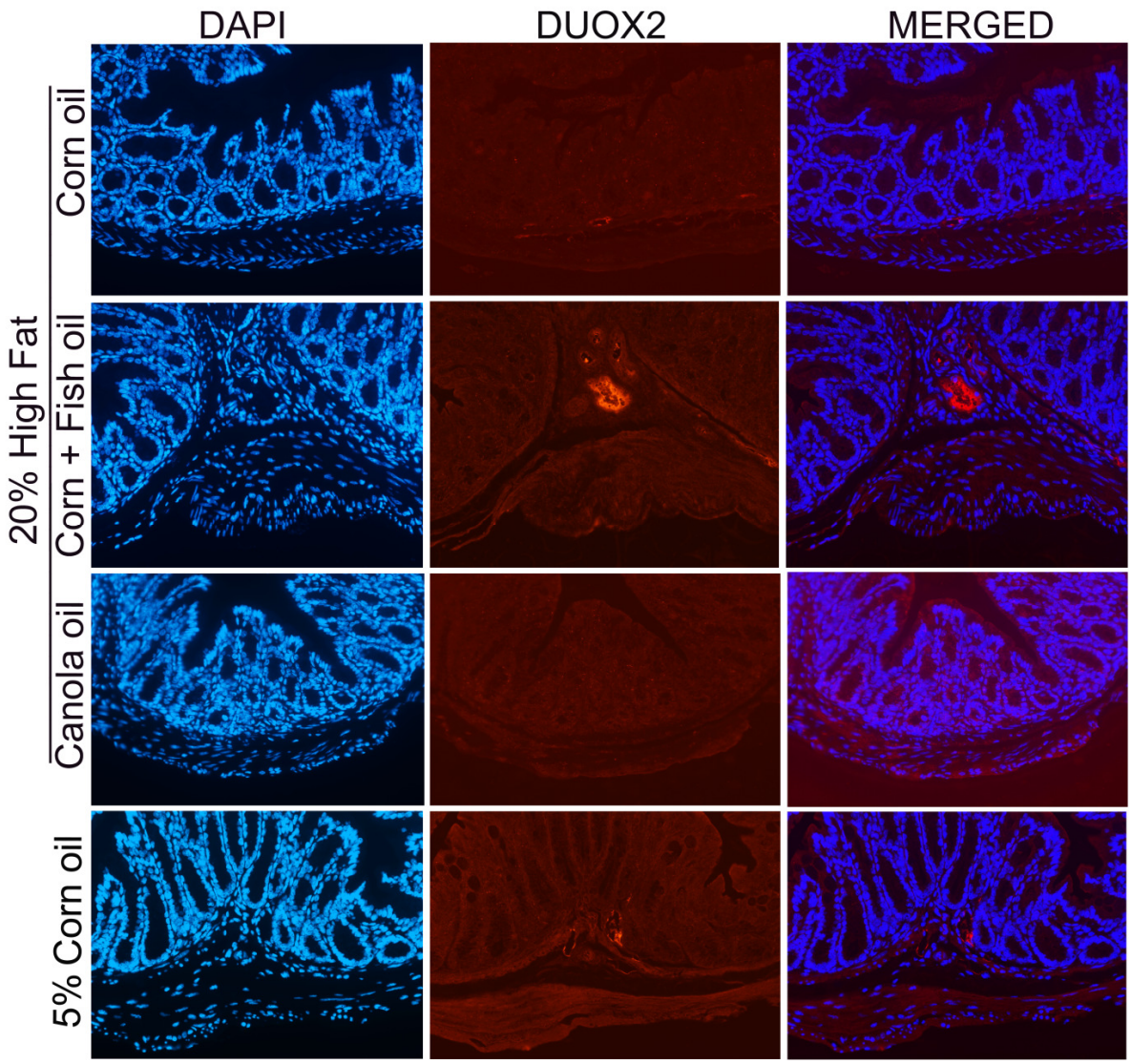

Figure 11: Fish oil increases DUOX2 enzyme expression which has the ability to increase the generation of hydrogen peroxides in vessels present in submucosa of the colon.
Our DUOX2 immunofluorescence result shows corn + fish oil (2nd row) fed mice have generated high levels of DUOX2 in blood vessels present in the submucosa of the colon. Mice fed with corn oil (1st row), canola oil (3rd row) and 5% corn oil (4th row) have expressed low or less of DUOX2 in the blood vessels in the sub mucosa region of the colon. We have observed manually DUOX2 expression in entire colon tissue sections of each mouse (each group had 5 to 6 mice). Above immunofluorescence images in each row represent respective diets groups. All above pictures were taken at 200x magnification.

