THE EFFECT OF DENOSUMAB, THE INHIBITOR FOR RECEPTOR ACTIVATOR NUCLEAR FACTOR KAPPA-B LIGAND (RANKL), ON DINITROBENZENESULFONIC ACID (DNBS)-INDUCED EXPERIMENTAL MODEL OF CROHN'S DISEASE

BY

AZIN KHAFIPOUR

A Thesis Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Immunology
University of Manitoba
Winnipeg, Manitoba

Abstract

Background: The pro-inflammatory mediator receptor activator of nuclear factor-kappa B ligand (RANKL) plays a major role in the development of rheumatoid arthritis; however, its role in inflammatory bowel disease is unknown. Genome-wide association meta-analysis for Crohn's disease (CD) identified a variant, near the TNFSF11 gene that encodes RANKL. Moreover, CD risk allele increased expression of RANKL in specific cell lines. This study aims to elucidate if the RANKL inhibitor Denosumab (Prolia™) can reduce the severity of experimental colitis via modifying gut microbiota dysregulation. **Methods:** CD-like colitis was induced via intrarectal administration of dinitrobenzenesulfonic acid (DNBS, 4mg/kg) dissolved in Ethanol (30%) to C57Bl/6 mice (n=12). One day before colitis induction, daily injection of Prolia (10mg/kg/d, intraperitonealy) was initiated and continued over four days. Vehicle mice received PBS1%. On the sacrifice day, inflammatory status was evaluated clinically. DNA was extracted from colonic mucosa and fecal samples, and V4 region of bacterial 16S rRNA gene was amplified and subjected to Illumina sequencing for microbiome analysis. Alpha- and beta-diversities were calculated in QIIME and subjected to SAS and PERMANOVA, respectively. Differences in clustering pattern of microbiota at the genus level were determined. Differences between were considered significant at $P<0.05$. **Results:** Disease severity, macroscopic score and pro-inflammatory cytokines (Il-6, IL-1β and TNF-α) were increased in DNBS/Ethanol-treated *vs.* vehicle mice. Prolia treatment decreased ($P<0.05$) only the pro-inflammatory cytokines. Prolia treatment also modified the alpha- and beta-diversity of colonic mucosa and fecal microbiota. DNBS/Ethanol group clustered separately ($P<0.05$) compared to the vehicle group, Prolia treatment attenuated the negative effects of DNBS/Ethanol. **Conclusions:** The development of colitis in DNBS/Ethanol model

was accompanied by disruption of gut microbiota. Preventative treatment with Prolia modulated intestinal inflammation and gut microbiota dysbiosis in a murine model of colitis. Our results provide a rationale for considering Prolia as a future potential therapy in CD, but more experimental and clinical studies need to be performed.

Acknowledgements

The first and always appreciation is to my beloved God, the creature of all opportunities and precious moments of "learning". Thanks for never leaving me alone and thanks for providing me with the best leaders, family members, and friends in all steps of my life. I appreciate all the hardness I faced in my path, without them I could never be this much stronger.

I would like to express my deepest appreciation to my supervisor, Dr. Jean-Eric Ghia for all his great academic guidance, supervision and continued support during my studies. His mentorship was paramount in encouraging me to not only grow as an immunologist but also as an independent thinker. Without his guidance and persistent help this dissertation would not have been possible.

I am also grateful of our collaborative lab supervisor, Dr. Ehsan Khafipour, the best leader from the day I knew him ;) He always pushed me toward the best pathways in my life. Undoubtedly, without his continuous guidance and support, I could not come this much further.

I am also indebted to Dr. Jude Uzonna for all his incredible support during my master program. Without doubt, he has been very influential in my professional and personal development. I am equally grateful for Dr. Hassan Marzban for his invaluable guidance and support in my project, which had lasting effect and helped me enormously.

I also wish to thank my colleagues, Mohammad Fazle Rabbi, Dr. Nour Eissa, Dr. Hongxing Wang for their guidance and help on my research. I would like to also acknowledge specifically Hooman Derakhshani due his technical assistance and advice, prominently assist my research. Last but not the least, I am truly grateful for the entire Department of Immunology and Department of Animal Science for providing me with the outstanding

training and environment for professional development. I would like to thank for the financial support provided by Children's Hospital Research Institute of Manitoba, Crohn's and Colitis Canada, and Natural Sciences and Engineering Research Council of Canada for which this work could not have been realized.

Dedication

Dedicated to the memory of my dad, Mohammad Hossein Khafipour, a man taught me the strength is always in kindness.

