EFFECTS OF VITAMIN D SUPPLEMENTATION ON INTESTINAL INFLAMMATION IN EXPERIMENTAL INFLAMMATORY BOWEL DISEASE

by

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A thesis submitted in conformity with the requirements for the degree Master of Science

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2012

Abstract

Vitamin D may have immunomodulatory effects in the intestine. Our objective was to determine if exposure to vitamin D mitigates intestinal inflammation in IL-10 KO mice. Mice were randomized to a diet containing 25 IU (low) or 5000 IU (high) of vitamin D/kg of diet in utero and offspring were maintained on the same diet or switched to the other diet at weaning. Fecal samples were collected at 3 months of age. Vitamin D did not affect intestinal inflammation in male and female mice and did not affect KC cytokine concentration or regulate colonic gene expression in male mice. Vitamin D modulated the gut microbiota in a sex-specific manner and depending on timing of exposure. Females in the HH group had significantly higher fecal counts of C. coccoides than the other vitamin D interventions. Therefore, vitamin D may favourably modulate microbiota composition without attenuating inflammation.
ACKNOWLEDGEMENTS

There are many individuals I would like to thank for their help and support throughout my M.Sc. program. First, I would like to thank my supervisors, Dr. Wendy Ward and Dr. Elena Comelli, for giving me this amazing opportunity. The amount of knowledge and experience I have obtained throughout this program has been insurmountable and I am greatly appreciative of this. You are both great mentors. Wendy, I would like to personally thank you for your positive encouragement throughout my degree and with my future plans. I would also like to thank you for the countless time spent on editing my work and always doing the edits so promptly. I have learned a great deal from this and feel I have become a better writer because of you. Elena, I would like to personally thank you for all your great advice with my project and the hours spent in your office going over my presentations and lab work. I would also like to thank you for expanding my knowledge in an area that was foreign to me prior to starting my M.Sc. degree.

I would also like to thank my advisory committee, Dr. Reinhold Vieth, for all of his insight into my project and for helping with my data analysis. I would like to thank our lab research associate, Jim Chen, for his assistance with my project, data analysis, and teaching me how to analyze my histology samples. Furthermore, I would like to thank the administrative staff, Louisa and Emeliana, for all their help in addressing my concerns.

My project and experience never would have been the same without the help and support from my lab members. First, to the Comelli lab: Raha, Natasha, Chris, Wen and Sophia. Thank you for teaching me the lab protocols, assisting in my project, and helping me with my many questions throughout this process. Especially to Raha and Natasha, for all their kind advice and wonderful friendship. To the Ward lab: Kristina, Jovana and Elsa –
your friendship and support is very appreciative. Especially to Kristina, for all your work with the animal study and your positive attitude! And to Jovana, for your advice and company in the lab office! I also must thank everyone from lab meeting – the Comelli, Archer and Bazinet labs. All of your suggestions and assistance with presentations has made me much more comfortable with public speaking, something I was always so nervous doing!

Finally, I would like to thank my boyfriend, friends and family. To Al, for always encouraging me to stay positive when I felt overwhelmed, and helping me through my many computer issues! I do not have words for how much I appreciate all you have done for me these past two years. I’d like to thank my friends for being supportive as well, and being there for me when I needed a distraction. To my sister, Karen, for your friendship and advice that came through our many e-mails and phone calls. Finally, to my mom and dad: for your emotional and financial support. I never would have been able to pursue my education and career goals without you and I am so fortunate to have such supportive parents. Thank you.
CONTRIBUTIONS

The maintenance and analysis of this study was contributed by a number of individuals. Andrea Glenn was solely responsible for the histological analysis, DNA and RNA extractions, qPCR experiments and all statistical analyses. Kristina Fielding and Andrea Glenn were responsible for breeding and care of the IL-10 KO mice. Jianmen Chen was responsible for serum and organ collection from the mice upon necropsy. Andrea Glenn, Raha Jahani, Natasha Singh, Christopher Villa and Wen Su assisted with collection of intestinal tissues and contents. Jianmen Chen prepared the proximal colon tissue for histology analysis. Christopher Villa assisted Andrea Glenn in analyzing KC concentrations of the proximal colon.
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LIST OF DEFINITIONS

**Dysbiosis** is a non health-compatible state of imbalance of the intestinal microbiota (bacteria and other micro-organisms) [1]

**Nutritional Programming** is the process through which exposure to environmental stimuli or insults during critical phases of development brings about permanent changes to the physiology or metabolism of the organism [2]

**Predictive-Adaptive Response** refers to the hypothesis whereby developing organisms are assumed to receive information about the quality of the external environment, and in response, formulate predictions as to future ecological conditions [3]
LIST OF ABBREVIATIONS

AI – adequate intake
AIEC – adherent invasive *Escherichia coli*
Ang-4 – angiogenin-4
BMC – bone mineral content
BMD – bone mineral density
CARD – caspase recruitment domain-containing protein
CD – Crohn’s disease
CD8+ – cluster of differentiation 8+
CFU – colony forming unit
DGGE – denaturing gradient gel electrophoresis
DRI – daily recommended intake
DSS – dextran sulphate sodium
EAE – experimental autoimmune encephalomyelitis
F/B ratio – Firmicutes/Bacteroidetes ratio
GI – gastrointestinal
IBD – inflammatory bowel disease
IFN-γ – interferon-gamma
IL – interleukin
KO – knockout
LITAF – lipopolysaccharide-induced tumor necrosis factor-alpha
MS – multiple sclerosis
NF-κB – nuclear factor kappaB
NOD – nucleotide oligomerization domain
PCR – polymerase chain reaction
PND – postnatal day
RANKL – receptor activator of nuclear factor kappaB ligand
RXR – retinoid X receptor
SCFA – short chain fatty acid
SPF – specific pathogen free
SSCP – single strange confirmation polymorphism
Th1 – T-helper 1
Th17 – T-helper 17
Th2 – T-helper 2
TNF-α – tumor necrosis factor-alpha
UC – ulcerative colitis
VDR – vitamin D receptor
VDRE – vitamin D responsive element
WT – wild type
CHAPTER ONE

INTRODUCTION
1.0 INTRODUCTION

The role of vitamin D in bone health and calcium homeostasis has been well established, but more evidence is suggesting it may have effects on the immune system and thus have a role in disease prevention. Within the immune system, 1,25-dihydroxyvitamin D (1,25(OH)₂D) inhibits T helper (Th)-1 type immune function in cells, induces T regulatory cells, and enhances phagocytosis by white blood cells [4]. Research has found links between vitamin D deficiency and diseases such as cancer, type-1 diabetes, multiple sclerosis (MS), and inflammatory bowel disease (IBD) [5]. The vitamin D hypothesis proposes that vitamin D regulates the development and function of the immune system, and that early life changes in vitamin D status can affect the immune response [6]. The identification of vitamin D receptors (VDRs) in immune cells sparked interest that vitamin D may play a role as an immune system regulator. Cantorna and colleagues [7, 8] have discovered that vitamin D deficiency accelerates the development of experimental MS and type-1 diabetes, and that treatment of 1,25(OH)₂D suppressed the development of these diseases. Studies have also shown that vitamin D deficiency or an absence of VDRs exacerbated intestinal inflammation in interleukin-10 knockout (IL-10 KO) mice (an experimental model of IBD), resulting in premature death compared to IL-10 KO mice with adequate vitamin D status [9, 10]. Zhu et al also discovered through microarray analysis that several TNF-α-associated genes were down regulated by 1, 25(OH)₂D treatment in the colon of IL-10 KO mice.

Interestingly, patients with IBD have low circulating levels of 25-hydroxyvitamin D (25(OH)D), in that they would be classified as vitamin D insufficient or deficient [11]. There are several reasons as to why vitamin D status may be low in patients with IBD, these include nutrient malabsorption, glucocorticoid therapy and living in northern climates where vitamin D synthesis from sunlight is limited [12, 13].
Overall, these findings indicate that vitamin D may play a role in the development and disease activity of IBD. Further investigation into how vitamin D supplementation can mitigate the development of IBD is warranted, particularly if early-life exposure to vitamin D can prevent disease progression. The objective of this study was to determine if early-life exposure to high levels of vitamin D (in utero and during suckling) could protect against intestinal inflammation in adulthood. In order to address this we investigated the effects of vitamin D supplementation on colonic inflammation severity, cytokine expression, gut microbiota composition and colonic gene expression in adult IL-10 KO mice that develop intestinal inflammation by 6-8 weeks of age.
CHAPTER TWO

LITERATURE REVIEW
2.0 LITERATURE REVIEW

2.1 Nutritional programming

Dietary habits in early life and subsequent stages of development may set a trajectory for higher or lower risk of chronic disease during adulthood. This concept is termed “nutritional programming”, and can be defined as the process through which exposure to environmental stimuli or insults during critical phases of development brings about permanent changes to the physiology or metabolism of the organism [2]. Evidence of in utero and early life exposure to nutritional insults has been well documented from the Dutch Famine during World War II [14]. In the Netherlands during 1944-45, official daily rations for the general population was well below recommended intakes, with the height of the famine allowing 400 to 800 calories of food a day. Children of pregnant females during the famine were found to have adverse health outcomes later in life such as coronary heart disease, type 2 diabetes and decreased renal function [15]. Women exposed to the famine were also at an increased risk of breast cancer and obesity later in life. Timing during gestation in which exposure to the famine occurred also influenced later health consequences. For example, offspring exposed to the famine in early gestation were linked with outcomes such as altered blood coagulation and coronary heart disease later in life [16].

The effects of nutritional programming have been largely studied using animal models, and these studies have provided insight into how maternal diet impacts the health of the mature offspring. These studies have shown that early life nutrition (such as in utero and early postnatal life) can influence outcomes such as blood lipids, blood pressure, body fatness, atherosclerosis risk, behaviour, learning and longevity later in life [4]. In utero exposure to fish oil supplementation in rats was beneficial in maintaining circulating glucose, insulin, cholesterol and homocysteine levels in adult offspring [17]. Further research examined the effects of a high or low protein diet during gestation in rats on
later hypertension in the adult offspring. A low protein diet during pregnancy suppressed
the renin-angiotensin system, reduced number of glomeruli, and resulted in glomerular
enlargement in newborns, as well as resulted in hypertension in adulthood [18]. Research
from our lab also found that neonatal exposure to genistein, an isoflavone found in soy
protein, resulted in higher bone mineral density (BMD) and bone strength in mice later in
adulthood [19]. These studies suggest that early nutrition may program disease outcomes
later in life.

2.1.1 Programming of the gut microbiota

Nutrition during the first few days of life can affect intestinal health, including the
composition of the gut microbiota. For example, infants that were exclusively formula-
fed were found to be more often colonized with Escherichia coli (E. coli), Clostridium
difficile (C. difficile), Bacteroides fragilis (B. fragilis) and lactobacilli than exclusively
breast-fed infants [20]. Breast-fed infants were shown to have less C. difficile and E. coli
in their feces compared with formula-fed infants at one month of age [21]. Bifidobacteria
and lactobacilli are considered the most important health-beneficial bacteria for the
human host, whereas bacteria such as staphylococci and E. coli are potentially pathogenic
[22].

Early life nutrition may play a possible role in the development of IBD. Individuals with
Crohn’s disease (CD) are less likely to have been breast-fed than controls. The mean
length of the breast-feeding period was also significantly shorter among CD patients
compared to the controls [23]. Breast-feeding may protect infants from IBD in infancy,
possibly due to the presence of immunoglobulins and antimicrobial peptides in breast
milk or that breast-feeding may stimulate the early development and maturation of the
infant intestinal mucosa [24]. This effect may be due to the growth factors in breast milk
that act as gastrointestinal trophic factors; or from delaying the infant’s exposure to cow’s
milk and the potential etiological constituents found in cow’s milk such as lactose and triacylglycerols. This indicates that early-life feeding practices have the potential to modulate the gut microbiota and influence later disease risk such as IBD.

2.1.2 Programming of bone development

Bone health has also been found to be affected by nutrition in early life. Infants fed a standard formula versus infants fed human milk, had higher rates of bone formation and turnover by 8-12 years of age [25], and decreased bone formation early in life is a risk factor of adult degenerative bone disease. Preterm infants fed mother’s milk also had a higher BMD after a 20-year follow-up [26]. Preterm infants are different than term infants in that they have underdeveloped mineralization of bone because preterm infants are born prior to or during the most rapid phase of fetal mineral accretion and thus are at an increased risk for metabolic bone disease [26]. However, these results raise the hypothesis that optimal early nutrition is important for reducing the risk of later bone disease. Maternal vitamin D insufficiency or deficiency during late pregnancy was also associated with a deficit in bone mineral accrual in offspring at nine years of age [27]. A reduction in both bone size and BMC was found in children of mothers with vitamin D insufficiency or deficiency without effects on childhood height or lean mass [27]. The study findings provide evidence that the intrauterine environment, such as maternal vitamin D status during pregnancy, is significantly correlated with bone mineral accrual at nine years of age. Bone abnormalities are observed in IBD patients, which may result from malabsorption of nutrients, drug treatments, the inflammatory process itself, or their combined effect [12]. However, some studies have reported bone abnormalities at diagnosis prior to any treatment for IBD [12]. Therefore, further investigation into the effect of early nutrition on intestinal health and IBD is warranted.
2.2 Inflammatory Bowel Disease

IBD is a chronic disorder of the gastrointestinal (GI) tract that may manifest as either Crohn’s disease (CD) or ulcerative colitis (UC) [28]. Clinical symptoms include weight loss, diarrhea accompanied by blood and abdominal pain. The hallmark of this disease is an uncontrolled inflammation of the intestinal mucosa, which may affect any region of the GI tract [13]. Individuals with IBD have an inability to down-regulate inflammatory responses in the GI tract compared to that seen in a healthy gut. Diagnosis of IBD is based on the presence of transmural or superficial patchy granulomatous infiltration and/or acute inflammatory cells [13]. The peak onset for IBD is 15 to 30 years of age, although it may occur at any age, and higher rates of the disease occur in people of Caucasian and Ashkenazi Jewish origin. This may be due to living in regions such as North America and Northern Europe (where exposure to sunlight occurs less) or from genetic susceptibility [13]. Canada has one of the highest rates of IBD in the world, and nationally, there are approximately 13.4 and 11.8 cases of CD and UC per 100,000 persons per year, respectively [29]. There is currently no cure for IBD, and the economic costs of IBD are significant – estimates from Canada suggest that IBD costs $1.8 billion each year. This includes hospitalizations, medications, physician visits and indirect costs such as loss of productivity [30].

The etiology of IBD is currently unknown. However, there are multiple factors that may contribute to the development of this disease [13]. These include environmental triggers, genetic factors, immunoregulatory defects and microbial exposure. Proposed environmental factors include “Westernization” (changes in lifestyle, such as diet, smoking, exposure to sunlight, pollution, and industrial chemicals in southern countries and Asia), sanitation, exposure to infection, occupation, diet and tobacco smoking [13]. IBD may have origins in early life as well, such as optimal nutrition during pregnancy and infancy. As previously mentioned, CD patients were less likely to be breast-fed than
controls [23] and another study also found that not being breast-fed was associated with an increased risk of CD in later life [31]. Moreover, children of mothers who consumed vitamins, iron and other mineral supplements three months before and during pregnancy had lower rates of UC than children of mothers who did not use such supplements [32].

2.2.1 Genetic risk

Genetic susceptibility to IBD has been long recognized. Having a sibling or parent with the disease is a significant risk factor for development of IBD [33]. To date, 71 genetic susceptibility loci have been identified, but the presence of many of these identified genes in unaffected individuals and only being present in a quarter of IBD patients highlights the complexity of a genetic basis for this disease [34]. The major gene involved in IBD that has been discovered is a polymorphism in the CARD15 gene, which encodes the nucleotide-binding oligomerization domain 2 (NOD2) protein [35]. In Caucasian populations, approximately 30% of CD patients carry one of three single nucleotide polymorphisms (SNP) on a single allele compared to 15% of healthy individuals [35]. However, the mechanism whereby defects in the NOD2 gene lead to the development of IBD remains unclear but NOD2 has been thought to control commensal bacteria in the gut [36].

2.2.2 Immune response

Defects in the immune system have been discovered in CD and UC patients. In patients with IBD, the individual’s immune system attacks its own body by triggering the CD8+ pathway of the Th cells. In healthy individuals, helper T-cells respond to immune challenges by determining which cytokines will allow the immune system to be most beneficial to the individual [28]. The immune response in CD is Th1/Th17 driven, whereas UC is Th2 driven (Figure 2.1). This immune response results in secretion of cytokines, which are key signals in the intestinal immune system that are responsible for
communication between cells, proliferation of antigen specific effector cells and mediating the local and systemic inflammation pathways [37]. Th1 cells secrete interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-12, IL-18, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [38] and this leads to chronic inflammation of the intestinal mucosa through biochemical and morphological changes in T cells [28]. TNF-α, which exerts its proinflammatory effects through the production of IL-1β and IL-6, is a key inflammatory mediator in the pathogenesis of IBD [28]. TNF-α disrupts the microenvironment of responding T-cells, which adversely affects many aspects of immune system development, immune response regulation and tissue development. Clinical studies have found a clinical improvement in CD patients treated with a human-mouse chimeric TNF-α neutralizing antibody (infliximab) [39]. However, infliximab treatment can lead to adverse side effects such as infusion reactions, delayed hypersensitivity reactions and formation of anti-double stranded DNA [40]. Overall, cytokines play an important role in the pathogenesis of IBD, and their manipulation through various drug treatments or dietary interventions has the potential to reduce disease severity and maintain remission.

2.2.3 Intestinal permeability

IBD has also been associated with an increased permeability of the epithelial lining of the gut resulting in continuous stimulation of the mucosal immune system [41]. Luminal bacteria have been found to intensify the permeability defect even further, which establishes a cycle of mucosal inflammation that allows for the uptake and translocation of bacteria. In healthy humans, the intestinal lining throughout the ileum and colon was found to be covered with mucus, which prevents bacteria from coming in contact with the mucosal surface [42]. In contrast, individuals with IBD had a dense coating of bacteria on the intestinal surface. The bacteria had adhered to epithelial cells, entered crypts and were sporadically found within cells [42]. Similar results were also found in a mouse
model study that induced intestinal inflammation by administering dextran sodium sulfate (DSS) in the drinking water of the mice. DSS altered the mucus thickness and permeability of the intestinal wall in the mice, mimicking human colitis [43]. Bacteria were shown to penetrate the inner mucus layer and reach the epithelial cells, triggering an inflammatory reaction. These observations suggest that altered properties or lack of the inner colon mucus layer may be an initial event in the development of colitis [43].
Figure 2.1: Cytokine Imbalance in IBD

Th1/Th17 immune response associated with CD disease; whereas Th2 immune response associated with UC colitis. Figure represents which cytokines are secreted by Th cells and that this results in a proinflammatory state. Treg: T-regulatory cells; IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; IL-13: Interleukin-13; IL-5: Interleukin-5; IL-17: Interleukin-17; IL-6: Interleukin-6, IFN-γ: Interferon-γ; TGF-α: Tumor necrosis factor-α; IL-12: Interleukin-12; IL-8: Interleukin-8
2.3 Gut microbiota

2.3.1 Microbiota in healthy humans

The intestinal microbiota is necessary for the development of the gut immune system by acting as the first line of defense against pathogens and in the immunoregulation of the GI tract [44]. Impaired defense against pathogens and defects in the immune system have been implicated in IBD, suggesting microbial exposure and microbial development may be involved in the pathogenesis of IBD [44]. The normal relationship between commensal bacteria and the host is symbiotic and it is hypothesized that exposure to commensal bacteria down-regulates the inflammatory response of the gut to the microbes and food antigens to which it is exposed [44]. In IBD, this tolerance is lost and exposure to luminal microbiota triggers an inflammatory response by the cells lining the mucosa and leads to a chronic, destructive, immune response [45].