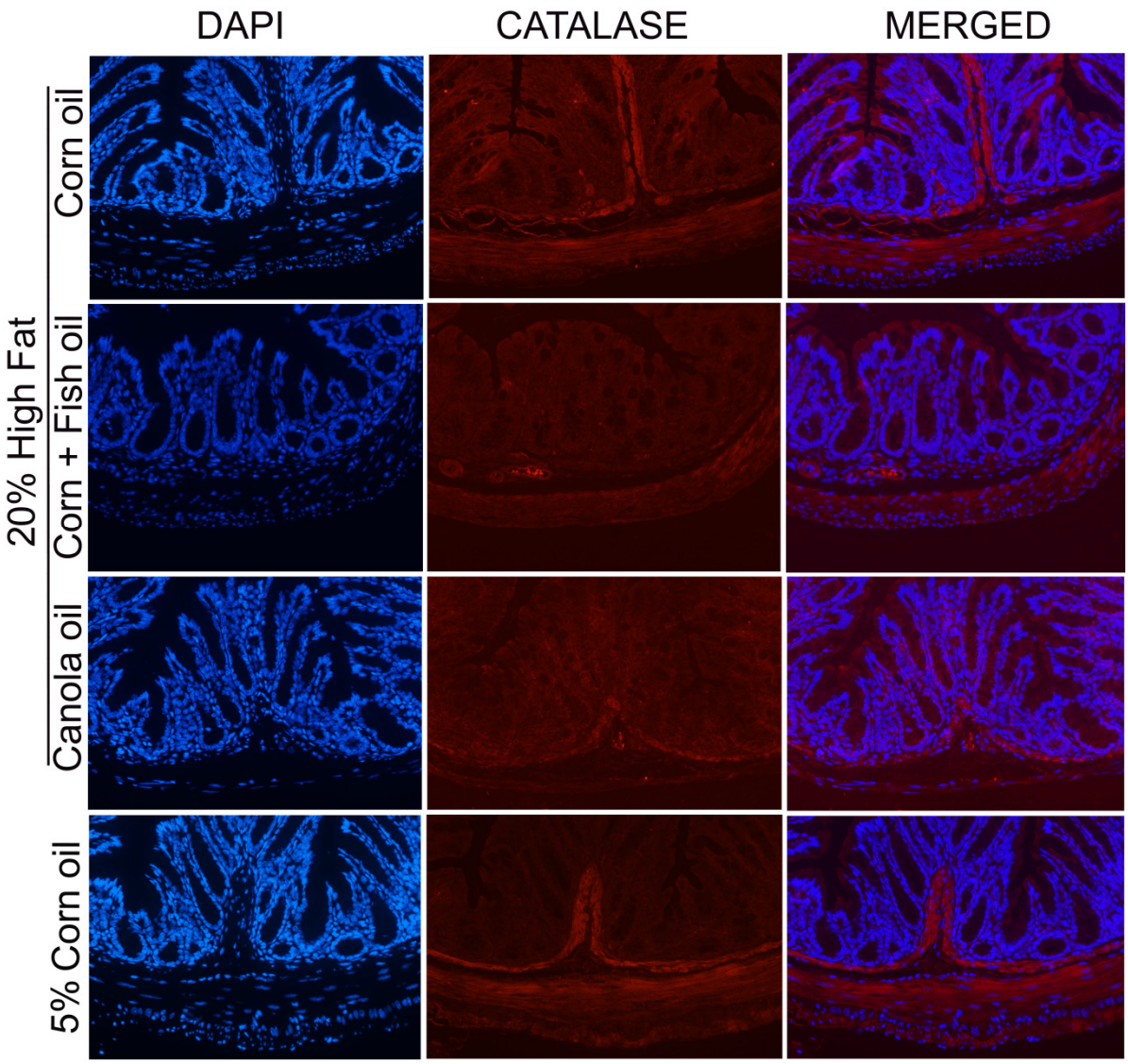

Figure 12: Fish oil minimizes the catalase expression in the colon.
Catalase immunofluorescence results in colon tissues suggest, fish oil added to corn oil shows low levels of catalase expression in the muscularis mucosa and circular muscle layer (2nd row); high levels of expression were observed in corn oil (1st row), 5% corn oil (4th row) and in canola oil (3rd row); relatively low levels of catalase activity were noticed compared to corn oil and 5% corn oil. We have observed manually catalase expression in entire colon tissue sections of each mouse (each group had 5 to 6 mice). Above immunofluorescence images in each row represent respective diets groups. All above pictures were taken at 200x magnification.