Dedicated to my strongest and kindest Mom, Firoozeh Maghsoodi, an absolute masterpiece in the journey of my life.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APC = Antigen presenting cell

AP1 = Activator protein-1

AU = Arbitrary unit

BALB = Bagg Albino

BLAST = Basic local alignment search tool

BMD = Bone mineral density

bp = Base pair

BW = Body weight

CD = Crohn's disease

CD4 = Cluster of differentiation 4

cDNA = Complementary DNA

CRP = C-reactive protein

d = Day

DC = Dendritic cells

DIA = Disease index activity

DNA = Deoxyribonucleic acid

DNBS = Dinitrobenzene sulfonic acid

dNTPs = Deoxyribosenucleotides

DSS = Dextran sulphate sodium

ECM1 = Extracellular matrix protein 1

ELISA = Enzyme linked immunosorbent assay

ER = Endoplasmic reticulum

GI = Gastrointestinal

GIT = Gastrointestinal tract

GWAS = Genome wide associated studies

h = Hour

H & E = Hematoxylin and eosin stain

HPLC = High quality reagents and chemicals

IBD = Inflammatory bowel disease

IEC = Intestinal epithelial cells

IFN = Interferon

IL = Interleukin

ILCs = Innate lymphoid cells

iNOS = Inducible nitric oxide synthase

i.p. = Intraperitoneally

i.r. = Intrarectally

KEGG = Kyoto encyclopedia of genes and genomes

LEfSe = Linear discriminant analysis with effect size

LOD = Limit of detection

LPS = Lipopolysaccharide

MAM = Mucosa-associated microbiota

MPO = Myeloperoxidase

N = Nitrogen

NCBI = National center for biotechnology information

NFκB = Nuclear factor kappa B

NK = Natural killer

nMDS = Non-metric multidimensional scaling

NO = Nitric oxide

NOD2 = Nucleotide-binding oligomerization domain 2

OPG = Osteoprotegerin

OTU = Operational taxonomic units

PAST = Paleontological statistics

PBS = Phosphate Buffered Saline

PCR = Polymerase chain reaction

PERMANOVA = Permutation multivariate analysis of variance

PICRUST = Phylogenetic investigation of communities by reconstruction of unobserved states

PLS-DA = Partial least square discriminant analysis

PMO = Postmenopausal osteoporosis

PRAR = Peroxisome proliferator-activated receptor

QIIME = Quantitative insights into microbial ecology

qPCR = Quantitative polymerase chain reaction

RANK = Receptor activator nuclear factor kappa-b

RANKL = Receptor activator nuclear factor kappa-b ligand

ROM = Reactive oxygen metabolite

RORγ = RAR-related orphan receptor gamma

rRNA = Ribosomal ribonucleic acid

qRT-PCR = Quantitative reverse transcriptase polymerase chain reaction

SAS = Statistical analysis system

SCFA = Short chain fatty acids

SED = Standard error of the difference between treatment means

SEM = Standard error of the mean

SRA = Sequence read archive

STAMP = Statistical analysis of metagenomic profiles

STAT3 = Signal Transducer and Activator of Transcription 3

TBP = TATA Box-Binding Protein

TGF = Transforming growth factor Th T helper

TLR = Toll like receptor

TNBS = Trinitrobenzene sulfonic acid

TNF = Tumor necrosis factor

TNFSF11= Tumor necrosis factor superfamily 11

Treg = T regulator

Tris-HCl = Tris hydrochloride

UC =Ulcerative colitis

Chapter One

1.1. Overview

Inflammatory bowel diseases (IBD), consisting of ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic inflammation and ulceration in the segments of the gastrointestinal tract (Nemati et al., 2017). The highest IBD prevalence worldwide was reported in 2011 were in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons) and North America (UC, 249 per 100,000 persons; CD, 319 per 100,000 persons), which provide evidence for association between this disease and industrialization (Molodecky et al., 2012). Furthermore, based on Moum and Zeng (1996) studies, there are peaks in the onset of UC and CD exacerbations in winter months in the Northern Hemisphere and the highest rate of prevalence have been reported in Scandinavia, Great Britain, and North America. This theory is thought to be related to vitamin D deficiency in IBD patients (Sands et al., 2009).