In healthy individuals, the intestinal microbiota is composed of 500 – 1000 different species and greater than 99.9% of the culturable bacterial populations are obligate anaerobes [46]. Six phyla of bacteria predominate the intestinal microbiota, which are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria and the uncultured group TM7 [46]. Highly represented genera from these phyla include Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Eubacterium, Peptococcus, Peptostreptococcus, Escherichia, and Veillonella [47]. Bacterial population density increases from the proximal small intestine (10^7 organisms per gram luminal contents) to the colon (10^{11} per gram of contents). The microbiota acts as a multifunctional organ that is responsible for the breakdown of otherwise indigestible plant polysaccharides, biotransformation of conjugated bile acids, degradation of dietary oxalates, and synthesis of certain vitamins [48]. The microbiota composition of healthy adults has been found to be stable over time and is host-specific [46].
Firmicutes, one of the dominant bacterial phyla, which include highly represented species such as *Clostridium leptum*, *Clostridium coccoides* and *Faecalibacterium prausnitzii*, are known to be one of the most abundant producers of the short-chain fatty acid, butyrate [49]. Butyrate has been found to be an essential regulator of gene expression, inflammation, differentiation, apoptosis and a major energy source for colonocytes [50]. Other predominant microbes in the gut are the *Bacteroides* species (from the Bacteroidetes group) [51]. *Bacteroides* is typically considered an opportunistic pathogen, as they generally maintain a beneficial relationship in the gut, but some species can be pathogenic if the organism escapes the gut [51]. *Bifidobacterium* (from the Actinobacteria group) is another dominant group of the flora that is considered a health promoting bacteria because of its production of lactic acid (which prevents growth of harmful bacteria) and is widely used as a probiotic agent [52]. Furthermore, Bifidobacteria strains have also shown to protect against the enterohaemorrhagic *E. coli* O157:H7 infection in mice [53]. This protective effect was thought to be through the production of acetate, providing evidence that Bifidobacteria may promote intestinal defense and protect the host from lethal infection. *E. coli* (from the Proteobacteria group) consists of approximately 0.1% of the bacteria in the colon, with most strains being harmless and involved in the production of vitamin K [54]. However, some serotypes of *E. coli* have been involved in urinary tract infections, neonatal meningitis and intestinal diseases [55].

### 2.3.2 Factors influencing the development of the microbiota

There are many factors thought to contribute to and influence the development of the microbiota. During the first year of life, the infant intestinal tract changes from sterile to dense colonization, resulting in a microbial composition similar to that found in the adult intestine. This first year of initial colonization represents a critical window in which deviations in colonization patterns may impact immune maturation [49].
2.3.2.1 Birth

The microbiota composition of infants of caesarean section has been reported to differ from that of infants delivered vaginally, both in the timing of colonization and in composition [56]. The fecal colonization of infants born by cesarean section was delayed and *Bifidobacterium* species of bacteria and *Lactobacillus* species of bacteria colonization rates reached the rates of vaginally delivered infants at 1 month and 10 days, respectively. Infants born by cesarean section were also less often colonized with bacteria of the *Bacteroides fragilis* group than were vaginally delivered infants [56]. Normal microflora (as seen in vaginally delivered infants) has immunostimulatory effects and well-functioning mucosal immunity is a prerequisite for health, because the mucosal surfaces are favored as portals of entry by most infectious agents, allergens, and carcinogens [56]. Children born via cesarean section were found to be at an increased risk of developing eczema, food allergies and asthma, [57] indicating that mode of delivery and subsequent gut microbial colonization may adversely impact health.

2.3.2.2 Infant feeding practices

Studies have found a lower abundance of Bifidobacteria and a higher abundance of aerobic bacteria in the microbiota of formula-fed infants compared to breast-fed infants [58]. Higher counts of *E. coli* and *C. difficile* were also more frequently found in infants who were formula-fed compared to breast-fed infants [20]. The differences in colonization between breast-fed and formula-fed infants may be due to the rich source of oligosaccharides found in human milk. These oligosaccharides are indigestible by human infants and act as prebiotics, as the fermentation of breast milk oligosaccharides promotes the growth of beneficial Bifidobacteria [59]. Interestingly, infants that received an infant formula enriched with oligosaccharides also had higher counts of Bifidobacteria [20].
2.3.2.3 Weaning

Weaning from breast-milk or formula also results in a change in the gut microbiota. The switch from a high fat liquid diet to solid polysaccharide-rich food results in a switch from a high population of facultative anaerobes (such as *E. coli*, enterococci and staphylococci) to obligate anaerobes, such as Bacteroidetes, *C. coccoides* and *C. leptum* [60].

2.3.2.4 Antibiotic use

Antibiotic use in the first year of life has also been found to influence gut microbiota composition. Oral use of antibiotics within the first month resulted in lower counts of Bifidobacteria and *Bacteroides fragilis* species [20, 61]. The use of antibiotics early in life has been associated with an increased risk of developing atopic diseases in children, such as asthma and eczema [61].

2.3.2.5 Age

Age has also been found to influence gut microbiota composition. Infants were found to have low levels of total bacteria compared to adults and seniors and the most abundant bacterial group in infants was *Bifidobacterium* [62]. Elderly were found to have high levels of *E. coli* and Bacteroidetes compared to adults. This change in the microbiota composition may be due to a reduction in GI transit time and digestive secretions [62]. Furthermore, the Firmicutes/Bacteroidetes ratio changed throughout the lifespan, with average ratios of 0.4, 10.9, and 0.6 for infants, adults and elderly, respectively.

Overall, factors in early life such as infant feeding, mode of delivery, weaning, antibiotic use, age and environment can influence the development of gut microbial composition and the developing immune system. This suggests that early life factors can play a role in gut health and immunity, which can ultimately influence later disease outcomes.
2.3.3 Dietary influences on gut microbiota after weaning

There are many dietary factors that may influence the composition of the intestinal microflora. As mentioned earlier, the first days of life when the bacterial population has not yet been established are important in programming the infant gut microbiota.

2.3.3.1 Carbohydrates

Dietary factors later in life may also alter the gut microflora. The fermentative breakdown of indigestible carbohydrates by the colonic bacteria results in the production of short chain fatty acids (SCFAs) which serve as a major energy source for the host. The three main SCFA end products are acetate, propionate, and butyrate [63]. Butyrate provides the majority of the energy requirement for colonocytes and is preferred over glucose, glutamine or ketone bodies.

2.3.3.2 Probiotics and prebiotics

The use of probiotics and prebiotics are the most well-known influences of microbiota composition. Probiotics are defined as “live microorganisms” which when administered in adequate amounts confer a health benefit on the host [64], and prebiotics are a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota [64]. Ingestion of both or either of these dietary supplements can increase the number of health-supporting bacteria such as Bifidobacteria, which may prevent pathogens from colonizing in the gut, reduce gut permeability, release antimicrobial peptides or modulate the immune system [65].

2.3.3.3 Plant-based diet

Research has also found that the gut microbiota composition differs between vegetarians and omnivores. One study in particular found that the high fibre diet common of vegans resulted in lower total counts of Bacteroides, Bifidobacteria, E. coli and other
Enterobacteriaceae species compared to omnivores [66]. The stool pH of vegans was lower than omnivores, which may be attributed to the high fiber intake typical of vegans. Gut bacteria breakdown of the indigestible fibers into SCFAs results in a lower pH environment. This may explain the lower abundance of *E. coli* and Enterobacteriaceae in vegans compared to omnivores as these bacteria favour higher pH environments [66].

### 2.3.3.4 Vitamins and minerals

Vitamin and mineral intake may also manipulate the gut microbiota composition. Iron fortification of biscuits (20 mg iron) that were fed to African children four times a week was found to increase fecal enterobacteria, such as *E. coli*, as well as deplete numbers of Bifidobacteria and lactobacilli [67]. This shift in the gut microbiota further resulted in increased gut inflammation. Furthermore, in a mouse model of ileal CD, oral replacement therapy with iron sulfate was found to trigger inflammatory processes that can increase disease activity [68]. Vitamin A deficiency in rats after weaning was also found to increase total bacteria and *E. coli* counts in intestinal contents and feces [69]. Vitamin D deficiency has also been found to alter gut microbial composition, with one study finding that vitamin D deficiency in mice resulted in an increase in colonic bacterial load compared to vitamin D sufficient mice [70].

### 2.3.4 Intestinal microbiota in rodents

The intestinal microflora of rodents resembles that of humans, and bacterial groups such as gram-negative *E. coli* and streptococci are the first to colonize, followed by lactobacilli and anaerobic spiral-shaped bacteria [44]. After weaning, bacteria such as *Bacteroides* and *Clostridia* species become the most dominant microbes [71]. Bibiloni and colleagues examined the large bowel microbiota of mice, and found that healthy wild-type mice were commonly colonized by bacteria such as lactobacilli belonging to the Acidophilus group and *Eubacterium ventriosum* [72]. Overall, the rodent microbiota has the same
dominant phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) as humans, and follows a similar developmental process [71] and mouse models are commonly used in gastrointestinal and microbiota research [73]. One study in particular compared the cecal microflora of mice at 4-weeks and 8-weeks of age [74]. At 4-weeks of age, the microbiota was classified into 6 phylogenetic groups whereas the 8-week old mice microbiota could be classified into 10 phylogenetic groups. The majority of the groups identified from the cecal microbiota were from the \textit{C. coccoides}, \textit{Bacteroides} and \textit{Lactobacillus} groups [74]. The intestinal microbiota of rodents may also differ between strains and environment. The cecal microbiota of two different strains of mice was analyzed, revealing significant differences in microbiota composition between the two strains [75]. Furthermore, the inbred mice of the same strain had more similar composition than out bred mice, suggesting genetic background is a significant factor in microbiota composition development. Environment is also a factor that determines microbiota composition in rodents. Differences in microbiota of the same strains of mice have been found between laboratory facilities [75]. Moreover, the same strain in the same facility, but in a different room, can also result in significant differences in microbiota composition [75].

2.3.5 Gut microbiota in IBD patients

Dysbiosis, or the shift from predominating “protective” bacterial species to “harmful species”, has been thought to lead to the colonization of the gut by bacteria that is more “proinflammatory” than the normal microbiota, and has been hypothesized as a cause of IBD [76]. Dysbiosis has been considered to be a part of IBD based on findings in animal models of colitis, as well as the treatment of IBD with probiotics and antibiotics in humans [76]. Using the application of molecular techniques, such as 16s rRNA sequences to enumerate and identify bacteria, studies have identified microbial diversity differences in healthy patients and patients with IBD.
Fecal microbiota has been shown to differ in IBD patients compared to healthy subjects [77]. Fecal samples were collected from 57 subjects with colitis and 27 healthy subjects, and microbiota composition was determined by real-time quantitative PCR (qPCR). Patients with active CD had fewer counts from the Firmicutes phylum compared to healthy subjects, and both \textit{C. leptum} and \textit{C. coccoides} were decreased in the colitis patients. \textit{Faecalibacterium prausnitzii} (a major species within the \textit{C. leptum} group) was significantly diminished in colitis patients compared to healthy patients [77]. Results also found a lower level of Bifidobacteria in the active colitis subjects compared to healthy subjects. Furthermore, the Firmicutes/Bacteroidetes ratio was reduced in patients with active IBD and infectious colitis compared to patients in remission and healthy subjects (Table 2.1). It was also shown that total bacteria, \textit{Bacteroides} and \textit{E. coli} did not differ between colitis and healthy patients [77]. The findings from this study suggest that Firmicutes, one of the dominant phyla of the microbiota, may play a protective role in gut integrity and the development of IBD.

Further research analyzed biopsy samples from 46 healthy subjects and 57 IBD patients to examine if there were any differences in the mucosa-associated colonic microflora [78]. Using 16S rDNA single strand confirmation polymorphism (SSCP) fingerprinting, cloning libraries and real-time PCR, the researchers found significant differences between the healthy controls and IBD patients. The main differences in the colonic microbiota were a 50% reduction in microbial diversity in CD patients and a 30% reduction in UC patients compared to healthy subjects [78]. The loss of diversity was largely ascribed to anaerobic bacteria such as the \textit{Bacteroides} species, \textit{Eubacterium} species, and \textit{Lactobacillus} species. Findings from this study suggest that there are also differences in the mucosa associated colonic microflora, as shown in fecal microbiota, in IBD patients compared to healthy subjects.
Another study that determined the microbiota composition of GI tissue samples from healthy and IBD patients using culture-dependent rRNA sequence analysis also found microbial differences between the two groups. In this study, UC and CD colon samples had fewer sequence types representative of *Bacteroides* species and the *Lachnospiraceae* subgroup of Firmicutes, and more sequences representative of *Proteobacteria* and the *Bacillus* subgroup of Firmicutes [44].

More recent research has continued to find dysbiosis of the gut microbiota in IBD patients compared to healthy controls. Walker and colleagues [79] took colonic mucosal biopsies from active CD and UC patients, and from healthy controls. Paired mucosal biopsies of inflamed and non-inflamed tissue were retrieved from the IBD patients. To determine bacterial composition, an in-depth sequencing of over 10,000 near full-length bacterial 16S rRNA genes and qPCR analysis were used. Results from this study found that samples from both inflamed and non-inflamed CD and UC patients had significantly lower counts of the Firmicutes phylum and higher counts of the Bacteroidetes phylum [79]. There was also a slight but not significant increase in Enterobacteriaceae in CD patients (detected in 5 out of 6 samples) compared to controls (detected in 2 out of 6 samples). Results of the qPCR analysis found that total bacterial load was significantly lower in the inflamed CD patients than in the inflamed UC patients; however, results from this study did not find a specific bacterial species or cluster of bacteria that was associated with inflamed tissue versus non-inflamed tissue [79].

Overall, the evidence shows that dysbiosis of the gut microbiota is present in IBD patients compared to healthy subjects. However, the bacterial species or groups that are causing this dysbiosis are not as clear. Many studies have found decreases in the Firmicutes phylum, particularly *C. leptum, C. coccoides*, and *F. prausnitzii* [44, 77, 79], but are finding increases, decreases or no change in the Bacteroides phylum (Table 2.1). An increase in Enterobacteriaceae has also not been found in all studies, which is
worthy of note because research has speculated adherent-invasive \textit{E. coli} (AEIC) as a possible causative agent for IBD [80]. The literature clearly shows that the intestinal microbiota plays a key role in IBD, but research has yet to determine a specific bacterial group or species in this disease; or if this dysbiosis is a cause of IBD or an effect of the disease course. Further research is warranted.
### Table 2.1 Microbiota composition of IBD patients

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Age &amp; Gender</th>
<th>Study Design</th>
<th>Results</th>
<th>Quantification of microbiota composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sokol et al., 2009</td>
<td>32 CD or 17 UC patients, 8 infectious colitis (IC) patients, 27 healthy subjects (HS)</td>
<td>Mean age &amp; gender (% male): CD: 38 (11%); UC: 39 (9%); IC: 34 (5%); HS: 36 (11%)</td>
<td>Case-control study; Fecal samples collected and microbiota analyzed by qPCR</td>
<td>Firmicutes group (<em>C. leptum</em> and <em>C. coccoides</em>), <em>F. prausnitzii &amp; Bifidobacterium</em> species, Firmicutes/Bacteroidetes ratio were lower in CD and infectious colitis compared to healthy subjects.</td>
<td>(Log10 Equivalent/g stool): Firmicutes: 9.8 ± 0.4 (CD), 10.8 ± 0.2 (HS); Bifidobacteria: 8.0 ± 0.4 (CD), 9.2 ± 0.7 (HS); F/B ratio: 1.3 (CD), 6.0 (HS)</td>
</tr>
<tr>
<td>Ott et al., 2004</td>
<td>26 CD or 31 UC patients, 46 HS</td>
<td>Age range: 16-82 years; gender (M/F): CD (9/17); UC (18/13); HS (16/30)</td>
<td>Case-control study; Biopsies taken from different segments of the colon and mucosa associated microflora analyzed by SSCP, cloning and PCR</td>
<td>Diversity of CD and UC reduced by 50% and 30%, respectively, compared to controls; diversity loss from decrease in <em>Bacteroides</em>, <em>Eubacterium</em>, and <em>Lactobacilli</em> species</td>
<td>Total bacteria did not differ between controls and CD/UC; 78% reduction in <em>Bacteroidetes</em> in CD, 45% decrease of <em>Enterobacteria</em> in CD patients</td>
</tr>
<tr>
<td>Frank et al., 2007</td>
<td>191 CD, UC patients, HS</td>
<td>N/A</td>
<td>Case-control study; GI tissue samples retrieved and analyzed by culture dependent rRNA sequence analysis</td>
<td>Depletion of commensal bacteria in CD and UC, particularly from the Firmicutes and Bacteroidetes phyla</td>
<td>10-fold decrease in total bacteria &gt;300-fold decrease in <em>Lachnospiraceae</em>, 50-fold decrease in <em>Bacteroidetes</em> in CD/UC patients</td>
</tr>
<tr>
<td>Walker et al.</td>
<td>6 CD, 6 UC patients, 5</td>
<td>Age range: 24-73; gender</td>
<td>Case control study; Paired mucosal</td>
<td>Decreases in Firmicutes group and increases in Bacteroidetes</td>
<td>Total bacterial load: only significantly lower in</td>
</tr>
<tr>
<td>al., 2011</td>
<td>HS</td>
<td>(M/F): CD (2/4); UC (5/1); HS (2/3)</td>
<td>biopsies of inflamed and non-inflamed tissue from IBD patients, plus controls; analyzed by clone-library analysis and qPCR</td>
<td>group in IBD patients; increases of Enterbactericeae group in CD patients. No bacterial species or cluster found to be associated with inflamed or non-inflamed samples</td>
<td>inflamed CD patients compared to inflamed UC patients</td>
</tr>
</tbody>
</table>

CD: Crohn’s disease; UC: ulcerative colitis; qPCR: quantitative polymerase chain reaction; HS: healthy subjects; SSCP: single strand confirmation polymorphism; GI: gastrointestinal
2.3.6 Microbiota in the IL-10 KO mouse (IBD-model)

Mouse models of IBD or acute colitis are commonly studied to research potential causes, triggers and treatments for the disease. Similar microbial composition has been observed in mouse models of IBD, such as the interleukin-10 knockout (IL-10 KO) mouse model. It is important to note that IL-10 KO mice that are germ-free do not develop IBD, in contrast to the same mice kept under specific pathogen-free (SPF) conditions or inoculated with a specific pathogen after gnotobiotic birth [81]. This finding highlights that bacteria are required for the initiation of intestinal inflammatory conditions such as IBD.