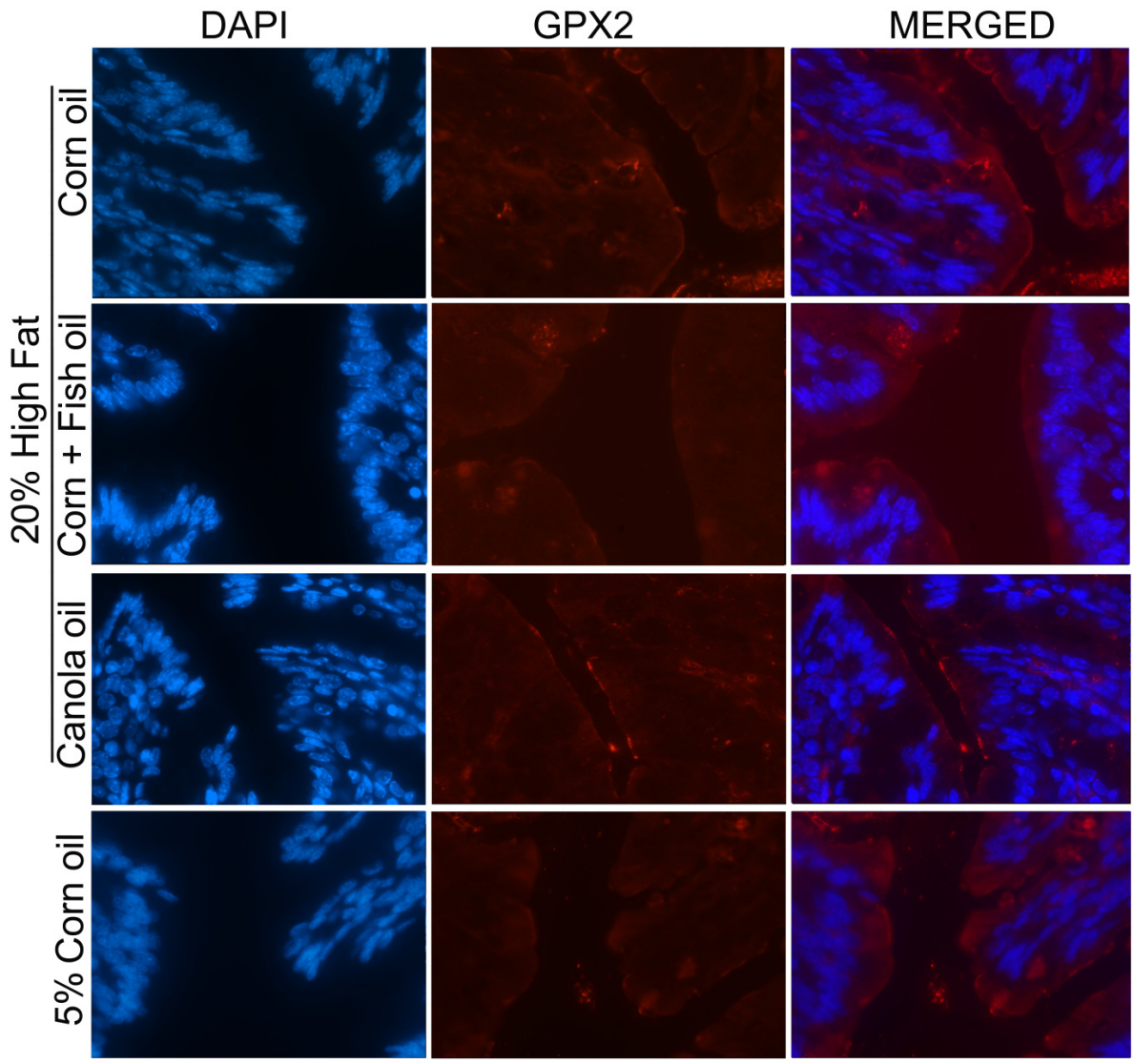

Figure 13: Regardless of type of fats, no changes in the expression of GPX2 in epithelium of the colon. Immunofluorescence of GPX2 of colon tissues shows there is no different in the expression of GPX2 in the colon epithelial cells [corn oil (1st row), corn oil+ fish oil (2nd row), 20% canola oil (3rd row), and 5% corn oil (4th row)]. We have observed manually GPX2 expression in entire colon tissue sections of each mouse (each group had 5 to 6 mice). Above immunofluorescence images in each row represent respective diet groups. All above pictures were taken at 600x magnification.