The total direct cost for IBD patients in Canada is estimated to be approximately between $7,210 CAD (95% CI $5,005 - $9,464) (Gibson et al., 2008). Although the causative etiology of IBD is not fully understood, extensive studies in the past few decades suggest impairments in the immune system, genetic susceptibility and environmental risk factors as well as gut microbiota dysbiosis as the main players in IBD pathogenesis (Kelsen et al., 2017; Lopetuso et al., 2017; Nemati et al., 2017; Rodriguez de Santiago et al., 2017; Rogler, 2017). According to the genome-wide association study (GWAS), 163 genes and genetic loci have been identified that can contribute to IBD pathogenesis (Abreu, 2013; Loddo et al., 2015; Liu

et al., 2016), from which approximately 30% are shared between CD and UC patients. For instance, in CD, GWAS identified a variant, near the gene TNFSF11 that encodes for receptor activator of nuclear factor kappa-B ligand (RANKL) (Boyce et al., 2007; Dixon et al., 2007; Ali et al., 2009; Krela-Kazmierczak et al., 2016). The CD risk allele increased expression of RANKL in specific cell lines (Franke et al., 2010; Sanseau et al., 2012). Although RANKL plays a major role in the development of rheumatoid arthritis by assisting the excessive osteoclastogenesis in such disease, its role in the development of IBD is not clear. Because of the implication of the RANK/RANKL in the pro-inflammatory pathway of the immune system, it is expected that this pathway can contribute to IBD (Boyce et al., 2007). This study aims to elucidate if Denosumab (Prolia™) as a RANKL inhibitor have any impacts on dinitrobenzosulfonic acid (DNBS)-induced colitis in an experimental setting through the regulation of immune activation and gut microbiota. If positive, RANKL inhibitor may potentially serve as a therapeutic approach in IBD, particularly in CD patients.

1.2. Literature Review

1.2.1. Overview of Inflammatory Bowel Disease

1.2.1.1. Definition of IBD

IBD, consisting two major types of idiopathic intestinal disorders: CD and UC, are characterized by chronic inflammation and ulceration in the segment of the gastrointestinal tract (Kelsen et al., 2017; Nemati et al., 2017). In CD, there is an incidence of transmural inflammation at the site of the ileum and the colon which in a discontinuous pattern may involve any parts of the gastrointestinal tract; while in UC the inflammation occurs at the mucosal layer and mostly involves the rectum with the possibility to affect a part or the entire colon in a continuously manner (Fiocchi, 1998; Fiocchi, 2005; Danese et al., 2011; Baumgart et al., 2012). CD is usually associated with intestinal granulomas, strictures, and fistulas, whereas the incidence of bowel perforation (rupture) and colorectal cancer occur more in UC (Abraham et al., 2009).

1.2.1.2. Epidemiology of IBD

Epidemiologic studies demonstrated a strong worldwide variation in the context of prevalence and incidence of IBD. The highest prevalence of IBD reported in 2011 was in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons) and in North America (UC, 249 per 100,000 persons; CD, 319 per 100,000 persons) illustrating a significant association between IBD and industrialized countries (Hanauer, 2006; Gibson et al., 2008). In contrast, Eastern countries having considerably lower incidence rates (Gibson et al., 2008; Hanauer, 2006). However, in the last two decades, studies reported a rising incidence rate in several developing regions including Asia, South America and Middle East suggesting an

evolution of IBD and an increased prevalence across geographic regions (Molodecky et al., 2012; M'Koma, 2013; Kaplan, 2015).

1.2.1.3. Etiopathology

1.2.1.3.1. Genetic Susceptibility in IBD

According to the genome-wide association study (GWAS), 163 genes and genetic loci have been identified that can contribute to susceptibility to IBD (Abreu, 2013; Loddo et al., 2015; Liu et al., 2016), from which approximately 30% are shared between CD and UC patients. Analyses of these genes showed that they are associated with several essential pathways implicated in intestine homeostasis, implying a role of genetic susceptibility in the mechanism of the pathogenesis of IBD (Doecke et al., 2013; Mondal et al., 2017). Some of these pathways include: barrier function, epithelial restitution, microbial defense, innate and adaptive immune regulation, reactive oxygen species generation, autophagy, endoplasmic reticulum (ER) stress, and metabolic pathways (Kaser et al., 2008; Mazmanian et al., 2008; Sollid et al., 2008). Up until now, more than 30 genes related to IBD susceptibility have been recognized of which some are only associated with CD (e.g. NOD2, ATG16L1, IRGM, PTGER4) or UC (e.g. ECM1), while others are shared between CD and UC patients (e.g. IL23R, STAT3) (Xavier et al., 2007; Kaser et al., 2008; Mazmanian et al., 2008; Sollid et al., 2008; Zhang et al., 2014).