One study found an altered composition of intestinal microbiota in the IL-10 KO mouse that had been infected with *C. rodentium*, *C. jejuni* or treated with DSS. A reduction in both the total number of intestinal bacteria and in bacterial diversity was found, particularly an overgrowth of the Enterobacteria species [82] (Table 2.2). Uninfected IL-10 KO mice in this study were also found to have less *Bacteroides* species present in the gut than their wild-type (WT) counterparts. Another study found that *Lactobacillus* and *Eubacterium* species were also rarely detected in germ-free IL-10 KO mice that were inoculated with microbiota from SPF mice [72] (Table 2.2). This study also found an increase in *Bifidobacterium animalis*, *Bacteroides* species, and *C. cocleatum* in the IL-10 KO mouse compared to WT mice [72].

Another study found that the IL-10 KO mouse also had reduced bacterial diversity compared to WT mice [83], with the two dominant bacteria in the gut being *Blautia producta* and *E. coli* (which was rarely detected in WT mice). Knoch et al [84] inoculated IL-10 KO and WT mice with a mixture of *Enterococcus faecalis* and *Enterococcus faecium* strains and found the caecal contents of IL-10 KO mice had reduced total...
bacteria numbers and *Bacteroides-Prevotella-Porphyronas* species compared to the WT mice (Table 1.2). More *Enterococcus* species were also found in the IL-10 KO mice compared to the WT mice [84]. Overall, it is evident that the microbiota composition of the IL-10 KO mouse model differs from their WT counterparts and is similar to what is found in patients with IBD [72]. However, research shows that the microbial composition of the IL-10 KO mice is not always consistent, such as some showing increases in *Bacteroides* species [82, 84] while others found a decrease in *Bacteroides* species [72]. The strains of mice used in these studies were not always consistent, indicating a potential reason to the differences in microbiota composition. Many of these studies did find a reduction in bacterial diversity as well as an increased number or overgrowth of potentially pathogenic bacteria, such as *E. coli*, in the IL-10 KO mice compared to WT mice (Table 1.2) suggesting that the bacterial community may play a role in the development of this disease.

### 2.3.7 Summary

In summary, the etiology of IBD is unknown, but the microbiota appears to be a contributing factor. Early-life nutrition may also be implicated in later IBD development. Vitamin D deficiency in early-life has been linked to poor bone outcomes later in life, and may also impact the gut microbiota. IBD is more prevalent in countries that have less exposure to sunlight (and therefore less vitamin D synthesis, with the exception of Australia and New Zealand), warranting further research into vitamin D’s role in IBD development and the concomitant gut microbiota.
Table 2.2: Microbiota composition of IL-10 KO mice

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Age &amp; Sample size</th>
<th>Study Design</th>
<th>Qualitative data</th>
<th>Quantification of specific bacteria</th>
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<tbody>
<tr>
<td>Lupp et al., 2007</td>
<td>WT and IL-10 KO mice (C57BL/6)</td>
<td>Age N/A; n = 4-6 mice per group</td>
<td>Randomized mice to be infected with <em>C. rodentium</em>, <em>C. jejuni</em> or chemically induced IBD through DSS. Microbial composition determined by FISH/PCR</td>
<td><em>C. rodentium</em>, <em>C. jejuni</em>, chemically, and genetically IBD resulted in depletion of indigenous microbiota and promoted overgrowth of the <em>Enterobacteriaceae</em> family. Uninfected IL-10 KO mice had depleted #’s of <em>Bacteroides</em> species compared to WT</td>
<td><em>E.coli</em>: $4.7 \pm 3.4 \times 10^7$/g colon in untreated IL-10 KO to $4.7 \pm 6.8 \times 10^8$/g colon in DSS treated IL-10 KO mice</td>
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<tr>
<td>Bibiloni et al., 2005</td>
<td>IL-10 KO mice (129 Sv/Ev; germ-free)</td>
<td>Age: 10-12 weeks prior to exposure n = 5-6 mice for each time point/group</td>
<td>Colon samples collected from germ-free mice and 2 weeks or 5 weeks after exposure to microbiota of SPF mice. Microbial composition determined by PCR/DGGE</td>
<td>Composition of microbiota changed as colitis progressed. <em>Bacteroides</em> species, <em>Bifidobacterium animalis</em>, <em>Clostridium cocleatum</em>, enterococci were more apparent in colitic mice. <em>Eubacterium ventriusum</em>, Lactobacilli species</td>
<td>Not determined</td>
</tr>
<tr>
<td>Study</td>
<td>Mouse Model</td>
<td>Age Details</td>
<td>Sample Collection</td>
<td>Microbial Composition</td>
<td>Results</td>
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<td>Wohlgemuth et al., 2009</td>
<td>IL-10 KO and WT mice (129/SvEv)</td>
<td>Age: 1,8,16,24 weeks of age n= 20 mice per group</td>
<td>Intestinal contents (caecum and colon) collected from mice at the 4 different ages. Microbial composition determined by DGGE/FISH</td>
<td>Reduced bacterial diversity in IL-10 KO mice compared to WT. Two dominant bacterial species in IL-10 KO mice were Blautia producta and E. coli. E. coli were rarely detected in WT mice.</td>
<td>Total bacteria #/g caecal contents reached $10^{10}$ at 8 weeks, decreased with age. E. coli #/g caecal contents reached $10^7$ at 8 weeks and almost $10^9$ at 16 weeks (IL-10 KO mice)</td>
</tr>
<tr>
<td>Knoch et al., 2010</td>
<td>IL-10 KO and WT mice (C57BL/6J)</td>
<td>Age: 7, 8.5, 10, 12, 14 weeks of age n = 4-6 mice per group</td>
<td>Mice randomly assigned to be inoculated with a mixture of 12 Enterococcus faecalis and Enterococcus faecium strains. Caecal samples collected at 5 different ages. Microbial composition determined by DGGE/PCR</td>
<td>Total bacteria were higher in caecum of 12-week old IL-10 KO mice compared to 7-week old IL-10 KO. Total bacteria was higher in WT mice than IL-10 KO. Bacteroides-Prevotella-Porphyromonas spp. was higher in WT mice. More Enterococcus spp. was present in IL-10 KO compared to WT mice</td>
<td>Not determined</td>
</tr>
<tr>
<td>Wohlgemuth et al., 2011</td>
<td>IL-10 KO and WT mice (129/SvEv)</td>
<td>Age: 24 weeks n = 5 mice per group</td>
<td>Colonic and cecal contents collected from mice. Microbiota composition determined by PCR/FISH</td>
<td>Firmicutes phylum in IL-10 KO mice represented by <em>B. producta</em>, <em>Ent. gallinarum</em>, <em>C. innocuum</em>, <em>Robinsoniella peoriensis</em> and <em>L. murinus</em>. Bacteroidetes sequences were 25% higher in WT mice. Total bacteria and Bacteroidetes-Prevotella group higher in WT mice than IL-10 KO mice. High proportion of <em>E. coli</em> in IL-10 KO mice, while none in WT mice. <em>E. rectale-C. coccoides</em> cluster were more abundant in IL-10 KO mice</td>
<td>Total bacteria: $10^8$ – $10^9$ bacteria/g fecal matter for IL-10 KO mice; <em>E. coli</em>: $10^7$ bacteria/g fecal matter for IL-10 KO mice</td>
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WT: Wild-type; IL-10 KO: Interleukin-10 knockout; DSS: dextran sodium sulphate; FISH: fluorescent in-situ hybridization; PCR: polymerase chain reaction; SPF: specific pathogen free; DGGE: denaturing gradient gel electrophoresis
2.4 Vitamin D

Vitamin D has been long recognized as the sunshine vitamin, and can be classified as one of two seco-steroids: cholecalciferol (vitamin D$_3$) or ergocalciferol (vitamin D$_2$) [85]. Vitamin D$_3$ is from sources such as animal products or sunlight, while invertebrates, fungi and plants produce vitamin D$_2$ by UV irradiation [5]. Optimal vitamin D levels (serum 25(OH)D > 37.5 nmol/l) in early life have been found to prevent rickets in children, due to vitamin D’s role in calcium homeostasis [85]. Vitamin D status during pregnancy can also influence the health of the developing fetus. Infants of mothers with poor maternal vitamin D stores have been found to have retarded fetal growth, decreased mineral accretion, reduced postnatal linear growth and reduced weight gain [86]. Early life vitamin D status has also been implicated in the development of Th1 autoimmune diseases, schizophrenia, brain tumors, and cardiovascular health [87-89]. Moreover, summer season of birth (associated with higher vitamin D levels) was associated with a lower risk of later development of CD [87]. This suggests that there are critical windows of exposure to adequate vitamin D levels during fetal maturation, but further research needs to be conducted to help define these results and better understand long-term health outcomes of maternal and early postnatal vitamin D status in chronic diseases.

2.4.1 Metabolism

Vitamin D$_3$ is formed from ultraviolet B rays when the skin is exposed to sunlight, which then causes the photolysis of 7-dehydrocholesterol to previtamin D$_3$ [5]. Previtamin D$_3$ is then rearranged into vitamin D$_3$. Vitamin D can also be obtained from food, but very few foods naturally contain vitamin D. Some oily fish, such as salmon and mackerel, are an abundant source of vitamin D, but most dietary vitamin D comes from consumption of fortified milk products [85]. Before vitamin D can aid metabolism of calcium, including
intestinal absorption of calcium, it must first be activated to 25-hydroxyvitamin D (25(OH)D) in the liver by the mitochondrial enzyme 25-hydroxylase. 25(OH)D is the best indicator of vitamin D status as this is the form vitamin D is found in the blood, and it has a half-life of 3-4 weeks [85]. 25(OH)D is then converted into its active form, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), in the kidneys by 1α-hydroxylase [5]. Once formed, 1,25(OH)$_2$D travels to the small intestine and bone, the main sites of regulating serum calcium levels (Figure 2.2). Specific receptors in the small intestine (vitamin D receptors (VDR)) bind 1,25(OH)$_2$D to activate genes that enhance intestinal calcium absorption [5]. VDRs and/or 1α-hydroxylase have been identified in a wide range of cells, such as: enterocytes, osteoblasts, activated T and B lymphocytes, β-islet cells, and cells in most organs (brain, heart, skin, gonads, prostate, breast), and mononuclear cells [90]. If dietary calcium needs are not being met, 1,25(OH)$_2$D interacts with bone-forming cells (osteoblasts), which then stimulate the formation of bone-resorbing cells (osteoclasts) [5]. This process is responsible for removing calcium stores from the bone, which are then transferred to the blood to maintain normal blood calcium levels. This process also explains the role of vitamin D in bone health and the development of osteoporosis [5].

2.4.2 VDR signaling

1,25(OH)D initiates its biological responses through binding to the VDR. VDR is a DNA-binding transcription factor that is widely distributed in over 38 tissues and forms a heterodimer with a retinoid X receptor (RXR) [91]. This VDR-RXR heterodimer recognizes vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes. The optimal VDRE resembles the estrogen responsive element half-site [92]. The 1,25(OH)D-VDR complex regulates the expression of at least eleven genes
involved in bone and mineral homeostasis, as well as genes responsible for healthy aging, cell life, cancer, the immune system and metabolism [92]. VDR-KO mice have been found to have enhanced colonic proliferation, indicating the importance of VDR to promote cell differentiation and apoptosis and in reducing the risk of age-related epithelial cancers of the colon [93]. In the immune system, 1,25(OH)D-VDR activates the innate immune system to fight infection, represses the adaptive immune system and lowers the risk of autoimmune disease [92].

2.4.3 Recommended intake

Dietary recommended intakes (DRIs) of vitamin D are different for different stages of the life cycle. Generally, vitamin D levels are considered sufficient if serum levels are greater than 75 nmol/L [5] (Table 2.3). Adequate intakes (AIs) are listed as 600 IU/day from birth through 70 years of age; 800 IU/day if older than 70 years of age; and 600 IU/day for pregnant and lactating women [94]. Maternal DRIs are increased during pregnancy to maintain increased maternal intestinal calcium absorption, and to ensure extra calcium availability for fetal skeletal growth [4]. The concentrations of 25(OH)D and 1,25(OH)2D are generally low in the fetal blood compared to maternal blood, but at birth, umbilical cord 25(OH)D levels are directly correlated with maternal levels [95]. The tolerable upper limit (UL) of vitamin D is 4000 IU/day. However, research is suggesting that recommended intakes of vitamin D should be increased [96]. Studies are demonstrating health benefits of higher intakes of vitamin D (such as 1000 IU/day) that result in serum levels of 25(OH)D greater than 75 nmol/L [97]. The potential benefits of this increased intake are, but not limited to, reduced fracture risk, greater tooth retention, reduced risk of colon cancer and improved lower extremity function. The Canadian Pediatric Society recently published a position statement suggesting that mothers during
pregnancy with less than 1000 IU/day of vitamin D may be inadequate in maintaining optimal serum levels of 25(OH)D for both mothers and infants, and that consideration should be given to supplementing 2000 IU/vitamin D/day to women during pregnancy and lactation [98]. The new focus of this increased recommended intake of vitamin D is in preventing associated childhood and adult disease.

2.4.4 Sex differences in effects of vitamin D

It may be important to investigate if vitamin D supplementation affects males and females differently, or has a stronger effect in one gender over the other as research has indicated sex differences in vitamin D effects, particularly in MS patients. Recent research has discovered that in an animal model of experimental autoimmune encephalomyelitis (EAE), which is the animal model for MS, that only female mice were responsive to the vitamin D$_3$ intervention [99]. Further research also found that female mice that were ovariectomized were now unresponsive to vitamin D treatment [100]. This study also discovered that vitamin D treatment effects returned with low estrogen diestrus level implants (0.1 mg) of 17$\beta$-estradiol. High levels of diestrus implants (0.36 mg) were not used as this dose inhibited EAE independently of vitamin D. It was also shown that vitamin D$_3$ and 17$\beta$-estradiol work together to increase 17$\beta$-estradiol production and VDR expression in the inflamed central nervous system of the EAE mice [100].

Similar results have also been found in humans. A study found that the immunomodulatory effects of vitamin D were significantly stronger in females than in males [101]. This finding was observed in MS patients as well as healthy subjects. Specifically, the effect of vitamin D on production of IFN-$\gamma$ and IL-17 was significantly decreased in females compared to males. While production of IL-10 was significantly
increased in females compared to males. The study also found that the female and male subjects had similar 25(OH)D and 1,25(OH)D levels [101]. The investigators tried to determine why females had a stronger inhibition of T cell proliferation and found that the T cells from females and males synthesized 1,25(OH)D at similar rates, but that females inactivated 1,25(OH)D at a slower rate, resulting in accumulation in self-reactive T cells. This is interesting as osteoporosis in post-menopausal women has been attributed to estrogen deficiency [102]. Estrogen deficiency was found to stimulate osteoclastogenesis through increasing production of TNF-α and receptor activator of nuclear kappa-β ligand (RANKL), thus indicating T cells contributed to bone loss induced by estrogen deficiency [102].

Vitamin D and estrogenic compounds may also work together in the colon. Observational research showed that women who have ever used hormone replacement therapy have a 20% reduction in colon cancer incidence [103]. As mentioned earlier, the protective effects of estrogens may be partly mediated by vitamin D. Studies have found that estradiol treatment increased VDR and 1-α-hydroxylase mRNA levels in rectal mucosal biopsies from postmenopausal women, as well as in colonic cell lines [104, 105]. Furthermore, the phytoestrogen genistein was found to enhance VDR expression in colon cancer cell lines [106].

This provides evidence that females may be more sensitive to the anti-inflammatory effects of vitamin D, due to the connection between estrogenic compounds, 1,25(OH)D degradation and VDR expression, especially those suffering from MS/EAE. The evidence showing similar effects in the colon and colon cancer cell lines suggests this may also occur in females with IBD, due to the similar inflammatory process between MS and IBD. IBD patients are also at a higher risk of developing colon cancer [107],
indicating estrogen may be involved in these diseases.
Table 2.3: Classification of vitamin D status [97, 108]

<table>
<thead>
<tr>
<th>Vitamin D Status</th>
<th>Serum 25(OH)D (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>&lt; 27.5 nmol/L</td>
</tr>
<tr>
<td>Insufficient</td>
<td>&gt; 27.5 nmol &amp; &lt; 50 nmol/L</td>
</tr>
<tr>
<td>Sufficient</td>
<td>&gt; 50 nmol/L</td>
</tr>
<tr>
<td>Optimal</td>
<td>&gt; 75 nmol/L</td>
</tr>
</tbody>
</table>
Figure 2.2: Vitamin D Metabolism
2.5 Vitamin D and IBD

2.5.1 Animal studies

There is increasing evidence that vitamin D plays a role in the development and severity of IBD, with the majority of evidence from animal models of IBD. Cantorna and colleagues [8] have conducted the most research in this area. In one study, IL-10 KO mice and wild-type (WT) mice were subjected to one of three experiments involving vitamin D supplementation. In experiment one, three-week old vitamin D deficient mice (dietary amounts not listed) were either maintained vitamin D deficient or subjected to an experimental diet which included 5 µg of vitamin D\textsubscript{3} per day (which is vitamin D sufficient). All animals were on a high calcium diet (1g/100g of diet). Normal calcium levels in the diet are 0.05g/100g of diet, and this was the amount used in our study. The severity of IBD development was compared to vitamin D deficient and vitamin D sufficient in IL-10 KO and WT mice [8]. In experiment two, three-week old vitamin D deficient mice were either maintained on the vitamin D deficient diet or were supplemented with 0.005 mcg of 1,25(OH)\textsubscript{2}D\textsubscript{3}/day, the activated form of vitamin D. The mice were then killed four weeks later. In experiment three, 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment was started at the first signs of IBD symptoms (such as diarrhea, which typically occurred around seven weeks of age). These seven-week old vitamin D deficient mice were then divided into two groups. One group was maintained vitamin D deficient and the other group was supplemented with 0.02 mcg of 1,25(OH)\textsubscript{2}D\textsubscript{3} for two weeks [8].

In experiment one, vitamin D deficient IL-10 KO mice began to die at seven weeks of age, and by nine weeks of age, 58% of the vitamin D deficient IL-10 KO mice had died. The vitamin D sufficient IL-10 KO mice and the vitamin D deficient WT mice appeared healthy, even at 13 weeks of age [8]. The vitamin D deficient IL-10 KO mice were also
more growth retarded than the other mice (Table 2.4). Results from experiment two showed that vitamin D deficient IL-10 KO mice had significantly more inflammation in their small intestine than the 1,25(OH)\(_2\)D\(_3\) supplemented mice. WT mice that were vitamin D sufficient or insufficient did not have any signs of inflammation in the small intestine [8]. In experiment three, inflammation of the small intestine of the IL-10 KO mice was reduced after two weeks of 1,25(OH)\(_2\)D\(_3\) treatment. Vitamin D deficient IL-10 KO mice also had a histology score (ranked 0-5; 0 being no inflammation and 5 being massive inflammation) of 3.0 ± 0.2, the vitamin D sufficient IL-10 KO mice had a histology score of 1.7 ± 0.4, and the WT mice of both groups had a histology score of 0 [8]. Serum vitamin D levels were not measured in this study, however, serum calcium (mmol/L) was measured. The IL-10 KO mice on the vitamin D deficient diets and 1,25(OH)\(_2\)D\(_3\) treated had serum calcium levels of 1.74 ± 0.28 and 3.00 ± 0.3, respectively. The WT mice on the vitamin D deficient and 1,25(OH)\(_2\)D\(_3\) treated had serum calcium levels of 1.67 ± 0.32 and 2.72 ± 0.32 (Table 2.5).