3.13 Summary of results

Our study demonstrated that fish oil (n3 PUFA) added into corn oil (n6 PUFA) enhanced the presence of beneficial microbes *Lactobacillus spp.* It also reduced harmful pathobionts such as Enterobacteriaceae, *Clostridium coccoides gp.,* and *Clostridia spp.* which was found in elevated levels in rich corn oil fed mice. We unexpectedly found that fish oil induced higher oxidative stress in the colon as evidenced by increased HNE and DUOX2 immunofluorescence staining and by reduced levels of catalase staining.

4 Chapter: Discussion

This is the first study to observe that the gut microbiota changes in response to dietary PUFAs. In our study we found that rich in corn oil diets (n6 PUFA) enrich the opportunistic pathogens such as Enterobacteriaceae, *Clostridium coccoides gp,* and *Clostridia spp.* in the GI tract. Meanwhile fish oil supplemented to corn oil reverses these patterns and also enriches beneficial microbe *Lactobacillus*. All high fat diets, regardless of the compositions, reduce the *Bacteroides* species and induce epithelial cell death. Dietary corn oil reduces the level of tissue-resident macrophages, and fish oil addition normalizes these levels similar to the normal chow group. Fish oil supplemented diets appear to reduced neutrophil infiltration. Finally, fish oil supplementations unexpectedly induce oxidative-stress, as shown by increased 4-HNE expression, dual oxidase 2 (DUOX2) NADPH oxidase expression and decreased catalase expression. Overall, our results suggest that there are differential effects of n6 and n3 PUFAs on the intestinal microbial ecology, where n6 PUFA enriches detrimental microbes and n3 PUFA enrich beneficial microbes. While we have specifically addressed how these microbial differences alter the host directly, we did find that there were also differential effects of n6 and n3 PUFAs on the host whereby n3 PUFA seems to induce oxidative stress in the colon.

The gut microbiota of Clostridium clusters IV and XIVa of Firmicutes promote production of short chain fatty acids in the colon (Louis et al., 2009; Louis et al., 2007). Especially butyrate is known to be essential for colon epithelial cells. It has been shown that butyrate may have anti-carcinogenic, anti-inflammatory properties (Hamer et al., 2008). In contrast, in rats, butyrate promotes hypersensitivity (Bourdu et al., 2005) and most of species of Clostridium cluster XIVa (phylum Firmicutes) are opportunistic pathogens or pathobionts (Lozupone et al., 2012). Bacteria such as *Clostridium coccoides gp, Clostridia spp.* and

Eubacterium rectale belong to Clostridium cluster XIVa of Firmicutes. These species are associated with pathogenesis of IBD (Duck et al., 2007) and IBS (Jeffery et al., 2011; Schoepfer et al., 2008). Similarly, *Clostridium difficile, C. perfringens*, *C.tentani*, *C. bolteae* and *C. symbiosum* are associated with systemic infection (Decousser et al., 2007). In addition, *C.clostridioforme* and *C. hathewayi* are involved in bacteremia and wound infection (Elsayed et al, 2004; Finegold et al., 2005). Furthermore, small intestine and mesenteric lymph nodes of IBD patients reportedly have subset species of Clostridium cluster XIVa. In addition, *C. bolteae* and *C. symbiosum* cause systemic infection through invading the gut mucosa (Decousser et al., 2007; Elsayed et al., 2004). We observed that *Clostridium coccoides gp.* and *Clostridia spp.* were enriched in the ileum of mice fed diets rich in corn oil (n6 PUFA), whereas fish oil supplemented with corn oil reduce the presence of each of these species. In the colon, we observed no significant changes in *Clostridium coccoides gp.* and *Eubacterium rectale* but the trend for *Clostridia spp.* was the same as in the ileum. Furthermore, we observed that canola oil has no significant role in the appearance of *Clostridium coccoides gp., Clostridia spp.* and *Eubacterium rectale* species in the gut. Another interesting observation of our study is that corn oil increases the Enterobacteriaceae in the colon. Surprisingly, the addition of fish oil into corn oil reverses the Enterobacteriaceae trend. Most pathogenic bacteria belong to the Enterobacteriaceae family. Bacterial cell walls, lipopolysaccharide (LPS), and peptidoglycan of pathogens are recognized by the pattern recognition receptors (PRRs) of the immune system in order to maintain the immune homeostasis in infection conditions. Furthermore, increased Enterobacteriaceae species have been observed during IBD, clinically and experimentally (Gophna et al., 2006; Lupp et al., 2007; Tjonneland et al., 2009). In addition, pathogenic microbes of Enterobacteriaceae such as *Salmonella spp*., *Sheigella spp*. and *E.coli,* cause enteric infection. Presence of endotoxin