A later study randomized four-week old IL-10 KO mice to one of two groups: a 1,25(OH)\(_2\)D\(_3\)-supplemented diet (0.005 mcg/day) or a vitamin D deficient diet (vitamin D amounts not listed, this is the same diet as previous study by Cantorna et al [8]. In other experiments, the mice were randomly assigned to one of four different diets: 1) low calcium (no added calcium), 2) low calcium + 1,25(OH)\(_2\)D\(_3\) (0.02 mcg/day), 3) high calcium (2.2%), or 4) high calcium (2.2%) + 1,25(OH)\(_2\)D\(_3\) (0.02 mcg/day). The mice remained on these diets until they were killed at nine weeks of age [9]. The most severe histopathology scores were from the mice in the low-calcium group, and that 1,25(OH)\(_2\)D\(_3\) treatment resulted in a decrease in the histopathology scores, regardless of dietary calcium. Treatment with 1,25(OH)\(_2\)D\(_3\) to vitamin D deficient IL-10 KO mice was
effective in increasing serum calcium levels and reducing IBD severity, regardless of
dietary calcium [9]. The low calcium group had a 100% incidence of IBD, while the low
calcium and 1,25(OH)\(_2\)D\(_3\) supplemented, and high calcium groups and 1,25(OH)\(_2\)D\(_3\)
supplemented groups had incidence rates of IBD of 63-64%. The mice treated with high
calcium and 1,25(OH)\(_2\)D\(_3\) had the lowest incidence rate of IBD (11.1%). LITAF gene
expression in whole colon tissue of the IL-10 KO mice was reduced by 1,25(OH)\(_2\)D\(_3\)
supplementation. Several genes associated with TNF-\(\alpha\) (IkB\(\varepsilon\), TNF-\(\alpha\) induced protein 2,
TNF-\(\alpha\) receptor 1, LITAF, TNF-\(\alpha\) itself) were down-regulated by 1,25(OH)\(_2\)D\(_3\) treatment
in the colonic tissue of IL-10 KO mice [9]. IkB\(\varepsilon\) is present in T cells, and is an inhibitor
of nuclear factor kappa B (NF-\(\kappa\)B), which is needed to activate TNF-\(\alpha\). Inhibition of
IkB\(\varepsilon\) would result in direct effects on the TNF-\(\alpha\) production via suppression of NF-\(\kappa\)B
[109]. The NF-\(\kappa\)B pathway is a 1,25(OH)\(_2\)D\(_3\) target in T cells, monocytes, and dendritic
cells. TNF-\(\alpha\)-inducing protein 2 is a TNF-\(\alpha\)-inducing protein produced by endothelial
cells and lymphoid tissues. The 1,25D\(_3\)-mediated inhibition of these genes suggests that
the TNF-\(\alpha\) pathway is an important in vivo vitamin D target [9]. LITAF is a transcription
factor that has been shown to bind to the TNF-\(\alpha\) promoter, and LITAF alone was able to
increase TNF-\(\alpha\) production [109]. 1,25D\(_3\) treatment inhibited the expression of LITAF in
the colons of IL-10 KO mice and the VDR/IL-10 KO mice with the most severe form of
IBD showed the highest expression of LITAF [9]. Overall, this study shows that the
TNF-\(\alpha\) pathway is central to IBD in the IL-10 KO mouse, and that 1,25(OH)\(_2\)D\(_3\)
treatment reduced the ability of the IL-10 KO mouse to produce and respond to TNF-\(\alpha\),
ultimately resulting in less severe IBD (Table 2.5).

A more recent study also found beneficial effects of vitamin D supplementation in a
mouse model of colitis. Three-week old C57BL/6 mice were either raised on a normal
diet (vitamin D sufficient) or on a vitamin D insufficient diet for six weeks (vitamin D amounts not listed). Colitis was then induced in the mice by administration of dextran sodium sulphate (DSS) in the drinking water [70]. Mice receiving only tap water served as controls. Results of this study observed that there was an increased severity of colitis in the DSS-treated vitamin D deficient mice relative to DSS-treated vitamin D sufficient mice (Table 2.4). Results revealed the presence of severe ulceration, granulation, and inflammation in DSS-treated mice on the vitamin D sufficient diet, but this was exacerbated even further in the DSS-treated vitamin D deficient mice [70]. Results also showed a significant increase in altered gene expression in vitamin D deficient mice colon tissue compared to vitamin D sufficient mice. One gene in particular, angiogenin-4 (Ang4), was down regulated in the vitamin D deficient mice. This was further confirmed by immunohistochemistry. Ang4 is an antimicrobial peptide that is involved in host control of enteric bacteria. Analysis of the presence of bacterial 16s rDNA in colon tissue also revealed higher levels of bacterial infiltration in vitamin D deficient mice compared with vitamin D sufficient mice, suggesting that vitamin D can regulate colonic genes that can directly affect the gut microflora [70]. These findings suggest that vitamin D deficiency has similar effects on DSS-induced colitis as VDR or CYP27B1 gene knockout, and that vitamin D deficient mice showed dysregulated colonic containment of enteric bacteria, which may be a key mechanism that predisposes these mice to colitis. The results also suggest that vitamin D deficiency alone is sufficient to alter expression of key colonic genes, which may increase susceptibility to colitis or enhance severity of the disease [70].

In another recent study using a DSS-colitis induced model, C57BL/6 mice were treated intra-rectally with 1 g/kg of BXL-62 (an anti-inflammatory VDR agonist) or with 0.3
The results of this study found that symptoms and signs of colitis in mice treated with BXL-62 were significantly lower compared to the treatment with \(1,25(OH)_2D_3\) (Table 1.5). The results observed that daily intra-rectal treatment of BXL-62 induced a significant improvement in stool consistency and in visible fecal blood, while \(1,25(OH)_2D_3\) treatment improved stool consistency only early on in the disease course, but that \(1,25(OH)_2D_3\) treatment did ameliorate the bloody stool score [110]. \(1,25(OH)_2D_3\) treatment also failed to improve colon lesions, while BXL-62 significantly decreased lesion extension in the left colon. The colon of \(1,25(OH)_2D_3\)-treated mice had less severe inflammation compared to controls, but BXL-62-treated mice had even less inflammation than their \(1,25(OH)_2D_3\)-treated counterparts. The results of this study demonstrate that BXL-62 presents a higher potency in ameliorating IBD symptoms compared to \(1,25(OH)_2D_3\) [110]. Although \(1,25(OH)_2D_3\) treatment reduced symptoms of colitis in the DSS-treated mouse model, results showed that VDR agonists might be more effective in treating IBD.

The importance of vitamin D receptors in controlling gastrointestinal immunity was also observed in another study in which IL-10 KO, VDR and IL-10 double KO (DKO), VDR KO, and WT mice were studied. DKO mice presented with the most severe IBD that involved the entire intestinal tract [10]. Cytokine expression was measured by RT-PCR in all mice, and WT mice did not express any cytokines in their colon. VDR KO mice expressed IL-1\(\beta\) in the colon as well as faint TNF-\(\alpha\) bands (Froicu et al., 2006). IL-10 KO mice expressed TNF-\(\alpha\), IL-2, and IFN-\(\gamma\) but not IL-1\(\beta\) in the colon, and DKO mice expressed two to three-fold higher levels of IL-1\(\beta\), IL-2, IFN-\(\gamma\) and TNF-\(\alpha\) compared with single KO mice. This study showed that the immune system alterations in the DKO mice are a severe form of IBD and over-expression of inflammatory cytokines in the
colon. The disease in IL-KO mice with functional VDRs is less pronounced, suggesting that VDR deficiency exacerbates IBD severity [10].

Overall, the results of these animal studies support the hypothesis that vitamin D treatment/supplementation can prevent or ameliorate signs and symptoms of IBD. The effects of vitamin D supplementation appeared to further positively influence IBD symptoms when in conjunction with high calcium. However, some symptoms of IBD were treated with $1,25(OH)_2D_3$ regardless of dietary calcium. VDR deficiency was also found to exacerbate IBD symptoms and VDR agonists were found to have a higher effect on IBD than vitamin D treatment. However, vitamin D treatment was still found to positively benefit mice with IBD symptoms. The serum vitamin D levels were only measured in one study, which makes comparing the results of each study difficult, particularly with my study that did measure vitamin D status of the animals.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain and Species</th>
<th>Sample size</th>
<th>Study Design</th>
<th>Dose</th>
<th>Findings</th>
<th>Serum 25(OH)D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lagishetty et al., 2010</td>
<td>C57BL/6 mice; control or treated with DSS</td>
<td>n = 16 mice per group</td>
<td>Fed diet for 6 weeks from weaning</td>
<td>Vitamin D sufficient or deficient (dose not provided)</td>
<td>Increased severity of colitis in DSS-treated vitamin D deficient mice.</td>
<td>Vitamin D-deficient DSS treated mice showed decreased levels (2.5 ± 0.1 vs. 24.4 ± 1.8 ng/ml or 6.26 vs. 61 nmol/L)</td>
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<td></td>
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<td>Ang4 down-regulated in vitamin D deficient mice</td>
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<td></td>
<td>Higher levels of bacterial infiltration in vitamin D-deficient mice compared to controls</td>
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<td></td>
<td></td>
<td>Vitamin D-deficient IL-10 KO mice: growth retarded compared to vitamin D sufficient mice (WT and IL-10 KO)</td>
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<td></td>
<td></td>
<td></td>
<td>Vitamin D deficient IL-10 KO mice began to die at 7 weeks of age</td>
<td></td>
</tr>
<tr>
<td>Cantorna et al., 2000</td>
<td>IL-10 KO mice; wild type mice (WT)</td>
<td>n = 10 vitamin D sufficient IL-10 KO; 26 vitamin D deficient IL-10 KO; and 20 WT mice</td>
<td>3-week old vitamin D-deficient mice maintained vitamin D-deficient or vitamin D sufficient</td>
<td>Vitamin D deficient or food with 5.0 mcg 1,25(OH)2D3</td>
<td>Not measured</td>
<td></td>
</tr>
</tbody>
</table>

1 Pregnant dams were fed vitamin D deficient diet to ensure weanlings would be vitamin D deficient by 5 weeks of age.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain and Species</th>
<th>Sample size</th>
<th>Study Design</th>
<th>Dose</th>
<th>Findings</th>
<th>Serum 25(OH)D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantorna et al., 2000</td>
<td>IL-10 KO mice; WT mice</td>
<td>n = 7 vitamin D deficient IL-10 KO; 9 vitamin D supplemented IL-10 KO; and 5 WT mice</td>
<td>3-week old vitamin D deficient mice(^1) divided into two groups</td>
<td>Mice maintained vitamin D deficient or supplemented with 0.005 ( \mu \text{g/d} ), 1,25(OH)(_2)D(_3).</td>
<td>Vitamin D-deficient IL-10 KO mice had significantly more intestinal inflammation than their vitamin D-sufficient counterparts</td>
<td>Not measured</td>
</tr>
<tr>
<td>Cantorna et al., 2000</td>
<td>IL-10 KO mice; wild type mice</td>
<td>n = 7/per group for IL-10 KO; 4/per group for WT mice</td>
<td>7-week old vitamin D-deficient mice(^1) (diarrhea symptoms) divided into two groups</td>
<td>Mice maintained vitamin D deficient or supplemented with 0.2 ( \mu \text{g/d} ), 1,25(OH)(_2)D(_3).</td>
<td>Intestinal inflammation was reduced 2 weeks after vitamin D-sufficient diet treatment in IL-10 KO mice</td>
<td>Not measured</td>
</tr>
<tr>
<td>Laverny et al., 2010</td>
<td>C57BL/6 mice; colitis induced by DSS administration</td>
<td>n = 10 mice/per group</td>
<td>Mice were divided into two groups 1 day before DSS administration</td>
<td>1. Treated with 1g/kg BXL-62 (anti-inflammatory VDR agonist), 2. Treated with 0.3g/kg 1,25(OH)(_2)D(_3), both</td>
<td>Weight loss and diarrhea was significantly lower in BXL-62 treated mice compared to 1,25(OH)(_2)D(_3) treated mice</td>
<td>Not measured</td>
</tr>
</tbody>
</table>
daily intra-rectal treatments | analysis, 1,25(OH)\textsubscript{2}D\textsubscript{3} failed to improve colon lesions

\hline

\textsuperscript{1}Pregnant dams were fed vitamin D deficient diet to ensure weanlings would be vitamin D deficient by 5 weeks of age
2.5.2 Human Studies

The strongest evidence for a role of vitamin D in IBD occurrence and severity has been found in mouse models of IBD. However, there is some evidence in humans suggesting vitamin D deficiency and/or supplementation may be implicated in this disease.

Studies have found that patients with IBD are vitamin D deficient or insufficient [11, 111]. One recent study found that at baseline, 70% of patients with quiescent CD were vitamin D deficient or insufficient [111]. This study also examined absorption of vitamin D in healthy and CD patients using a novel vitamin D bioavailability test and found that CD patients had on average a 30% decrease in their ability to absorb vitamin D [111].

A geographical variation in the incidence of IBD (according to latitude of residence) has also been discovered. One prospective study examining data from the Nurses’ Health Study found that incidence of IBD was significantly lower among the women who lived in more southern latitudes compared to women living in northern latitudes in the United States, with residence later in life (>30 years) being more strongly related to risk [112]. This finding suggests that environmental factors, such as sunlight exposure, may be involved in the development of this disease. Another prospective study that also examined data from the Nurses’ Health Study examined vitamin D status in relation to risk of IBD. The results found that a higher predicted serum plasma level of 25(OH)D significantly reduced the risk of incident for CD, but not UC colitis, in women [113]. Overall, these studies suggest that vitamin D may be an important mediator in the pathogenesis of IBD but it is currently not known if vitamin D deficiency is a cause or secondary outcome of the disease.

To date, there has only been one clinical trial published investigating the effects of vitamin D supplementation on disease activity in IBD. In this randomized, double-blind, placebo-controlled trial, 94 CD patients were randomized to receive either 1200 IU of
vitamin D or a placebo per day, for one year [114]. Supplementation with vitamin D increased serum vitamin D levels and reduced the risk of relapse from 29% in the placebo group to 13% in the supplemented group. While this difference was not statistically significant, the finding does suggest that vitamin D supplementation may be beneficial to those suffering from CD. The promising evidence from animal studies, prospective data and the clinical trial provide a basis for more research to be conducted in this area, and for larger clinical trials to further elucidate the potential benefits of vitamin D in this disease.

2.6 Mouse model

The interleukin-10 KO (IL-10 KO) mouse has been studied extensively to investigate potential drug and dietary treatments for IBD, such as vitamin D [8]. The IL-10 KO mouse was used in this study as these mice will spontaneously develop inflammatory bowel disease (IBD) like symptoms after weaning (approximately 6-8 weeks of age) that resembles CD found in humans [10]. IL-10 is an anti-inflammatory cytokine in which the function of T-regulatory cells depends on [115]. This dysregulation of the immune system leads to failed or impaired T cells and an excessive generation of interleukin-12 (IL-12) and interferon-γ (IFN-γ), which ultimately leads to IBD like symptoms, such as chronic intestinal inflammation [116]. Like patients with CD, patchy ulceration, transmural acute and chronic inflammation and epithelial hyperplasia develop in the IL-10 deficient mice [117]. As previously discussed, the IL-10 KO mouse also has significant alterations in the species and levels of bacteria colonizing the gut compared to control mice raised in the same environment, and this has also been seen in CD patients. IL-10 KO mice also have been shown to have increased levels of tumor necrosis factor-α (TNF-α) [118] and both IFN-γ and TNF-α have been shown to disrupt the intestinal epithelial barrier. A disrupted intestinal epithelial barrier allows increased antigenic uptake and continuous stimulation of the mucosal immune system [116]. This has also
been seen in patients with CD [119]. Elevated levels of the proinflammatory mediators IL-1β and IL-6 have also been seen in the IL-10 KO model [120], and these proinflammatory markers have also been observed in humans with IBD [35].

IL-10 KO mouse models do not develop chronic colitis when raised in germ-free environments; therefore, conventional housing was required in this study for IBD to progress [121]. This model is also preferred over other mouse models, such as invasive chemically-induced colitis models, as the mice do not develop dramatic symptoms such as diarrhea [121]. This mouse model is ideal for this study because along with intestinal inflammation, research from our lab discovered that IL-10 KO mice are prone to impaired bone metabolism [120]. Impaired bone metabolism, such as lower bone mineral density (BMD), has also been seen in humans with IBD [122]. Our lab investigated the effects of vitamin D supplementation on bone strength during intestinal inflammation in this model to determine if supplementation could also improve bone outcomes in these mice. Based on these findings, the IL-10 KO mouse model can be used for studying intestinal inflammation, and we used this model to determine if early intervention with vitamin D can attenuate or prevent intestinal inflammation in male and female mice.
CHAPTER THREE

RATIONALE, OBJECTIVES AND HYPOTHESES
3.0 RATIONALE, OBJECTIVES AND HYPOTHESES

3.1 Rationale

IBD dramatically lowers the quality of life of affected individuals and there is no cure for this disease [30]. Individuals with IBD are also at an increased risk of premature death, because IBD is a life-long disease requiring medications and often frequent surgeries [30]. Each year, 114 deaths in Canada are attributed to CD and UC. The economic burden for direct and indirect costs for IBD is estimated to be $1.8 billion each year [29]. Therefore, a nutritional intervention aimed at preventing and protecting against this disease is essential. The role of vitamin D in autoimmune diseases is beginning to be established [7, 8] and based on the research findings, it is anticipated that supplementation with vitamin D in utero and during early life will down-regulate the expression of genes related to inflammation in the colon and ultimately attenuate intestinal inflammation.

Understanding the role of vitamin D in intestinal microbial composition is in its infancy. This thesis research will observe how vitamin D may modulate the gut microbiota in a disease model state. Furthermore, our study investigated the dietary form of vitamin D₃ to determine if the same effect can be obtained as what was found with the pharmacological form (1,25(OH)₂D₃). Another novel aspect of this study is the prevention aspect, determining if early nutrition can influence gastrointestinal health and microbial composition, as well as protect against disease. Positive findings from this study may provide an important base for prospective studies in humans.
3.2 Objectives

- **Study 1:** To determine if exposure to high levels of vitamin D *in utero* and throughout adulthood prevents and protects against the development of intestinal inflammation by modulating the inflammatory response and gut microbiota in the IL-10 KO mouse model.

- **Study 2:** To determine potential mechanisms of action of vitamin D supplementation on markers of the inflammatory process and intestinal health the proximal colon in the IL-10 KO mouse model.

3.3 Hypotheses

- **Study 1:** Because beneficial effects of dietary and pharmacological doses of vitamin D after weaning on intestinal inflammation have previously been reported, it is hypothesized that IL-10 KO mice exposed to dietary supplemental levels of vitamin D early in life (*in utero* and suckling and continued throughout adulthood) will be protected against the development of intestinal inflammation. I hypothesize that vitamin D exerts its beneficial effects through decreasing proinflammatory cytokines and supporting health-promoting species in the gut microbiota.

- **Study 2:** Because vitamin D has been found to regulate many genes involved in intestinal health, inflammation and microbial control, I hypothesize that vitamin D supplementation will down-regulate genes associated with inflammation and up-regulate genes associated with intestinal health and microbial control.
CHAPTER FOUR

MATERIALS AND METHODS
4.0 MATERIALS AND METHODS

4.1 Animals and Diet

Six breeding pairs of 129/SvEv IL-10 KO mice were received from Dr. Richard Fedorak, University of Alberta. Upon arrival, the breeding pairs were caged together in standard environment conditions (12 hour light: 12 hour dark cycle, room temperature of 23°C) and fed AIN93G diet (which contains vitamin D levels of 1000 IU/kg of diet) \( \text{(Table 4.1)} \). Incandescent lighting was used in the room instead of fluorescent lighting to ensure that the room was free of UVB radiation. Female offspring were weaned at postnatal day (PND) 29 and randomized to one of two diets: 1) vitamin D level of 5000 IU/kg diet (high vitamin D) or 2) vitamin D level of 25 IU/kg diet (low vitamin D). Mice were weaned a week later than usual (PND 21) because these mice are much smaller and frailer than WT mice. At 7-8 weeks of age, female offspring were mated harem style (3 females per 1 male). This duration of time prior to mating allowed the female mice to obtain appropriate levels of serum 25(OH)D that were high (200 nmol 25(OH)D/L) or low (30 nmol 25(OH)D/L). Male offspring consumed the same diet as the females during breeding and females consumed their respective diet during pregnancy and lactation. Pregnancy was determined by heavier body weight and overall appearance of the females. Pups born from the vitamin D high or low mothers were weaned at PND 29 and randomized to either continue on the same diet that their mother received through pregnancy and lactation or the other diet studied, resulting in four vitamin D interventions: High/High (HH), High/Low (HL), Low/High (LH) and Low/Low (LL) \( \text{(Figure 3)} \). At PND 29, pup gender was determined by anogenital distance. Dams were euthanized and tissues collected once pups were weaned. Food intake and body weights were obtained once a week. Mice were studied until necropsy at 3 months of age \( \text{(Figure 4.1)} \), which represents young adulthood in mice \( (n = 15 - 25/group/gender) \). The dietary vitamin D levels were selected based on a study conducted by Fleet et al [123]. In this study, mice were fed AIN93G diets containing 400, 1000, 5000, 10000, or 20000 IU.
vitamin D/kg diet from weanling until ten weeks of age, at which time they were euthanized and serum and tissues were collected [123]. Feeding mice 5000 IU vitamin D/kg diet resulted in 200 nmol/L serum 25(OH)D (representing high 25(OH)D) and feeding mice 25 IU vitamin D/kg diet resulted in serum 25(OH)D levels of approximately 35 nmol vitamin D/L (representing low vitamin D status). Therefore, 5000 IU and 25 IU/kg/diet were chosen for this study.

Fecal samples were collected prior to necropsy at 3 months of age. The fecal samples were stored at -80°C for later investigation of microbial composition. The mice were euthanized by carbon dioxide and cervical dislocation. Blood was collected via cardiac puncture immediately upon death. Following this, the whole intestine was immediately removed. The large intestine was cut in two halves corresponding to ascending (proximal) and descending (distal) colon. The distal 1/8th of the small intestine corresponding to the ileum, the proximal 1/8th corresponding to the duodenum and caecum were also collected. Contents were washed out with ice-cold sterile saline solution (0.9% NaCl) and stored at -80°C for microbial composition analysis. A one cm portion from the distal colon, proximal colon, distal ileum and proximal duodenum were cut and fixed in 10% phosphate-buffered formalin for inflammation severity analysis. The remaining intestinal tissues were cut in half and snap frozen in liquid nitrogen, and then stored at -80°C for later analysis of gene expression in the proximal colon.

4.2 Analysis of diets

To confirm that the insufficient (low) and supplemented (high) intervention diets had the appropriate levels of vitamin D, samples of the two diets were sent to Maxxam Analytics for analysis (Mississauga, ON, Canada). Analysis was performed in a blinded manner as the diets were labeled as ‘A’ or ‘B’ vitamin D. The vitamin D level of the low diet was undetected as the analysis had a reportable detection limit of 200 IU vitamin D/kg diet.
The vitamin D level of the high diet was 4,730 IU vitamin D/kg diet, close to the desired amount of 5,000 IU vitamin D/kg diet.

4.3 Sex specific analysis

In this study, analyses of histological inflammation severity, serum 25(OH)D and the gut microbiota composition were measured in both male and female IL-10 KO mice. IL-8 concentrations and microarray analysis were further outcomes measured in male mice. We chose to do these further analyses in the male mice to complement another study conducted in our lab. Raha Jahani examined the gut microbiota composition and gene expression in healthy male mice and discovered 104 genes differentially expressed between the high and low vitamin D3 interventions. Therefore, we wanted to determine if similar results would be found in a disease model; and further investigate if exposure to high vitamin D in early-life could regulate gene expression later in life.
Table 4.1: Composition of AIN93G purified rodent diet and modified AIN93G diet containing high or low levels of vitamin D

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude Protein</td>
<td>%</td>
<td>17.9</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>%</td>
<td>7.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>%</td>
<td>4.8</td>
</tr>
<tr>
<td>Moisture</td>
<td>%</td>
<td>7.0</td>
</tr>
<tr>
<td>Ash</td>
<td>%</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>%</td>
<td>0.70</td>
</tr>
<tr>
<td>Lysine</td>
<td>%</td>
<td>1.48</td>
</tr>
<tr>
<td>Methionine</td>
<td>%</td>
<td>0.56</td>
</tr>
<tr>
<td>Cystine</td>
<td>%</td>
<td>0.30</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>%</td>
<td>0.21</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>%</td>
<td>1.76</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>%</td>
<td>1.14</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>0.96</td>
</tr>
<tr>
<td>Tyrosine</td>
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<tr>
<td>Threonine</td>
<td>%</td>
<td>0.76</td>
</tr>
<tr>
<td>Valine</td>
<td>%</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/kg</td>
<td>5000</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg/kg</td>
<td>1561</td>
</tr>
<tr>
<td>Potassium</td>
<td>mg/kg</td>
<td>3600</td>
</tr>
<tr>
<td>Sodium</td>
<td>mg/kg</td>
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</tr>
<tr>
<td>Magnesium</td>
<td>mg/kg</td>
<td>507</td>
</tr>
<tr>
<td>Iron</td>
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</tr>
<tr>
<td>Zinc</td>
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<td>Manganese</td>
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<td>10</td>
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<tr>
<td>Copper</td>
<td>mg/kg</td>
<td>6.0</td>
</tr>
<tr>
<td>Iodine</td>
<td>mg/kg</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>IU/g</td>
<td>4.00</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>IU/g</td>
<td>1.00 or 0.025 or 0.0005</td>
</tr>
<tr>
<td>Alpha-Tocopherol</td>
<td>IU/g</td>
<td>75.00</td>
</tr>
<tr>
<td>Thiamine</td>
<td>mg</td>
<td>5.0</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Unit</td>
<td>Value</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>mg</td>
<td>6.0</td>
</tr>
<tr>
<td>Niacin</td>
<td>mg</td>
<td>30</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>mg</td>
<td>15.0</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>mg</td>
<td>6.0</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>mg</td>
<td>2.0</td>
</tr>
<tr>
<td>Biotin</td>
<td>mcg</td>
<td>200</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>mcg</td>
<td>25.0</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>mcg</td>
<td>750</td>
</tr>
</tbody>
</table>

**Gross Energy**  
Kcal/gm  
3.80

† Vitamin D content of unmodified AIN93G diet  
†† Vitamin D content of modified low vitamin D AIN93G diet  
††† Vitamin D content of modified high vitamin D AIN93G diet
4.4 Intestinal Histology Assessment

Intestinal tissue samples that were fixed in 10% phosphate-buffered formalin were embedded in paraffin. Five µm sections from the proximal colon were stained with hematoxylin and eosin (H&E) for light microscopy (400x power). Slides were analyzed blinded to vitamin D intervention (A.G.), and the degree of intestinal inflammation was assessed on a scale of 0-4 as previously described and validated [120]).

0 represents no inflammation, 1 represents minimal evidence of inflammatory infiltrate, 2 represents significant evidence of inflammatory infiltrate (such as cryptitis and crypt abscesses), 3 represents significant evidence of inflammatory infiltrate with goblet cell depletion, and 4 represents severe inflammation characterized by widespread infiltration with inflammatory cells, mucosal thickening, submucosal cell infiltration, a decrease in goblet cells and destruction of architecture [120]. A minimum of 3 sections (one transverse, two longitudinal) per proximal colon and 5 fields per section (15 scores in total) were examined to determine the mean histological inflammatory score for each mouse.

4.5 Proinflammatory Cytokines

To quantify KC, one-half segment of colonic tissues from the male mice were individually homogenized in PBS buffer supplemented with protease inhibitors (pH 7.4) and centrifuged at 4°C at 1800 rpm for 10 min. The Quantikine Colorimetric Sandwich ELISA kit, specific for KC (Quantikine Mouse CXCL1/KC Immunoassay, R&D systems, Minneapolis, MN), was employed following the manufacturer’s instructions. Protein levels for KC were measured in duplicates and expressed relative to total protein. Protein absorbance was measured using a microplate reader set at 450nm and concentrations were calculated from a pre-constructed standard curve.
4.6 Microbiota Composition Analysis

DNA was extracted from the fecal and colonic contents samples using the E.Z.N.A. Stool DNA Isolation Kit (Omega, USA), following manufacturer’s instructions modified to include a lysozyme digestion step at 37°C for 30 minutes. DNA from fecal samples of female and male mice at 3-months of age was extracted from each of the four groups (study 1). DNA from proximal colon contents of males at 3-months from the HH and LL groups were also extracted. DNA concentration and purity were spectrophotometrically assessed (OD260/280, 260/230), using Thermoscientific’s Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Microbiota compositions of fecal and colonic contents were determined by quantitative Real-Time PCR (qPCR) using an Applied Biosystems’ 7900 HT thermocycler equipped with a 384-wells block (Applied Biosystems, USA). 10 ng of DNA was used in each qPCR reaction (total volume: 10µl) together with the TaqMan Gene Expression Master Mix and custom made TaqMan assays targeting specific 16S rRNA sequences for total bacteria, Bacteroidetes, Bifidobacteria, Clostridium coccoides and Clostridium leptum (Table 4.2) (Applied Biosystems, USA). Power SYBR Green Master Mix and SYBR Green assays targeting 16s rRNA sequence for Escherichia coli was also used (Table 4.2). The amplifications were performed as follows: 2 min at 50°, 10 min at 95°, then 95° for 15 seconds and 60° for 60 seconds. For the SYBR Green assays, a disassociation step was added at the end of the amplification to confirm primers specificity (95° for 15 seconds, 60° for 15 seconds and 95° for 15 seconds). qPCR results were translated into bacteria counts using pre-constructed standard curves and were subsequently transformed to log10 colony forming units (CFU) per gram of wet feces/colonic contents for statistical analysis. The added Clostridium leptum and Clostridium coccoides counts were used as a proxy for Firmicutes.
Table 4.2: TaqMan/SYBR Green assays (primers and probe sets) used in this study

<table>
<thead>
<tr>
<th>Target Bacteria</th>
<th>Primers and probes sequences (5’ – 3’)</th>
</tr>
</thead>
</table>
| Total Bacteria [124]        | Forward: CGGTGAATACGTTCCCGG  
Reverse: TACGGCTACCTTGTTACGACTT  
Probe: CTITGACACCGGCCCCGTC |
| Bifidobacteria [125]        | Forward: CGGGTGAGTAATGCCTGACC  
Reverse: TGATAGGACGCGCCACCTCCA  
Probe: CTCTGGAAACCGGTTG |
| Bacteroidetes [125]         | Forward: CTTTCGATGGATAGGGGT  
Reverse: CACGCTACTTGGCTGGTTCAG  
Probe: AAGTCCCACATTG |
| *Clostridium coccoides* [125] | Forward: GACGCCGCGTGAGGGA  
Reverse: AGCCCCAGCCTTTGACCTAC  
Probe: CGGTACCGTAATAGGAGA |
| *Clostridium leptum* [125]  | Forward: CTTCCGTCGCGCGAAGTAA  
Reverse: GAATTAACACATCCTCACTTGCTT  
Probe: CAAATAAGCAGAATTCACC |
| *Escherichia coli* [125]    | Forward: CATGCCGCGTGATGAAGAA  
Reverse: CGGGATACGTAATGAGCAA  
Probe: CTTGTACACACCGGCCCCGTC |
4.7 Serum Vitamin D

Serum 25(OH)D levels were measured using the automated IDS-iSYS 25OHD chemiluminescence immunoassay (Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA), following the manufacturer’s instructions for use. 200 µl of serum was used for each sample. The reportable range of this assay is 12.5-350 nmol 25(OH)D/L. This analysis was performed in the lab of Dr. Vieth at Mount Sinai Hospital, Toronto, ON.

4.8 RNA extraction

Total RNA was extracted from whole proximal colons using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA, USA), following the manufacturer’s protocol and stored at -80°C. The concentration and purity of RNA samples were spectrophotometrically assessed (OD_{260/280}, OD_{260/230}), using Thermoscientific’s Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Quality of the RNA samples was further confirmed by an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

4.9 Microarray analysis

Twenty-four RNA samples from male mice, 6 from each dietary vitamin D intervention group, were selected for microarray analysis based on RNA Integrity Number (RIN), which is a measure of RNA quality, determined with an Agilent Bioanalyzer. Samples selected for microarray had a RIN of 6.5 or higher. Two hundred ng of the RNA samples were labeled using Illumina TotalPrep-96 RNA Amplification kit (Illumina Inc., San Diego, CA) as per amplification protocol and 1.5 ng of the generated cRNA from these samples were hybridized onto the Mouse WG-6 V2 Bead chips representing the mouse whole genome (Mouse Ref-8 v2.0). Microarrays were hybridized, washed and scanned according to the manufacturer’s protocols. Microarray data was checked for overall quality using the LUMI package in Bioconductor and R (version 2.14.1). For data
analysis, raw expression data was imported into Genespring v11.5.1 for analysis and the data was normalized using a quantile normalization function along with a “per probe” median centered normalization. All data analyses were performed on log2 transformed data. There were a total of 45,281 probes on the Illumina Mouse Whole Genome (version 2, release 6) array. Planned comparisons between diet switch groups were considered separately using false discovery rate corrected T-tests (Benjamini and Hochberg FDR q<0.05) or regular t-tests (P<0.05) if the former produced no significant results. In order to remove probes showing no expression overall, a filtering was applied such that only probes that were in the upper 80th percentile of the distribution of intensities in 80% of either of the groups under statistical comparison were allowed to pass through this filtering. Unsupervised hierarchical cluster or clustering of significant sets of results was performed using a Pearson centered distance metric under average linkage rules. Microarray experiments and statistical analysis of the data was performed by Carl Virtanen at the University Health Network Microarray Centre, University of Toronto.

4.10 Statistical Analysis

Statistical analyses were performed using Sigma Stat (Version 3.5, Jandel Scientific, Chicago, Illinois). Results are expressed as mean ± SEM and statistical significant was set at p < 0.05. Litter size and total litter weight were analyzed by Student’s t-test. IL-8 concentration data was analyzed by one-way ANOVA as this analysis was conducted in male mice only. Other data was analyzed by two-way ANOVA, within sex, with mother’s treatment and pup’s treatment as main effects. Repeated-measures two-way ANOVA was used for analyses of body weight of the male and female mice. Bacterial counts are expressed as log_{10} 16S rRNA copy numbers/g of wet feces or intestinal contents. Differences were assessed by Bonferroni’s test for post-hoc analysis.
4.11 Sample Size Calculation

Sample size needed to determine an effect of the intervention on the primary outcome, intestinal inflammation severity score, was calculated using data from control and IL-10 KO mice that were part of a previous study by our group [120]. The following parameters were used: $\alpha = 0.05$, $\beta = 0.8$, standard deviation of 0.68 and a minimum detectable difference of 1.63. The required sample size was calculated to be 5 per group per sex.

Using data from a study by Cantorna et al [8], the required sample size needed to detect differences in the intestinal inflammation severity score was calculated as 10 per group using $\alpha = 0.05$, $\beta = 0.8$, a standard deviation of 1.06 and a minimum detectable difference of 1.7. We used a larger sample size than calculated for some outcomes in this study to reduce potential littermate effects. A sample size of 10-13 per group per sex was used in this study for all parameters except the microarray analysis.
Randomized ♀ mice to 1 of 2 vitamin D interventions 3 weeks prior to pregnancy 
(n = 28)

5000 IU of vitamin D$_3$/kg diet (High) (n = 14)

25 IU of vitamin D$_3$/kg diet (Low) (n = 14)

Randomized ♀ and ♂ from each litter to continue on mother’s diet or switch to other diet 
(weaning to 3 months of age)

High/High HH

High/Low HL

Low/High LH

Low/Low LL

n = 15/group/sex

Fecal samples collected throughout the study

Necropsy and tissue collection at 3 months

1. Histological assessment
2. KC concentration
3. Gut microbiota composition

Figure 4.1: Study Design
CHAPTER FIVE

RESULTS & DISCUSSION STUDY 1:

EFFECT OF VITAMIN D SUPPLEMENTATION IN UTERO THROUGHOUT ADULTHOOD ON GROWTH AND INTESTINAL INFLAMMATION IN IL-10 KO MICE OFFSPRING
5.0 STUDY ONE

ABSTRACT

Vitamin D may have immunomodulatory effects in the intestine and potential to target the gut microbiota, and microbial dysbiosis has been implicated in IBD. The objective of study one was to determine if exposure to high levels of vitamin D mitigates intestinal inflammation in the IL-10 KO mouse model of IBD. Mice were randomized to a diet containing 25 IU (low) or 5000 IU (high) of vitamin D/kg of diet in utero until weaning when mice were randomized to the same diet or switched to the other diet and studied until 3 months of age. Colon inflammation severity and KC levels (males only) were assessed by histological analysis and ELISA, respectively. 25(OH)D levels and fecal microbiota composition were determined by a chemiluminescence immunoassay and qPCR, respectively. Vitamin D was not found to affect litter size, body weight growth and intestinal inflammation in male and female mice. Vitamin D also did not affect KC concentrations in males. Vitamin D significantly increased serum 25(OH)D in male and female mice. Vitamin D modulated the gut microbiota in a sex-specific manner, with the diet switch groups being most affected. Females in the HH group had significantly higher counts of \textit{C. coccoides} than the other vitamin D interventions. Clostridia are major butyrate producers and promote T-regulatory cell activity in the colon. Therefore, vitamin D may favourably modulate microbiota composition without attenuating inflammation in IL-10 KO mice.
5.1 RESULTS

5.2 Litter Characteristics

There were no significant differences between the high and low vitamin D groups in litter size, gender ratio and litter weight at PND 14 (Table 5.1).