systemic circulation is called endotoxemia and is commonly caused by gram negative bacteria of the Enterobacteriaceae (Hurley, 2009). These bacterial LPS bind to epithelial cells and activate NF-κ B resulting in production of pro-inflammatory cytokines and infiltration of macrophages and neutrophils in the infected area (Ogawa et al., 2008; Stecher et al., 2012). Interestingly, dietary corn oil (rich in n6 PUFA) is associated with an increased incidence of IBD (Tjonneland et al., 2009). We observed a high level of Enterobacteriaceae in the mice fed corn oil. Overall, corn oil diets are associated with increased pathobiont bacteria, which suggest that this diet may be associated with an increased risk to intestinal disease. In contrast, fish oil can reverse the presence of pathobionts.

SFB species is involved in Th17 immune responses associated with a mucosal surface. It has been shown that Th17 cells can be induced in the small intestines of germ free mice which have been colonized with SFB (Ivanov et al., 2009) or gram negative bacteria such as *C. rodentium* and *Kebsiella pneumoniea* (Happel et al., 2005). Others have shown that SFB protects against pathogens like *C. rodentium* by induction of Th17, IL-22 and epithelial production of bactericidal protein (Ivanov et al., 2009; Zheng et al., 2008). In addition, SFB induces MHC II molecules in the intestinal epithelium and selectively induces $CD4^+$ T cells that produce IL-22 and IL-17 (Ivanov et al., 2009; Umesaki et al., 1999). In our results, corn oil tends to increases the SFB species and addition of fish oil brought this species levels back to normal when compared to 5% corn oil (normal chow). Our SFB results provide evidence that corn oil promotes SFB in the gut, which could increase pathogenic immune responses in the gut in the context of IBD. However, this could also leave the colon more resistant to infection by pathogens like *C. rodentium*. Our lab is currently pursuing the effects of these microbial changes.

In this study, fish oil supplementation enriches beneficial *Lactobacillus* species in the gut. Interestingly, our *Lactobacillus* result resemble those of other studies which show an increase in species of *Lactobacillus* with n3 PUFA consumption and a decrease with n6 PUFA consumption (Pachikian et al., 2011; Ringo et al., 1998). This is important because *Lactobacillus spp.* are known to improve mucosal barrier function (Hamer et al., 2008; Ivanov et al., 2009; Schlee et al., 2008). For example, the human β-defensin 2 (hBD2) anti-microbial peptide is induced by *Lactobacillus* species such as *Lactobacillus acidophilus* PZ1138*, L. fermentum*, and *L paracasei* subsp. *paracasei* (Schlee et al., 2008). In addition, *L. plantrum, L. delburki* and *L. acidophilus* induce mucin secretions in the murine colonic epitheliaum (Caballero-Franco et al., 2007). Furthermore, these species modulates cytokine production, dendritic cells maturation, and also important for T cell maturation (Christensen et al., 2002). *Bifidobacterium* species also have well known beneficial properties. A reduced level of these species is observed in IBD, IBS, and other autoimmune diseases (Collado et al., 2007; Collado et al., 2009; Sokol et al., 2009). In addition, an extra-cellular protein, secreted by *Bifidobacterium*, promotes the GALT and mucosal barrier functions. Strains such as *Bifidobacterium longum* subsp. *longum* NCC2705, *Bifidobacterium breve*, *Bifidobacterium dentium*, and *Bifidobacterium longum* subsp. *infantis* produce serpin, an extra cellular protein which inhibits neutrophils and pancreatic elastase (Ivanov et al., 2006). Uncharacterized protein from probiotic cocktail of *Bifidobacterium* enhances epithelial tight junction proteins in order to maintain the barrier functions (Ewaschuk et al., 2008). Furthermore, *Bifidobacterium* play a role in the immune system development by producing extra cellular metabolites such as serpin which reduces the infiltration of neutrophils during conditions of inflammation, they also produce conjugated linoleic acid which activates peroxisome proliferator activated receptor (PPARs) (Benjamin et al., 2009; Coakley et al., 2003; Ewaschuk et al., 2008;

Ivanov et al., 2006; Marques et al., 2010). Administration of *Bifidobacterium* species, which induces IL-10, in animal colitis models, results in a reduction of inflammation (Jeon et al., 2012), epithelial damage, and pro-inflammatory markers (Philippe et al., 2011), shaping the colitogenic microbes which have ability to induces colitis (Veiga et al., 2010). In our results, all high fat diets enriched with beneficial Bifidobacteria compared to low fat (5%corn oil). An enrichment of beneficial microbes (*Lactobacillus* and *Bifidobacterium*) in fish oil supplemented to corn oil was associated with the reduction of inflammation may evident by the decreased level of infiltrated neutrophils. Neutrophils are one of the first inflammatory cell types to respond to inflammation.