5.3 Serum Vitamin D

The serum 25(OH)D levels of the HH (mean 96 nmol/L) and LH (mean 97 nmol/L) groups were not significantly different from each other, but were significantly higher (p < 0.001) than the serum 25(OH)D levels of the HL (mean 24 nmol/L) and LL (mean 22 nmol/L) groups. The HL and LL groups were not significantly different from each other (Figure 5.1). There were no significant differences in serum 25(OH)D levels between male and female mice.

5.4 Body Weight Growth

There were no significant differences in body weight among the four vitamin D interventions in both male and female mice (Figure 5.2).
<table>
<thead>
<tr>
<th></th>
<th>High Vitamin D (n = 20)</th>
<th>Low Vitamin D (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter weight PND 14 (g)</td>
<td>6.85 ± 0.34</td>
<td>6.72 ± 0.24</td>
</tr>
<tr>
<td>Litter size (#)</td>
<td>6 ± 0</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>Gender Ratio (# females)</td>
<td>3 ± 0</td>
<td>3 ± 0</td>
</tr>
</tbody>
</table>

† Data are presented as mean ± standard error mean
Figure 5.1: Serum 25(OH)D (nmol/L): n= 5/group/sex

*** p < 0.001

† HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
Figure 5.2: Body weights

† Data are presented as mean ± standard error mean. (n = 15 – 25 group/sex)

† Sex determination and ear tagging were performed at weaning, body weights prior to PND 21 were calculated as the mean pup weight/litter for each group. † HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
5.5 Intestinal inflammation severity

Evidence of inflammation such as lymphocyte infiltration, goblet cell depletion, cryptitis, crypt abscesses and complete destruction of architecture was observed in the mice studied. Examples of each inflammatory score from the IL-10 KO mice in this study are shown in Figure 5.4. There were no significant differences in the proximal colon intestinal inflammation severity between the four vitamin D interventions in the male mice and female mice (Table 5.4.1). The male mice had significantly higher inflammatory scores than the female mice (p < 0.01). Representations of average scores are found in Figure 5.4.3. The dams’ intestinal inflammation was also scored and there were no significant differences between the vitamin D interventions (Table 5.4.2). The dams’ inflammation severity was significantly higher than the male and female mice (p < 0.001 for both). Representations of average scores are found in Figure 5.4.3. No correlation was found between dams and their pups severity scores (Figure 5.4.4).
### Table 5.4.1: Offspring Proximal Colon Inflammation Severity Scores

<table>
<thead>
<tr>
<th></th>
<th>HH</th>
<th>HL</th>
<th>LH</th>
<th>LL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n = 13/group)</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.40 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Females (n = 13/group)</td>
<td>1.4 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>1.40 ± 0.3</td>
</tr>
</tbody>
</table>

† Data are presented as mean ± standard error mean.

### Table 5.4.2: Dams’ Proximal Colon Inflammation Severity Scores

<table>
<thead>
<tr>
<th></th>
<th>High Vitamin D</th>
<th>Low Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dams (n = 12/group)</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

† Data are presented as mean ± standard error mean
Figure 5.4: Histological assessment of colonic inflammation

(A) Score 0: no inflammation with normal crypt and goblet cells (arrow); m: mucosa; s: submucosa; ms: muscularis. H&E, 400X. (B) Score 1: minimal evidence of inflammatory infiltrate (arrow), H&E, 400X. (C) Score 2: significant evidence of inflammatory infiltrate (arrow), H&E, 400X. (D) Score 3: significant evidence of inflammatory infiltrate with goblet cell depletion (arrow), H&E, 400X. (E) Score 4: severe inflammation characterized by widespread infiltration with inflammatory cells, formation of crypt abscesses (arrows), mucosal thickening, submucosal cell infiltration, a decrease in goblet cells and destruction of architecture, H&E, 400X.
Figure 5.4.3: Histological representation of average colonic inflammation scores

(A-C) Representing scores between 1 and 2 (average score for male and female mice in HH, HL, LH and LL groups), with evidence of inflammatory infiltrate (arrows), H&E, 400X. (D-E) Representing scores between 2 and 3 (average score for female dams in high and low vitamin D groups), with evidence of inflammatory infiltrate and formation goblet cell depletion (arrows), H&E, 400X.
Figure 5.4.4: Correlation of Dams and Pups Severity Scores
† Pink indicates dam on high vitamin D, blue indicates dam on low vitamin D
5.6 Males KC concentration in proximal colon

There were no significant differences in whole proximal colon KC concentrations between the four vitamin D interventions (Figure 5.5). These concentrations are highly elevated compared to what would be found in a healthy mouse model, as KC was undetectable in normal colon tissue in parallel experiments conducted in our labs.).

![Figure 5.5: Proximal colon IL-8 concentration: n = 10/group](image)

5.7 Microbiota Composition

5.7.1 Total bacteria counts in the feces

There were no significant differences in total bacteria counts between the vitamin D
interventions in the male mice (Figure 5.6.1) (mean CFU/g wet feces: HH: 10.9; HL: 10.9; LH: 10.8; LL: 11.1). In the female mice, the HH group had significantly higher counts than the HL group (p < 0.001) and LH group (p < 0.05). The LL group also had significantly higher total bacteria counts than the HL group (p < 0.001) (Figure 5.6.1) (mean CFU/g wet feces: HH: 11.1; HL: 10.4; LH: 10.8; LL: 11.0).

5.7.2 Bacteroidetes counts in the feces

In the male mice, the HH group had significantly more Bacteroidetes than the LH group (p < 0.05). The LL group had significantly higher counts than the LH group (p < 0.001) and the HL group (p < 0.01) in the male mice (Figure 5.6.2) (mean CFU/g wet feces: HH: 9.5; HL: 9.2; LH: 9.0; LL: 9.8). In the female mice, the HH group had significantly more counts than the HL group (p < 0.01) and the LL group had significantly higher counts than the HL group (p < 0.05) (Figure 5.6.2) (mean CFU/g wet feces: HH: 9.6; HL: 9.0; LH: 9.5; LL: 9.6).

5.7.3 C. leptum counts in the feces

In the male mice, the LL group had significant higher C. leptum counts than the LH group (p < 0.05) (Figure 5.6.3) (mean CFU/g wet feces: HH: 7.6; HL: 7.3; LH: 7.0; LL: 7.9). In the female mice, there were no significant differences between groups (Figure 5.6.3) (mean CFU/g wet feces: HH: 8.5; HL: 7.6; LH: 8.4; LL: 7.8).

5.7.4 C. coccoides counts in the feces

In the male mice, the HH group had significantly more C. coccoides counts than the HL group (p < 0.05) (Figure 5.6.4) (mean CFU/g wet feces: HH: 6.7; HL: 5.6; LH: 6.0; LL: 6.4). In the female mice, the HH group had significantly higher counts than the HL group (p < 0.01), the LH group (p < 0.01) and the LL group (p < 0.05) (Figure 5.6.4) (mean CFU/g wet feces: HH: 7.5; HL: 5.0; LH: 5.5; LL: 5.9).
5.7.5  Bifidobacteria counts in the feces

There were no significant differences in the Bifidobacteria counts between the vitamin D interventions in the male mice (Figure 5.6.5) (mean CFU/g wet feces: HH: 8.2; HL: 7.9; LH: 8.2; LL: 8.5). In the female mice, the HH group had significantly more counts than the HL group (p < 0.001) and the LH group (p < 0.05). The LL group also had significantly more counts than the HL group (p < 0.01) (Figure 5.6.5) (mean CFU/g wet feces: HH: 8.3; HL: 6.7; LH: 7.3; LL: 8.0).

5.7.6  *E. coli* counts in the feces

In the male mice, the HH group had significantly higher *E. coli* counts than the LH group (p < 0.001). The LL group had significantly more counts than the HL (p < 0.05) and LH groups (p < 0.001) (Figure 5.6.6) (mean CFU/g wet feces: HH: 7.9; HL: 6.8; LH: 5.9; LL: 8.1). There were no significant differences in *E. coli* counts between the vitamin D interventions in the female mice (Figure 5.6.6) (mean CFU/g wet feces: HH: 7.8; HL: 6.8; LH: 7.3; LL: 7.6).

5.7.7  Firmicutes/Bacteroides ratio in the feces

In the male mice, the HH group had a significantly higher F/B ratio than the HL group (p < 0.001) and the LH group (p < 0.001). The LL group also had a significantly higher F/B ratio than the HL group (p < 0.001) and the LH group (p < 0.001). (Figure 5.6.7) (Mean log ratio: HH: 0.4; HL: -2.0; LH: -2.0; LL: 0.3). In the female mice, the HH group had a significantly higher F/B ratio than the HL group (p < 0.001) and the LH group (p < 0.001). The LL group also had a significantly higher F/B ratio than the HL group (p < 0.001) and the LH group (p < 0.01) (Figure 5.6.7) (mean log ratio: HH: 1.0; HL: -1.5; LH: -0.9; LL: 0.4).
Figure 5.6.1: Total bacteria counts in the feces

Figure 5.6.2: Bacteroides counts in the feces

n = 12/group/sex * indicates p < 0.05, ** p < 0.01, *** p < 0.001  
† HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
Figure 5.6.3: *C. leptum* counts in the feces

Figure 5.6.4: *C. coccoides* counts in the feces

n = 12/group/sex * indicates p < 0.05, ** p < 0.01  † HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low

Mom’s diet: p = 0.872
Pup’s diet: p = 0.002
Interaction: p = 0.534

Mom’s diet: p = 0.959
Pup’s diet: p = 0.428
Interaction: p = 0.042

Mom’s diet: p = 0.283
Pup’s diet: p = 0.040
Interaction: p = 0.004
Figure 5.6.5: Bifidobacteria counts in the feces

Figure 5.6.6: *E. coli* counts in the feces

n = 12/group/sex * indicates p < 0.05, ** p < 0.01, *** p < 0.001 ↑ HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
Figure 5.6.7: F/B ratio in the feces: n = 5-10/group/sex

** indicates p < 0.01, *** p < 0.001

† HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
5.7.8  Proximal colon content composition of the male mice

Only the HH and LL vitamin D interventions were analyzed for the proximal colon contents, as an examination to determine if the composition of the proximal colon contents and feces were different from each other. There were no significant differences in counts for total bacteria, Bacteroidetes, *C. leptum*, *C. coccoides*, Bifidobacteria and *E. coli* in the vitamin D interventions (Figure 5.6.8). There were also no significant differences between the feces and proximal colon contents in the counts of the bacteria groups analyzed. The mean CFU/g wet feces are as follows: Total bacteria: HH: 10.8, LL: 10.9; Bacteroidetes: HH: 9.2, LL: 9.2; *C. leptum*: HH: 7.5, LL: 7.8; *C. coccoides*: HH: 6.7, LL: 6.6; Bifidobacteria: HH: 8.2, LL: 8.4; *E. coli*: HH: 7.6, LL: 7.6.

5.8  Correlation data

Inflammation severity scores, all bacteria groups and KC concentration data were correlated using Spearman’s rho. Bacteroidetes counts were positively correlated with inflammation score (Figure 5.8). All other parameters were not correlated.
Figure 5.6.8: Microbiota composition of the proximal colon contents:

n = 12/group † HH: High/High; LL: Low/Low
Figure 5.6.8: Microbiota composition of the proximal colon contents:

n = 12/group † HH: High/High; LL: Low/Low
Figure 5.8: Log_{10} Bacteroidetes counts vs. inflammation severity scores

Correlation coefficient: 0.270; p < 0.05; n = 73
5.9 DISCUSSION

The main objective of this study was to determine if supplementation with vitamin D, particularly in early life, could attenuate colonic inflammation in the IL-10 KO mouse. This study demonstrates that vitamin D supplementation did not provide protection against colonic inflammation, as there were no significant differences in the inflammation severity scores between the four vitamin D interventions studied. Female mice were found to have lower inflammation severity scores than males; and dams’ had higher severity scores than male and female offspring. Vitamin D did not affect growth of either male or female mice or KC concentration of male mice. However, vitamin D modulated the gut microbiota. Specifically, the mice exposed to switching vitamin D diets had the most gut microbiota changes. Interestingly, the female mice in the HH group had higher C. coccoides counts than female mice exposed to any level of low vitamin D.

5.9.1 Histological analysis

The finding that dietary vitamin D supplementation did not attenuate intestinal inflammation in this study is novel. Cantorna and colleagues [8], who conducted the most prominent research in this area and conducted the first study to discover beneficial effects of vitamin D on disease in IL-10 KO mice (C57BL/6 strain), found that vitamin D treatment ameliorated intestinal inflammation. However, this study had administered dietary vitamin D while Cantorna and colleagues administered a much higher dose of the biologically active form (pharmacological form) of vitamin D (1,25(OH)2D3). 1,25(OH)2D3 likely exerts different or more potent effects than vitamin D, which could indicate why no significant improvements in inflammation were seen.

Furthermore, the area examined in Cantorna’s study was the jejunum, while this study analyzed the colon. Interestingly, Cantorna and colleagues described their animal facility in which the mice were bred as specific pathogen free (SPF). A SPF facility is free from certain pathogens, all bedding/cages are sterilized and the food is irradiated, indicating a “cleaner” facility than
conventional facilities. Dr. Ralf Kuhn, who developed this mouse model, discovered that IL-10 KO mice reared in SPF conditions only exhibited inflammation and lesions in the proximal colon [117]. Thus, analyzing if vitamin D supplementation could mitigate inflammation in the colon should be the main outcome to consider in Cantorna’s study. Given that the mice were raised in a conventional animal facility and because CD commonly affects the colon, analyzing the colon for inflammation in this study was key.

The average severity score for the four vitamin D interventions indicates mild inflammation was present in adult offspring. The inflammation showed to be “patchy” with areas of normal colon histology and inflammation (ranging from little inflammatory infiltrate to complete destruction of colonic architecture) appearing in all of the mice studied. This is a typical feature of CD. It is worthy to note that the average scores of this study were similar (averages between 1 and 2) to those found previously in our lab in mice (129/SvEv IL-10 KO mice) that were 13 weeks of age [126].

Female mice had lower inflammation scores than male mice in this study. This is interesting as female and male mice were found to be affected equally [117]. However, a previous study also found that male IL-10 KO mice (strain not specified) had more severe disease outcomes, as 100% of the male mice had died by 4 months of age [127]. The female mice had moderate disease during this time, and 50% of the female mice were still alive at 4 months of age [127]. One possible reason for this finding could be the higher levels of estrogen in female mice. Estrogen receptors (ERs) are found in the epithelial cells of the GI tract, indicating potential effects of this hormone on the gut. Estrogen has been demonstrated to have anti-inflammatory properties and has been found to mitigate intestinal inflammation in various rodent models of IBD (HLA-B27, DSS colitis and dinitrobenzene sulfonic acid (DNBS) colitis) [128]. Furthermore, oral treatment with genistein, a soy isoflavone, (100 mg/kg b.w. – mimicking levels obtained during pregnancy) for 14 days reduced expression of markers of inflammation in a rat model of trinitrobenzenesulfonic acid (TNBS)-induced colitis [129]. It would be interesting to determine if estrogen or isoflavones could also attenuate inflammation in the IL-10 KO mouse.
The dams of the offspring in this study also had significantly higher severity scores than both the male and female offspring. One potential reason for this could be that the dams were older (~16 weeks) than the offspring, allowing more time for disease to progress. Kuhn and colleagues found that weight loss and anemia started to develop at approximately 3-4 weeks of age in IL-10 KO mice (mixture of strains C57BL/6 and 129/Ola), and at 7-11 weeks of age, 75% of the IL-10 KO mice were affected [117]. Kuhn et al also discovered that at 3 months of age, 30% of the animals had died. No offspring in our study had died; however, 3-4 dams were euthanized due to the development of rectal prolapse. Although estrogen has been found to have anti-inflammatory effects and IBD may be better controlled during pregnancy (when estrogen levels are high), IBD may also flare up during a first pregnancy or postpartum [130]. This suggests pregnancy may also adversely affect disease severity in the dams.

5.9.2 KC levels in proximal colon

IL-8, or the mouse KC homologue, is a potent chemoattractant for neutrophils that is synthesized in response to bacterial products. An outcome measure of this study was to explore if supplementation with vitamin D, particularly in early life, could decrease inflammation through attenuating KC levels in male IL-10 KO mice. The data demonstrate that vitamin D supplementation did not affect KC concentration in whole proximal colon in male mice. Only male colonic tissue samples were analyzed to complement another study conducted in our lab, which also investigated colonic KC concentrations in male mice.

Previous research has found elevated mRNA and protein KC expression in colon of IL-10 KO mice than this study has found (mean of 11000 pg/g compared to 2500 pg/g). However, the mice in the other study were 4 weeks older and had more severe disease [127]. Moreover, the male mice (who had more severe illness than the females) had higher KC concentrations than the female mice. It would be interesting for future studies to determine if female mice also have lower KC concentrations compared to the male mice.
1,25(OH)₂D₃ was found to suppress IL-8 expression in a human melanoma cell line [131] but not in a human monocytic cell line [132]. Furthermore, supplementation with vitamin D₃ did not attenuate IL-8 protein expression in the serum of healthy human subjects [133]. However, other inflammatory markers, such as TNF-α and IL-12, were suppressed by 1,25(OH)₂D₃ in vitro and following vitamin D₃ supplementation in humans [133].

IL-8 expression has been found to be positively correlated with the histological grade of active inflammation in IBD patients in both the large and small intestine [134]. I found no correlation between IL-8 concentration and severity score in this study. However, IL-8 presence in the previous study was determined by in situ hybridization, examined under a light microscope and was found to be highly represented in the severely inflamed areas [134]. KC concentration was determined in the whole proximal colon using ELISA; therefore I cannot determine if KC concentration was higher in areas of severely inflamed tissue. The inflammation was found to occur in a patchy or non-continuous manner throughout the colon and this data represents an overall KC concentration of the whole proximal colon.

### 5.9.3 Microbiota composition

This study sought to determine if vitamin D supplementation could modulate the gut microbiota to a phenotype that can be considered health supporting compared to vitamin D deficiency. Research has found that the gut microbiota of healthy humans is compromised mainly of the Firmicutes and Bacteroidetes phyla [135]. Moreover, healthy subjects had high numbers of Bifidobacteria, and an inverse correlation with Bifidobacteria and abdominal pain was found [135]. My data demonstrates that vitamin D does modulate the gut microbiota and that males and females respond differently to supplementation. Specifically, total bacteria counts were decreased in the HL and LH groups of female mice. Bacteroidetes counts were decreased in the HL and LH groups of both male and female mice compared to the HH and LL groups. *C. leptum* counts were lower in the HL and LL groups compared to the HH group in female mice. *C.
coccoides counts were lower in the female mice exposed to low levels of vitamin D pre- or post-weaning. Bifidobacteria counts were lower in the diet switch groups of the female mice, and E. coli counts were lower in the diet switch groups of the male mice. In addition, the F/B ratio was decreased in the diet switch groups of both male and female mice. Based on the gut microbiota composition findings, it appears that female mice are more responsive to vitamin D than the male mice.

This study found that total bacterial counts were decreased in the diet switch groups (HL and LH) in the female mice. The only other study to date examining vitamin D deficiency on the gut microbiota in a mouse model of IBD, Lagishetty et al [70], found that mice that were vitamin D deficient had lower counts of total bacteria in the presence or absence of chemically induced IBD. Male mice total bacterial counts were not affected in this study. The diet switch groups (in both males and females) had lower counts of Bacteroidetes compared to the HH and LL groups. Bacteroidetes are one of the four main phyla within the fecal microbial community and comprise most of the Gram-negatives. The counts of Bacteroidetes increase sharply at weaning, when diet switches to solid food [136]. Interestingly, it has been considered that a decrease in Bacteroidetes may be correlated with low-grade endotoxemia in mice [137]. Lipopolysaccharide (LPS) is an endotoxin and a constituent of the cell membrane in Gram-negative bacteria that is released in the GI tract upon death of these bacteria [137]. Death of Bacteroidetes species and subsequent release of LPS can result in secretion of proinflammatory cytokines and inflammation [137]. Therefore, the decrease in Bacteroidetes counts in the diet switch groups could be a result of Bacteroides species death. Interestingly, Bacteroidetes counts were positively correlated with inflammation severity scores in this study (Figure 5.8). This suggests that higher counts of these bacteria may lead to more severe inflammation, providing further evidence that increased LPS can result in inflammation.

C. coccoides counts were highest in the HH group of female mice, and that any exposure to low levels of vitamin D (HL, LH and LL groups) resulted in decreased amounts of this species.
Clostridium bacteria are another large component of the gut microbiota and are major butyrate producers [138]. Clostridium species from the C. coccoides and C. leptum groups have been found to promote intestinal T regulatory cell activity in mice [139]. The species were found to increase activity of transforming growth factor-β (TGF-β) and IL-10. Moreover, mice exposed to a cocktail of Clostridium species were more resistant to experimental models of allergy and intestinal inflammation [139]. This indicates that boosting amounts of Clostridia in the gut could reduce susceptibility to chronic disease. However, if Clostridium species increase IL-10 activity, this process would be blocked in the IBD-model studied here and could explain why no improvements in intestinal inflammation occurred.

The diet switch groups also had lower counts of Bifidobacteria species in the female mice. Bifidobacteria are another dominant group of the endogenous gut microbiota that is considered a health promoting bacteria because of its production of lactic acid (which prevents growth of harmful bacteria) and acetate (which may promote intestinal defense by epithelial cells) [53], among other properties. It is not known if lactate and acetate production is protective against intestinal inflammation; however, increased counts of Bifidobacteria may be beneficial for disease outcomes.

The diet switch groups in our study had decreased counts of E. coli (male mice only), and certain strains of E. coli can cause intestinal inflammation and septicemia [55]. E. coli are also Gram-negative bacteria, indicating more lysis or death of these bacteria in the diet switch groups could release more LPS into the gut and increase inflammation. In contrast, certain strains of E. coli are used as probiotics and may suppress anti-inflammatory cytokines [140]. Moreover, commensal E. coli are involved in production of vitamin K which is essential for blood clotting, and beneficial for bone health [141]. Further investigation into the specific species or strains that are increased or decreased in this study would provide insight into how these results could be beneficial or detrimental to intestinal health. However, IBD patients have been found to have 3-4 higher log counts of E. coli in biopsy samples compared to healthy controls [142], indicating
higher counts of *E. coli* in this study may be unfavorable.

The F/B ratio was calculated in this study as a marker of dysbiosis, as a lower ratio has been found in IBD patients compared to healthy individuals [77]. I found a decreased F/B ratio in the diet switch groups of both the male and female mice compared to the other vitamin D interventions. A decreased F/B ratio has been found in IBD patients [77], and all mice in this study had low F/B ratios similar to what is found in these IBD patients (below 1.5) indicating dysbiosis is present in all of the mice studied. The lower F/B ratio found in the diet switch groups may suggest that even greater dysbiosis exists in these mice.

This is the first study to investigate vitamin D supplementation on gut microbiota composition in an IBD model. Our lab has previously analyzed vitamin D supplementation on gut microbial composition in healthy CD-1 male mice and found that exposure to low vitamin D resulted in a developmental decrease in the colonic Firmicutes/Bacteroidetes ratio and a lower colonic bacterial abundance in comparison to their high vitamin D counterparts [143]. However, diet switch groups or female mice were not previously examined in our lab. Currently, the literature indicates vitamin D deficiency may increase total bacterial counts in healthy and DSS-induced colitis mouse models [70] and that vitamin D deficiency in humans may be correlated with increased total bacterial and Bacteroidetes counts [144].

A possible explanation for the microbial differences discovered in the diet switch groups could be a predictive-adaptive response. This theory argues that the fetus responds to cues during pregnancy in anticipation of the likely long-term future environment [3]. Therefore, if the mice had developed in and adapted to either a high or low vitamin D environment, changing this environment at weaning may “shock” the system and be detrimental to the host. If a predictive-adaptive response is occurring, then this is the first study to show a nutritional programming effect on the gut microbiota. This would be an interesting unexplored avenue to investigate. However, understanding the implication of the alteration of the gut microbiota composition is
still in its infancy, and drawing conclusions on the effects of the microbiota differences observed is difficult. Further research into specific strains, as well as into the effect of changes in diet on microbiota composition is needed. The mechanisms in which vitamin D may influence gut microbiota composition are unknown, but vitamin D is thought to increase production and activation of antimicrobial peptides, thereby increasing pathogen recognition and clearance [145]. Another novel finding of this study is the sex differences in the gut microbiota composition. Sex differences in the effects of vitamin D have previously been found, but not in the context of gut microbiota composition, warranting further research.
CHAPTER SIX

STUDY 2 RESULTS & DISCUSSION:

VITAMIN D SUPPLEMENTATION ON COLONIC GENE EXPRESSION IN IL-10 KO MALE OFFSPRING
6.0 STUDY TWO

ABSTRACT

Vitamin D has been found to have anti-inflammatory properties and to be involved in regulating the expression of genes involved in intestinal health. In particular, vitamin D has been found to regulate the expression of tight junction proteins of the intestinal epithelium and influence the production of Angiogenin 4, an intestinal antimicrobial peptide. The objective of study two was to determine potential mechanisms of action of vitamin D on intestinal inflammation in male IL-10 KO mice. However, since we did not examine any differences in intestinal inflammation in study one, a new objective would be to determine why there were no changes in inflammation. The same male IL-10 KO mice were used in both study one and two. Mice were randomized to a diet containing 25 IU (low) or 5000 IU (high) levels of vitamin D/kg of diet in utero until weaning and then were either maintained on the same diet or switched to the other diet studied until necropsy at 3 months of age. Whole proximal colon was collected and a whole genome microarray analysis was conducted. Vitamin D was found to have no effect on gene expression in the male mice. Therefore, vitamin D supplementation does not regulate gene expression in the male IL-10 KO mouse suggesting that its effects on gut microbiota are not mediated by differential gene expression in the host. Since IL-10 is knocked out in this model, we were not able to determine if vitamin D mediates its actions through IL-10. Given there were no differentially expressed genes, this indicates that the mice may be unresponsive to vitamin D supplementation.
6.1 RESULTS

6.2 Colonic gene expression of male mice

Differential gene expression in the whole thickness colonic tissue of male mice between the four vitamin D interventions (n=6/group) was assessed by whole genome microarray analysis. For the post-weaning diet groups this resulted in 31,439 probes expressed and for the pre-weaning groups comparison this resulted in 31,447 probes. Our analysis showed that there was no clustering of genes within each vitamin D group or any differentially expressed genes between the vitamin D interventions (Figure 6.1).
Figure 6.1: Hierarchical clustering analysis

The dendrogram represents the degree of similarity between samples based on the global gene expression profiles. Numbers indicate each mouse within each group (n = 6/group).

† HH: High/High; HL: High/Low; LH: Low/High; LL: Low/Low
6.3 DISCUSSION

The outcome measure of the second study was to explore effects in which vitamin D may regulate intestinal inflammation and the gut microbiota in male IL-10 KO mice, or why there were no significant differences in intestinal inflammation. There was no clustering of genes within groups indicating vitamin D supplementation or deficiency in utero, throughout pregnancy and in adulthood did not alter gene expression in the colon.

Previous research investigating the effects of the pharmacological form of vitamin D, 1,25(OH)₂D₃, and calcium in this model (C57BL/6 strain) found that both 1,25(OH)₂D₃ and calcium down-regulated production of TNF-α [9]. 1,25(OH)₂D₃ alone was also found to down-regulate TNF-α-related genes and decrease colitis. This indicates that dietary vitamin D₃, which was used in this study, may not be strong enough to down-regulate genes involved in inflammation and that a pharmacological dose of 1,25(OH)₂D₃ may be needed.

Interestingly, previous research that examined gene expression through microarray analysis of colons in IL-10 KO mice (from C57BL/6 background) inoculated the mice with a complex intestinal microbiota or cultures of Enterococcus species to provide more consistent inflammation of the intestinal mucosa [84, 146]. Additional researchers also inoculated IL-10 KO mice with Enterococcus species as only mild inflammation was found in 12-week old mice raised in conventional conditions [147]. These studies investigated the effects of arachidonic and eicosapentaenoic acid-enriched diets on colonic gene expression and histology and found that these fatty acids changed gene expression in this mouse model [84, 146]. Perhaps inoculating mice in this study with bacterial cultures used in these studies would have provided a more consistent and severe form of intestinal inflammation, allowing us to study if a more severe form and consistent form of inflammation is required to observe beneficial effects of vitamin D supplementation.

Moreover, a study investigating the effects of the VSL#3 probiotic mixture on gene expression in IL-10 KO mice (strain not specified) discovered differentially expressed genes
and a reduced histopathology score in the caecum, but not in the colon, between the control and probiotic treated mice [148]. The researchers explained that the finding of no effect in the colon may be due to the differences in microbial retention and colonization between the gut segments studied, or that there was a lower level of inflammation in the caecum. It would be interesting to determine if beneficial effects on inflammation severity and gene expression would be found in other areas of the gut in this study and if inflammation is more or less severe in these areas. However, the explanation of no effect in the colon due to increased inflammation is not in concordance with the studies that inoculated the mice to increase intestinal inflammation.

This study investigated the effects of a food or food constituent (vitamin D) on gene expression. This type of research presents a challenging limitation, as it has been thought that there are relatively small effects of dietary interventions on physiological parameters and the effects of nutrition on gene expression patterns can be hard to detect [149]. A study investigating the effects of kiwifruit extract on colonic gene expression in IL-10 KO mice (C57BL/6J background) found subtle, in any, differentially expressed genes between their interventions studied [150]. Nutrition research can also be challenging as each nutrient investigated can have numerous targets with different affinities and specificities, and require very high concentrations to elicit an effect or reach toxic levels [149]. This is the opposite of pharmacology, where a single drug is used at a low concentration that acts with a relatively high affinity and selectivity to a limited number of biological targets. Given this knowledge, a higher dose of dietary vitamin D, or the pharmacological form of vitamin D, may be needed to observe an effect on gene expression.

However, previous research from our own lab found that the same dietary vitamin D interventions resulted in differences in gene expression in a healthy mouse model [143]. The results of study two are also surprising as vitamin D is a regulator of hundreds of genes through binding with the VDR. Vitamin D has been found to regulate numerous genes involved in intestinal health (such as mucins, tight junctions and antimicrobial peptides) and
the immune system [145]. This finding indicates that the IL-10 KO mice in this study were unresponsive to vitamin D. Perhaps the disease-state or lack of IL-10 in the mice may explain why no effect on gene expression was found. Future investigation into dietary vitamin D and IL-10 production is needed.
CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS
7.0 GENERAL DISCUSSION

This research has shown that supplementation with vitamin D does not attenuate inflammation or regulates gene expression in the colon of IL-10 KO mice. This indicates no beneficial or adverse effects as vitamin D supplementation did not improve health in this model (Figure 7.0). Vitamin D also does not affect growth of these mice. This may be a potentially beneficial effect as low levels of vitamin D (which have been found in IBD patients) would not affect growth of patients (Figure 7.0). However, findings from this study indicate that vitamin D impacts the gut microbiota and vitamin D supplementation resulted in higher serum 25(OH)D concentrations. This indicates a potentially beneficial effect, as although the modulation of the gut microbiota is not known to be beneficial or detrimental to health, an increase in the C. coccoides counts in the HH group is likely a beneficial effect. An increase in serum 25(OH)D in the supplemented group is also a beneficial effect as this shows that supplementation can increase serum levels in this mouse model of intestinal inflammation, and subsequently could in IBD patients as well (Figure 7.0). Additionally, timing of exposure (beginning pre-pregnancy) to vitamin D supplementation did not mitigate inflammation, regulate gene expression or affect growth; but modulated the gut microbiota. Exposure to vitamin D supplementation post-weaning also determined serum vitamin D levels at 3 months of age.

7.1 Vitamin D Dose and Diet

One possible reason for finding no inhibition of intestinal inflammation in this study is the vitamin D doses and diet used. The vitamin D doses for this study were 25 IU kg/diet or 5000 IU kg/diet. These doses were chosen based on a study conducted by Fleet et al, in which mice that received 25 IU kg/diet and 5000 IU/kg diet had a serum 25(OH)D level of 35 nmol/L and 200 nmol/L, respectively [123]. In this study, HL and LL had mean serum 25(OH)D levels of 23 nmol/L and the LH and HH mice had mean serum 25(OH)D levels of 97 nmol/L. The low vitamin D levels in this study are a bit lower than what was expected, but were similar to Fleet’s findings. However, the high vitamin D groups’ levels were 100
nmol/L less than expected. While the serum vitamin D levels of the high vitamin D group are
within the optimal range (> 75 nmol/L), perhaps achieving serum vitamin D levels closer to
200 nmol/L would have shown beneficial effects on inflammation. Previous studies [8-10]
did not measure serum 25(OH)D, thus making comparisons to these studies difficult.

Furthermore, previous research investigating the effects of vitamin D in this model used the
biologically active form of vitamin D, 1,25(OH)₂D₃, and this study administered dietary
vitamin D₃. Vitamin D, as either D₃ or D₂, does not have significant biological activity. It
must be metabolized within the body to the hormonally active form before it can exert its
effects. The VDR binds several forms of vitamin D, but its affinity for 1,25(OH)₂D₃ is 1000
times that for 25(OH)D, which explains their relative biological potencies. This suggests that
dietary vitamin D (at least in the doses used in this study) may not be powerful enough to
exert beneficial effects on intestinal inflammation. Clinically, this may be an important
finding as it indicates that vitamin D supplementation may not be beneficial to those suffering
from IBD, and that the hormonal form of vitamin D may be needed to treat and mitigate
symptoms in patients. This finding is of concern as dietary vitamin D is a much more cost-
effective and safe intervention to provide to animals and patients. Use of 1,25(OH)₂D₃ can
lead to hypercalcaemia, which includes symptoms such as pain, conjunctivitis, anorexia,
fever, chills, thirst, vomiting and weight loss [151]. Cantorna and colleagues (2000) measured
serum calcium levels to determine if hypercalcaemia was present in the IL-10 KO mice. The
study indicated that serum calcium levels normal for mice is 2.00 – 2.75 mmol/L. Their
findings showed that the mean serum calcium levels for the 1,25(OH)₂D₃ treated IL-10 KO
mice was 3.00 mmol/L, which indicates that hypercalcaemia may be present in these mice.
This finding was not addressed in their study.

Cantorna et al [8] did administer dietary vitamin D in one experiment (5 µg/d or 200 IU/d) to
vitamin D-deficient IL-10 KO mice and discovered that adding vitamin D back to the diet
increased growth of the mice and decreased mortality compared to the mice who remained on
the deficient vitamin D diet. Other parameters were not examined in this experiment. This is also substantially more dietary vitamin D than the mice in our study received (our mice in the supplemented group consumed approximately 15 IU/d). Moreover, our study administered the supplemental vitamin D diet earlier in life than Cantorna and colleagues (3 weeks prior to pregnancy in our study vs. at weaning). Since other parameters were not measured in this experiment, and since there were no differences in growth or had mortality of any mice, this indicates that even 25 IU/vitamin D/kg diet (approximately 0.75 IU/d per mouse) is adequate for growth and longevity in the IL-10 KO mice.

Interestingly, Cantorna and colleagues also mentioned that because 1,25(OH)\(_2\)D\(_3\) treatment of other experimental autoimmune diseases (Type 1 diabetes, EAE) was more effective when calcium was high, all mice in their study were fed a high calcium diet (1 g/100 g diet or 1% of diet). This is substantially higher than the calcium amount used in our study (5000 mg/kg or 0.5% of diet). Further research by Cantorna’s group [9] found that high dietary calcium (2.2% of diet) independent of 1,25(OH)\(_2\)D\(_3\) treatment decreased TNF-\(\alpha\) production; and the IL-10 KO mice fed the high calcium and 1,25(OH)\(_2\)D\(_3\) had the mildest form of IBD. In addition, a high calcium diet (4800 mg/kg diet) compared to a low calcium diet (1200 mg/kg diet-mimicking the Western diet) administered to HLA-B27 transgenic rats (which spontaneously develop colitis) inhibited intestinal permeability, histological severity scores, IL-1\(\beta\) production and diarrhea [152]. It is hypothesized that calcium protects against intestinal infections of food-borne bacterial pathogens and precipitates irritating bile and fatty acids [152]. This reduces cytotoxicity of the feces and may ultimately decrease inflammation in the gut. Interestingly, the high calcium diet administered in this study is similar to the amount of calcium in our study. However, the use of a high calcium diet may have in some measure provided the beneficial effects seen with the 1,25(OH)\(_2\)D\(_3\) treatment shown in previous research. It would be interesting to explore if higher levels of dietary calcium are needed to see beneficial effects of dietary vitamin D on inflammation in IL-10 KO mice, or decreasing
calcium levels in the diet to determine if the calcium is protecting against inflammation in this mouse model.

Absorption of vitamin D occurs in the small intestine, indicating that only small amounts of vitamin D$_3$ may reach the colon and be locally converted to 1,25(OH)$_2$D$_3$. This may provide another explanation of why no effects of vitamin D were observed. A recent study developed an analogue of 1,25(OH)$_2$D$_3$ that can be directly delivered to the colon [153]. β-glucuronides of vitamin D were synthesized, and the release of 1,25(OH)$_2$D$_3$ only occurred when the compound encountered β-glucuronidase. β-glucuronidase is an enzyme produced by Bacteroides species in the lower intestinal tract. In Goff et al’s study, β-glucuronides of 25(OH)D and 1,25(OH)$_2$D$_3$, as well as 1,25(OH)$_2$D$_3$ alone were administered to the DSS mouse model of colitis [153]. Administering β-gluc-25(OH)D (8.67 nmol/day) itself was ineffective in treating colitis in these mice. Administering 1,25(OH)$_2$D$_3$ and β-gluc-1,25(OH)$_2$D$_3$ (both 120 pmol/day) resulted in reduced fecal blood scores and colon lesions. However, 1,25(OH)$_2$D$_3$ itself resulted in hypercalcaemia while β-gluc-1,25(OH)$_2$D$_3$ did not. Moreover, combining β-gluc-1,25(OH)$_2$D$_3$ and β-gluc-25(OH)D together further improved disease outcome without inducing hypercalcaemia [153]. The study discovered that β-gluc-1,25(OH)$_2$D$_3$ was more than 100 times more active on colon tissues than the native hormone (1,25(OH)$_2$D$_3$). This study further provides evidence that 1,25(OH)$_2$D$_3$ is required to mitigate colonic inflammation, and administering analogues that directly target the colon may be a more beneficial and safe route to explore. It would be interesting to determine if the two analogues used in this study could mitigate inflammation in the IL-10 KO mouse model. However, appropriate doses of β-gluc-1,25(OH)$_2$D$_3$ need to be administered as high doses (600 pmol/day) also resulted in hypercalcaemia [153].
7.2 IL-10 KO Mouse Model

The IL-10 KO mouse is a well characterized and suitable model for studying therapeutic interventions for IBD. The immune response of these mice is Th1-driven, and pathological changes of inflammatory infiltrate are seen in the mucosa and submucosa of the GI tract [117]. Epithelial hyperplasia, crypt abscesses and focal ulcers also occur in these mice. Furthermore, the colitis is accompanied by manifestations such as weight loss, abnormally high white blood cell counts, splenomegaly and anemia [154]. The IL-10 KO mouse model has been treated successfully with a variety of nutritional and pharmacological interventions such as probiotics (129/Ola & C57BL/6 background) [155], soluble fiber/resistant starch (C57BL/6J strain) [156], omega-3 fatty acids (C57BL/6J strain) [84] and antibiotics (129/SvEv strain) [157].