In our study, fish oil + corn oil group had significantly increase macrophages similar to normal chow and corn oil group had lower macrophages compared to normal chow or fish+ corn oil group. In addition, a rich corn oil diet reduces the spleen lymphocyte function in rats (Kollmorgen et al., 1979). Diets rich in n6 PUFAs alter the membrane structure and signal of the immune cells in order to recruit low levels of macrophages in the colon (Calder, 2009; Stulnig et al., 2001). Macrophages subset M1 and M2 are important in tissues repair and injury. M1 (classically activated) are pro-inflammatory and M2 (alternatively activated) are anti-inflammatory (Kigerl et al., 2009). The ratio of M1/M2 has a vital role in cell repair and injury. Since there are pro-inflammatory and anti-inflammatory macrophages it is possible that the protective macrophages have been depleted in the corn oil group. The fish oil supplemented group had levels of macrophages similar to normal chow. It could be possible that fish oil increases anti-inflammatory macrophages. More work is required to determine if the M1 or M2 macrophage ratio was altered in the diet groups. CD86 (M1 macrophages) is expressed primarily on dendritic cells, resting monocytes and macrophages cells whereas CD206 (M2 macrophages) is a specific antibody also known as macrophages mannose receptor (MMR), which is expressed

during infection and inflammation conditions. Therefore, specific immunofluorescence staining with CD86 and CD206 will reveal the ratio of M1 and M2 macrophages.

The epithelial cell integrity mainly depends on cell death and cell regeneration in order to maintain mucosal homeostasis (Wong et al., 1999). Any defects in the GI epithelial cell generation leads to reduction in absorption, known as mucosal atrophy and higher new cell generation leads to in hyper secretion, known as hyperplasia which increases the risk of cancer (Rao et al., 2010). In addition, cell death is also considered as a marker for oxidative stress because highly generated free radicals attacking DNA resulted in DNA fragmentation (Rao et al., 2010; Timmons et al., 2012). In the present study, fish oil added to corn oil diet reduced the death of colon epithelial cells after *C.rodentium* infection (data not shown), reduced the intestinal crypt length (data not shown), and reduced the neutrophils and increases macrophage in the colon. Reduced level of epithelial cell death in fish oil fed mice suggests that fish oil alters epithelial cell generation during the course of infection and mechanism behind this alteration is not well understood. More studies are needed to establish the roles of fish oil on gut homeostasis.

Unexpectedly, we found that fish oil supplementation increases the oxidative status in the colon of the GI tract. In the context of oxidative stress, HNE is a reactive lipid peroxide product that adducts and affects the transduction signal and possesses a mutagenic property (Burcham, 1998). This product is generated by free radicals degrade the membrane components such as n6 PUFA and arachidonic acid which are the fatty acid component of the cell membrane (Burcham, 1998; Esterbauer et al., 1990). In our study, n3 PUFA was found in fish oil, canola oil and corn oil. Fish oil contains arachidonic acid, EPA and DHA, which may compete with n6 PUFA for the Δ desaturase enzyme to incorporate into the cell membrane. This incorporation led to higher HNE generation in the muscularis mucosa and submucosa of the colon.