IL-10 KO mice of the 129SvEv (the strain used in our study) and BALB/c background were found to have the most severe form of IBD, with mice from the C57BL/6J background having the least severe disease outcome [158]. At 3 months of age, 100% of 129SvEv IL-10 KO mice were affected, had an average inflammation severity score of 11.2 (out of 20) and 67% had signs of colorectal adenocarcinomas. At 3 months of age, 57% of C57BL/6J IL-10 KO mice were affected, had an average inflammation severity score of 2.9, and there were no signs of colorectal adenocarcinomas [158]. Therefore, the strain used in this study was the most appropriate to investigate the most severe form of IBD in which all mice would be affected.

Although the strain of mice used in this study was found to have the most severe form of IBD at 3 months of age, I observed only mild inflammation in the colon. A possible explanation of the observed mild inflammation could be the animal facility in which the mice were bred and raised. Previous studies [157] using the same strain of IL-10 KO mice and that had raised the mice in SPF conditions observed severe inflammation in the colon. The scale used for histological severity analysis in these studies was ranked out of 10 (and this study out of 4),
making the scores hard to compare. However, an average score of 7.6 (out of 10) was found previously in the same strain [157], which would be considered a more severe score than an average of 1-2 (out of 4) as observed in this study. Furthermore, germ-free IL-10 KO mice (129/SvEv strain) monoassociated with three different types of bacteria (*E. coli, Enterococcus faecalis* or *Pseudomonas flourescens*) developed dissimilar disease outcomes in terms of severity and timing [159]. Specifically, IL-10 KO mice monoassociated with *E. coli* developed mild inflammation in the caecum after 3 weeks. Mice monoassociated with *Enterococcus faecalis* developed distal colitis at 10-12 weeks of age; while *Pseudomonas flourescens* monoassociated mice remained disease free [159]. While the mice in our study were not initially germ-free and were raised in a conventional animal facility, this indicates that the bacterial population present from the facility, staff members or researchers; and facility sanitation practices could affect disease outcomes in this model. This may possibly explain the differences in disease severity between studies from other research groups, or even from within the same facility at different time points. Interestingly, the previous study conducted in our lab analyzing the same strain of IL-10 KO mice had similar inflammatory severity scores as our study. Although the studies were almost 10 years apart and had different investigators, the strain of mice and animal facility were the same. This indicates that strain and environment may strongly influence disease outcome in this experimental model of IBD. Interestingly, our lab found that flaxseed oil supplementation did not attenuate colonic inflammation compared to corn oil [126], indicating that another nutritional intervention administered in doses attainable in humans through diet also did not decrease colonic inflammation in this mouse model.

Furthermore, the inflammation severity was highly variable between mice. No correlation was found between the dam and offspring severity scores (**Figure 5.4.4**). Some mice were found to have similar inflammation severity scores as their siblings (for example, one litter had three pups which all had a score of 1.4 and another litter had three pups in which all had a score between 0.6-0.9), this was not the case for all litters in which more than one pup from
the same litter was examined for histological analysis. Other litters had severity scores ranging from 1 to 3. This former finding is beneficial as it limits litter effects; however, the variability may make detecting effects of a nutritional intervention difficult. More consistent and severe inflammation (through bacteria inoculation) could be considered for future studies. Likewise, since this study observed sex differences in disease severity, both sexes should be investigated to help understand the pathogenesis and treatment of IBD. Furthermore, controlling for stage of estrus cycle should be considered in future studies.

IL-10 is an anti-inflammatory cytokine that prevents excessive tissue damage caused by bacterial and viral infections, as well as proinflammatory responses [115]. Vitamin D has been found to affect IL-10 production. Treatment of mouse mast cells in vitro with 1,25(OH)$_2$D$_3$ induced IL-10 secretion and up-regulated IL-10 mRNA expression [160]. 1,25(OH)$_2$D$_3$ also increased IL-10 production in human Treg cells [161]. Vitamin D supplementation (2000 IU D$_3$/day) for 9 months increased serum IL-10 production in congestive heart failure patients [162]. Furthermore, psoriasis (disorder characterized by inflammation and abnormal epidermal proliferation) patients have been found to be deficient in IL-10 [163], and interestingly, topical vitamin D is a common treatment for psoriasis. Based on these findings, vitamin D may increase secretion and activity of IL-10, and this effect cannot be seen in our model of IBD. Therefore, the immune response of these mice may not represent a “normal” response to vitamin D. However, IL-10 is not the only immune system target of vitamin D [6] and beneficial effects of vitamin D have previously been found in this model [8, 9]. Therefore, it is more likely that type of vitamin D (dietary versus pharmacological form) administered is the explanation for the lack of attenuation of inflammation in the IL-10 KO mouse model.

7.3 Vitamin D Supplementation in Early Life

The hypothesis that dietary factors may imprint on the fetus or developing offspring and contribute to later health is termed nutritional programming [2]. Low prenatal and early-life
vitamin D status has been associated with later health outcomes such as asthma, allergies, osteoporosis, schizophrenia, type 1 diabetes and MS [90]. To my knowledge, this is the first study investigating the effects of early-life vitamin D supplementation and deficiency in a mouse model of IBD. Currently, the evidence linking vitamin D status during early-life and later CD risk is season of birth. Summer season of birth was associated with a lower risk of developing CD later in life [87], suggesting that late gestation and early postnatal life vitamin D status is the most crucial for later IBD development.

Our findings indicate that vitamin D status in early-life does not affect later IBD symptoms in the IL-10 KO mouse. Low birth weight has been associated with low prenatal vitamin D status [164] but this was not the case in our study as no differences in body weight of the offspring were found. Season of birth and childhood sun exposure has also been associated with later MS risk, and this hypothesis was tested in a mouse model of prenatal vitamin D deficiency in the EAE model of MS. Unexpectedly, the adult offspring born to vitamin D deficient dams, when compared to controls, developed a milder form and delayed progression of MS [165]. The mice were switched to the vitamin D sufficient control diet after birth. The researchers explained that the offspring that had known an in utero vitamin D-deficient environment were highly sensitive to the ex utero vitamin D sufficient diet, and thus had milder disease later in life. Previous findings from the same group found that permanent detrimental brain changes were only observed when vitamin D was brought back to the diet after weaning, and not immediately after birth [166]. This does not appear to be the case in terms of intestinal health, as diet switching at weaning did not affect later health outcomes in our study.
7.4 LIMITATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

7.4.1 Limitations

There are several limitations to this study that need to be addressed.

1) Only one region of the GI tract (proximal colon) was examined. Previous research has found that 1,25(OH)$_2$D$_3$ attenuated inflammation in the jejunum [8]. Therefore, the effects of vitamin D cannot be determined if they are site-specific or affect other regions of the GI tract differently.

2) Only one time point was examined, therefore the time in which the disease process started or if vitamin D delayed disease progression cannot be determined.

3) It cannot be determined if vitamin D regulates IL-10 expression since IL-10 is knocked out in this model. Previous research has shown that vitamin D can increase IL-10 activity [160]. Therefore, a normal immune response may not be occurring.

7.4.2 Future Directions for Animal Studies

For future studies investigating the effects of vitamin D supplementation in the IL-10 KO mouse model, several factors should be taken into consideration. Examining histological inflammation severity of various regions of the GI tract would be beneficial to determine if the actions of vitamin D are site-specific. Moreover, if vitamin D’s effects are GI region-specific, this indicates only modest improvement in overall disease outcomes in CD (which can affect any region of the GI tract) but may be beneficial to UC (which affects the colon only).

Future studies should consider using global microbial profiling, such as the use of DGGE or 16S rRNA sequencing, in addition to qPCR to provide more insight into the specific species or strains that have been modulated by vitamin D. Global profiling also allows for a global unbiased view of the microbiome, although it will not provide quantification, in which qPCR could be used to quantify the global microbial profiling outcomes. Observing more bacteria groups will also determine if vitamin D supplementation can modulate other bacterial groups.
Investigating the effects of the vitamin D interventions throughout life in this model would also be an interesting avenue to explore. Collecting tissues at different time points if possible (starting pre-weaning every week until necropsy) and examining histopathological severity and inflammatory markers (such as other proinflammatory cytokines besides KC) would allow us to determine at what age the disease process started. This will also allow us to examine if vitamin D supplementation delayed disease progression. It would also be interesting to determine if vitamin D supplementation could affect mortality in these animals.

Future studies should also examine additional vitamin D doses. Higher doses that result in the targeted serum level of 200 nmol/L should be used to establish if higher dosing is needed to observe a beneficial effect, and to determine if dietary vitamin D$_3$ can mitigate intestinal inflammation in safe amounts (no signs of hypercalcaemia). In addition, lower doses of dietary vitamin D$_3$ should be given to examine the lowest dose possible that these mice can survive on. Furthermore, the vitamin D requirements for mice have not been determined. The common AIN93 and AIN76 diets contain 1000 IU/kg/diet, but this may represent a considerable excess [167]. Investigation into serum 25(OH)D levels required for healthy mice is needed, and when this level is known, dietary amounts could be manipulated based on mouse needs rather than human needs. To determine if IL-10 is needed in order to see an effect of vitamin D, subsequent and parallel studies using other mouse models of IBD should be considered.

7.4.3 Future Directions for Human Studies

These studies were conducted in a mouse model; therefore it is unclear how these findings may relate to humans. Clinical trials with humans are needed to truly determine how vitamin D can impact disease of IBD patients. However, animal models can help us explore underlying mechanisms of vitamin D in the inflammatory process.
While our study insinuates that vitamin D supplementation does not attenuate inflammation, there is still evidence from animal and human research that suggests otherwise. Specifically, the one clinical trial to date found decreased relapse rates in the vitamin D supplemented group [114]. Further clinical trials with larger sample sizes need to be conducted. Analyzing other parameters besides disease activity index and relapse rates should also be considered (such as other inflammatory markers and histological analysis of biopsy samples if possible). Determining the gut microbiota composition of IBD patients receiving vitamin D supplementation would be novel, as this parameter has yet to be explored.

Retrospective cohort and prospective studies are needed to determine if early life vitamin D status can be associated with later disease risk of IBD. Studies that monitor mother’s supplement use and sun exposure pre-, during and post- pregnancy; as well as infant feeding practices and supplementation would be useful. The mothers and children could then be followed-up periodically throughout life to determine cases of IBD.

Drug treatment options for IBD are costly and have adverse side effects. Therefore, determining if supplementation with vitamin D can reduce reliance on these treatments would be a cost-effective and safe way to induce remission in IBD patients. Future studies in humans should explore doses required to observe beneficial effects whilst animal studies should elucidate the potential mechanisms of action. Together, the findings from animal and human studies will provide a more comprehensive understanding of vitamin D’s effect on intestinal health.

7.5 CONCLUSIONS
STUDY 1:

1) Dietary vitamin D does not attenuate inflammation of the colon in IL-10 KO mice as indicated by no differences in histology or IL-8 concentrations.

2) Exposure to supplemental vitamin D during early life did not result in better disease outcomes than IL-10 KO mice exposed to insufficient vitamin D in early life.

3) Dietary vitamin D modulates the gut microbiota composition in a sex specific manner. Specifically, in the female mice, total bacteria, Bacteroidetes and Bifidobacteria counts were lower in the diet switch groups. *C. coccoides* counts were greater in the HH group compared to all other groups studied in the female mice. In the male mice, Bacteroidetes and *E. coli* counts were decreased in the diet switch groups. In addition, the Firmicutes/Bacteroidetes ratio was decreased in the diet switch groups in both male and female mice. These changes in the gut microbiota did not influence inflammation. This is the first study to observe sex differences and effects of programming on the gut microbiota.

STUDY 2:

1) The colonic gene expression of male IL-10 KO mice was not altered by their vitamin D status.
Exposure to vitamin D supplementation (in utero, pregnancy and adulthood) in Il-10 KO mice

**Serum Vitamin D:**
- Supplementation increased serum levels by -70 nmol/L

**Gene expression:**
- No differences in male mice

**Colonie inflammation:**
- No differences in histological assessment
- No differences in Il-8 concentrations

**Gut microbiota:**
- Variety of differences between groups and sexes
- Diet switch groups most affected

**Body weight:**
- No differences

Potential Beneficial effect

No beneficial or adverse effect

No beneficial or adverse effect

Potential beneficial effects

Potential beneficial effect
CHAPTER EIGHT

REFERENCES
8.0 REFERENCES


Appendix 1:


**Vitamin D supplementation results in higher numbers of Clostridium coccoides in the feces of female but not male mice with intestinal inflammation**

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Vitamin D may have immunomodulatory effects in the intestine and potential to target the gut microbiota. Our objective was to determine if exposure to supplemental levels of vitamin D mitigates intestinal inflammation in interkeukin-10 knockout (IL-10 KO) mice. Mice were randomized to a diet containing 25 IU (low levels) or 5000 IU (supplemental levels) of vitamin D/kg of diet *in utero* until necropsy at 3 months of age when colonic and fecal samples were collected. Colon inflammation severity and IL8 levels (males only) were assessed by histological analysis and ELISA, respectively, and fecal microbiota composition was determined by qPCR. Vitamin D had no effect on IL-8 levels in males, or body weight and inflammation severity in either gender. Vitamin D had no effect on microbiota composition in males, but females in the supplemental group had higher (p<0.05) counts of *Clostridium coccoides* than females in the low group. All other bacteria measured were unaffected. Moreover, female mice had lower (p<0.05) colonic inflammation scores and more (p<0.05) *C. coccoides* than males. Clostridia are major butyrate producers and promote Treg cell activity in the colon. Therefore, vitamin D may favourably modulate microbiota composition without attenuating inflammation in female IL-10 KO mice.

Funding sources: Dairy Farmers of Canada, NSERC and Ontario Graduate Scholarship
Appendix II:


**Higher Bifidobacteria counts in male offspring exposed to supplemental levels of vitamin D in utero and during suckling in IBD-prone mice**

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Vitamin D deficiency has been linked to an increased risk of inflammatory bowel disease (IBD), and microbial dysbiosis has been implicated in IBD. Our objective was to determine if exposure to supplemental levels of vitamin D can favourably modulate microbiota composition pre-inflammation in the male interleukin-10 knockout (IL-10 KO) mouse that spontaneously develops intestinal inflammation at 6-8 weeks of age. The mice were randomized to a diet containing 25 IU (low group) or 5000 IU (high group) of vitamin D/kg of diet in utero and during suckling, and fecal samples were collected at 5 weeks of age. Fecal microbiota composition was determined by qPCR. Mice in the supplemental group had higher (p = 0.01) counts of Bifidobacteria than mice in the low group. Total bacteria, Bacteroidetes, *Clostridium leptum*, *Clostridium coccoides* and *Escherichia coli* counts were unaffected. Bifidobacteria have been found to sustain intestinal homeostasis and inhibit Th1-driven inflammation and may favourably alter gut microbial composition to a more health-promoting phenotype. Further investigation is needed to determine if this results in protection against developing intestinal inflammation.

Funding sources: Dairy Farmers of Canada, NSERC and Ontario Graduate Scholarship
Appendix III:

Abstract presented at Digestive Disease Week, San Diego, CA, May 19-22, 2012

**Vitamin D deficiency in utero through adulthood results in an inflammation-prone colonic gene expression profile in healthy CD-1 mice while IL-10 knock-out mice are not responsive**

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**Background:** Vitamin D signaling through its nuclear hormone receptor VDR has emerged as a pluripotent regulator of biological functions far beyond its canonical roles in calcium metabolism. In the realm of gastrointestinal physiology, many genes integral to the intestinal barrier, such as mucins, tight junctions, and antimicrobial peptides contain vitamin D responsive elements, attesting that their expression may be directly regulated by vitamin D. In addition, vitamin D is an important factor in immune cell development and immunomodulation. Vitamin D and VDR are involved in the development of specific intestinal intraepithelial lymphocytes and their deficiency has been implicated in exacerbation of symptoms in inflammatory bowel disease (IBD). **Objective:** The aim of this study was to investigate effects of exposure to low or supplemental levels of vitamin D from early life to adulthood on vitamin D status and intestinal gene expression signature in both healthy (CD-1) and IBD (IL-10 KO) mouse models. **Methods:** CD-1 and 129/SvEv IL-10 KO were randomized to a diet containing 25 IU (low level) or 5,000 IU (supplemental level) of vitamin D₃/kg of diet *in utero* until necropsy at 3 months when serum and colon samples were obtained from the male offspring (n=15-23/group). Colonic inflammation in the IL-10 KO mice was assessed histologically. Serum 25 (OH) vitamin D levels and colonic gene expression profiles in the CD-1 and IL-10 KO mice were determined by chemiluminescence immunoassay and whole genome microarray, respectively. **Results:** There was no significant difference in the colonic inflammatory score among the low vitamin D and supplemented IL-10 KO mice. The serum 25(OH)D levels were significantly higher in supplemented CD-1 (120.1 nmol/L ±3.1) and IL-10 KO mice (93.8 nmol/L ±2.7) in comparison to their low vitamin D CD-1 (13.8 nmol/L ±0.78) and IL-10 KO (23.0 nmol/L ±2.2) counterparts. A total of 104 genes, not including VDR, were differentially expressed between CD-1 mice on low
and supplemented vitamin D diets. In particular, expression of genes encoding for extracellular matrix proteins and immunoglobulins were increased and those involved in detoxification processes were decreased in low vitamin D compared to supplemented CD-1 mice. The colonic gene expression profiles of IL-10 KO mice were not altered by their vitamin D status. **Conclusions:** Continuous dietary vitamin D supplementation from conception onwards determines higher serum 25(OH)D levels in both healthy and IL-10 KO mice in adulthood, suggesting that vitamin D uptake is not impaired in IBD. While vitamin D deficiency in healthy CD-1 mice resulted in an inflammation-prone gene expression profile, the IL-10 KO mice were not responsive to vitamin D; therefore, vitamin D may be preventative in healthy animals but not in this genetic model of IBD.

Funding sources: Dairy Farmers of Canada, NSERC and Ontario Graduate Scholarship