Dual oxidase 2 (DUOX2) is NADPH oxidase protein which involves in generation of H_2O_2 as a maker for oxidative and inflammation conditions. H_2O_2 generation via dual oxidase (DUOX2) was noticed in the submucosa of the colons of mice fed with fish oil. This oxidase plays a role in killing microbes during infection. Lacking this enzyme is associated with more susceptibility to infection (Rada et al., 2008; Rokutan et al., 2008). Higher levels of H_2O_2 production may be due to a higher level generation of free radicals which is presumably involved in the production of H_2O_2 in the submucosa of the colon. In contrast, catalase is a peroxisome-specific marker protein of the catalase family which converts H_2O_2 to H_2O in oxidative and inflammation conditions. Catalase activity is important to decompose harmful H_2O_2 and water (Gaetani et al., 1996; Mueller et al., 1997). This enzyme expression at low levels was observed in the submucosa of the colon of mice fed with fish oil. Therefore, these observations suggest that fish oil enhances the production of HNE lipid peroxides production and increases H_2O_2 generation while lowering catalase antioxidant activity. Meanwhile, GPX2 is a homolog of selenium-containing antioxidant enzyme of glutathione family. This enzyme prevents the lipid per-oxidation of cell membrane, and reduces lipid hydroperoxides into alcohols, and reduces H_2O_2 to H_2O. The GPX and glutathione synthesis pathway has not been observed in obligate anaerobes such as *Bifidobacterium*, and catalase enzymes are present in aerobes and in facultative anaerobes but not in obligate anaerobes (Brioukhanov et al., 2004). In our experiment we have not seen any differences in the expression of GPX2 due to diet. Furthermore, Lactic acid bacteria (LAB) are sensitive to reactive oxygen species and lack the superoxide dismutase and catalase genes. But co-expression of these genes, from other species such as *sod A* from *Streptococcus thermophiles* and *kat A* from *L. sakei,* enhances the oxidative resistance of *Lactobacillus rhamnosus* (An et al., 2011). This collective evidence possibly suggests that

beneficial microbe *Lactobacillus spp*. alters gene expression system in the host's oxidative environment in order to survive in the GI tract. Another assumption is that these species can share their anti-oxidant genes in order to survive in the altered oxidative environment of the GI tract. Although not determined here, either the host's altered redox status favored the growth of oxygen-tolerant microbes *Lactobacillus spp*. or that the change in the microbes actually altered the host's oxidative responses. Overall, a fish oil (rich in n3 PUFA) supplemented diet with corn oil (rich in n6 PUFA) modulates oxidative responses of the gut but the specific contributions of the microbes involved were not determined here.

We also examined *Bacteroides spp*., which has been associated with consumption of carbohydrates (polysaccharides). In addition, high fat diets associated with obesity and reduced levels of *Bacteroides* species were observed in obesity animal models (Mozes et al., 2008; Murphy et al., 2010) as well as in obese people (Ley et al., 2006). We obsreved *Bacteroides spp*. levels were lowered in the ileum and in the colon of mice fed all high fat diets (corn oil, corn + fish oil and canola oil). This observation is supported by a study showed that a fish oil (n3 PUFA) diet has an inhibitory effect on the *Bacteroides spp*. in mice (Conlon et al., 2009; Thompson et al., 1995). However, low fat diets (5% corn oil) favor the growth of *Bacteroides spp.* compared to all high fat diets (corn oil, corn + fish oil and canola oil). Our previous study demonstrated that fecal transfer from disease resistant mice to disease susceptible mice alters the colonic pathology and immune status of disease susceptible mice with high level of *Bacteroides spp.* (Ghosh et al., 2011).Therefore, lower levels of *Bacteroides spp*. in the high fat fed mice suggest that may lack in resistant against disease. A specific species we examined from the Bacteroidetes phylum was *Bacteroides fragilis.* This species has enterotoxigenic properties and is associated with colon cancer (Sinkovics, 2012). In contrast, this species has been shown to

induce IL-10 and synthesis of polysaccharide A (PSA) which is an immune-modulator (Mazmanian et al., 2005). In our study, no differences in *Bacteroides fragilis* were observed in colon of mice fed all high fat diet and low fat. Furthermore, *Bacteroides fragilis* were not significant in the ileum of all high 20% fat diet fed mice but this species was only detected in two mice of the 5% corn oil group. In addition, *Enterococcus* species are opportunistic and nosocomial pathogens (Jett et al., 1994) they generate superoxides in vitro (Huycke et al., 2001) and produce hydroxyl ions when incubated with the colonic content of rats (Huycke et al., 2002). *Enterococcus faecium* was highely observed in patients with GI inflammatory disease (Kang et al., 2010). We observed that *Enterococcus* species were not affected by the content of high fat diets (corn oil, corn + fish oil, and canola oil). Thus, we conclude that high fat PUFA diets have no impact on the colonization of these two *Entrococcus* species in the gut.

Overall, our study demonstrates that high fat diets have an impact on intestinal gut microbiota. We observed that corn oil (n6 PUFA) increases pathobionts and that fish oil supplementation reverses this tendency and can enrich beneficial microbes. These microbial changes potentially affect host responses, including immune cell infiltration, where corn oil reduces macrophages and, again, fish oil supplementation reverses this effect. Finally, fish oil supplementation induces oxidative stress but more studies are required to determine the mechanism and significance behind this observation.

5 Chapter: Conclusion, significance and future

5.1 Conclusion

Dietary corn oil increases the opportunistic pathogens (Enterobacteriaceae and Clostridium cluster) whereas fish oil supplementation of corn oil reduces theses species and increases beneficial bacteria (*Lactobacillus*) which corresponds to alter in their gene expression against higher levels of oxidative status in the gut. Canola oil alters the GI microbiota similar to the corn oil group. Furthermore, HNE expression in the canola oil group were observed to be similar to those of the fish oil supplemented group but different from those of the corn oil group.

5.3 Significance of finding

Dietary n6 and n3 PUFA differentially affect the intestinal microbes and associated host responses. Diets high in PUFAs (n6 and n3) are prevalent in the Western world; additionally, fish oil pills are commonly consumed and many foods are supplemented with fish oil (DHA & EPA of n3 PUFA). Therefore, the composition and proportion of dietary PUFAs in Western diets alters the GI tract homeostasis.

5.2 Future directions

Ex vivo and *in vitro* study of isolated immune cells (macrophages and neutrophils) from different regions (spleen, bone marrow and blood) from these high fat diets fed mice will reveal mechanisms of n3 and n6 PUFAs effects on immune function. Also, studying different cell expression molecules (cytokines) and membrane stability will give us more through understanding of dietary oils in promoting host health.

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Appendices

Appendix A : Basal mix compositions

Formula	Corn oil	Corn + fish oil	Canola oil
Casein	240	240	240
DL-Methionine	3.6	3.6	3.6
Corn starch	150	150	150
Sucrose	298.8	298.8	298.8
Cellulose	50	50	50
Calcium carbonate	3.6	3.6	3.6
Mineral Mix[1]	42	42	42
Vitamin Mix [2]	12	12	12

Formula per Kg of all PUFAs high fat diets in gm, Mineral Mix[1] - AIN-76 (170915), Vitamin Mix [2]- Teklad (40060).

Appendix B : Macronutrient compostions

Macro nutrient	Corn oil (gm % wt/wt)	Corn + fish oil (gm % wt/wt)	Canola oil (gm % wt/wt)	5% Corn oil (gm % wt/wt)
Protein	21.2	21.2	21.2	22.6
Carbohydrate	44.4	44.4	44.4	51.6
Fat	20.0	20.0	20.0	5.2
Total Energy	4.53 Kcal/gm	4.53 Kcal/gm	4.53 Kcal/gm	3.41 Kcal/gm

Detailed macro nutrient composition and total energy per gm of diet according to manufacturer Harlan Teklad Basal Mix, TD.88232, USA.

Appendix C: Importance of bacteria selection

	Bacterial Primer	Reason to choice	References
1	*Bacillus spp.*	Beneficial properties as a probiotic	(Abriouel et al., 2011; Hamdache et al., 2011)
2	*Lactobacillus spp.*	Immune modulators and beneficial properties as a probiotic	(Foye et al., 2012; Ohashi et al., 2009)
3	*Enterococcus faecium*	Elevated level found inh IBD	(Kang et al., 2010).
4	*Enterococcus faecalis*	Involved in generation of super-oxides and associated with colon cancer	(Huycke et al., 2001; Huycke et al., 1996; Huycke et al., 2002)
5	*Clostridium coccoides gp.*	High in IBS patients and associated with IBD	(Lozupone et al., 2012; Duck et al., 2007; Jeffery et al., 2011; Schoepfer et al., 2008)
6	*Eubacterium rectale*	Butyrate producing bacteria	(Duncan et al., 2007; Hamer et al., 2008; Louis et al., 2009)
7	*Clostridia spp.*	associated with IBD	(Jeffery et al., 2011; Schoepfer et al., 2008)
8	SFB	Immune modulator,Th17 cells maturation	(Ivanov et al., 2008; Umesaki et al., 1999)
9	*Bacteroides spp.*	Reduced level associated with high fat diets and obesity	(Hildebrandt et al., 2009; Mozes et al., 2008)
10	*Bacteroids fragilis*	Associated with colon cancer	(Sears, 2009 ; Sinkovics, 2012)
11	*Enterobacteriaceae*	Pathobiont and associated with IBD and	(Lupp et al., 2007; Zheng et al., 2008 Seksik et al., 2003)
12	*Bifidobacterium spp.*	Immune modulators and beneficial properties as probiotic	(Ivanov et al., 2006; Jeon et al., 2012)