1.2.1.3.2. Environmental Factors

The (Derakhshani, Tun, et al., 2016)changing pattern in global prevalence of IBD suggests a role of environmental factors in the pathogenesis of IBD (Ali et al., 2008). One of these factors has been described as “Hygiene hypothesis”. In that context, a higher rate of IBD

pathogenesis has been described in high socioeconomic countries, where there is lack of exposure of people to certain microbial agents (both pathogenic and non-pathogenic) during infancy and childhood because of stringent sanitation conditions. Overall, the hygiene hypothesis suggests the insufficient development of the immune system that leads to increased rates of IBD and other dysfunctional immune-related diseases, such as asthma development (Hanauer, 2006; Ali et al., 2008; Mazmanian et al., 2008). Moreover, this lifestyle is also associated with Western diet, stressful job-related lifestyle as well as higher chance of exposure to pollution and industrial chemical in urban areas compared to rural sites (Xavier et al., 2007; Manzel et al., 2014; Vasquez, 2014). Drinking and smoking habits are also known as potential factors in the pathogenesis of IBD (Molodecky et al., 2012; Legaki et al., 2016).

1.2.1.3.3. Dysfunctional Immune Response in IBD

The immune inflammatory response, a host defense mechanism in the case of injury or infection, is stratified into two main steps: activation of the innate immune response followed by an activation of the adaptive immune response.

In the last several decades, studied demonstrated the contribution of dysregulated inflammatory molecules to the immunopathogenesis of IBD. Most considered IBD as a consequence of an abnormality at the level of the adaptive immune response, while GWAS revealed a variety of target genes that point toward a dysfunctional innate immune response (Cader et al., 2013). Proven efficacy of anti-TNF-α drugs, a crucial player in regulating innate inflammatory response underscores the importance of the established therapeutic interventions targeting the defective innate immune system (Rahimi et al., 2007). But it needs to be mentioned that adaptive immune cells can release tumor necrosis factor (TNF)-α.

Disrupted intestinal epithelial barrier integrity leads to mucosal susceptibility by enhancing toll-like receptor activity directed against commensal bacteria, pathogenic invaders or environmentally derived antigens, which results in activation of a series of innate inflammatory response cascades. In the context of chronic colonic inflammation, defect at the epithelial level can be caused either by genetic perturbations of expressing antimicrobial peptides in the epithelium layer or by an imperfection in controlling the intracellular bacteria within phagocytic cells, such as antigen presenting cells (APC) like dendritic cells or macrophages (Barcelo-Batllori et al., 2002; Roda et al., 2010; Bar et al., 2013). Following APC activations and production of a large amount of pro-inflammatory cytokines, not only the innate components including macrophages/monocytes, neutrophils, and dendritic cells will be activated but this will also lead to the recruitment activation and differentiation of naïve T-cells into effector T helper (Th) cells, polarizing them to a Th1, Th2, or Th17 profile, or activating B cells. These cytokine-mediated interactions also activate reactive oxygen metabolite (ROM) cascade and chemotaxis, causing further oxidative stress, which alters gut homeostasis and results in aggravation of the inflammation initiated tissue damage (Frink et al., 2007). Several pro-inflammatory markers play a key role in during the over-activated immune response during IBD. This includes interleukin (IL)-1, IL-6, IL-18, TNF, members of IL-12 family (IL-12, -23, -27, -35), interferon (IFN)-α, and IFN-β produced by APCs, such as dendritic cells (DCs) and macrophages, all reported during the development of IBD (Park et al., 2017). Although prototypically CD and UC were considered as T helper Th1 or Th2-associated diseases, over the time other pathways including the Th17, Th22, and T regulatory cell cytokines have been identified as a common factor in both disease phenotypes, highlighting the role of IL-22 and IL-17A/IL-23 axis (Monteleone et al., 2001; Strober et al.,

2007; Neurath, 2008; Park et al., 2017). The last axis is mostly secreted by innate lymphoid cells (ILCs) but as a controversial role as an effector or protector for IL17 in different models of IBD (Yang et al., 2008; O'Connor et al., 2009; Eken et al., 2014). Understanding the basic characteristic of the major subsets of clusters of differentiation (CD) 4+ and CD8+ T helper cells, encompassing Th1, 2, 9, 17, 22 and Tregs will help us to investigate their role in interactions between innate and adaptive immunity in the gut of IBD patients. IFN-γ produced by natural killer and DC in response to intracellular pathogens or commensal bacteria induces Th cell polarization and Th1 differentiation through activating STAT1, 4 (Signal transducer and activator of transcription1,4) transcription factors that lead to activation of the lineage-specific transcription factor T-bet (Becker et al., 2006; Chaudhry et al., 2009; Hisamatsu et al., 2013; Park et al., 2017). Th1 production is vital for cell-mediated immunity through the production of several signature cytokines including IFN-γ, TNF, and IL-2. As a fact, it has been recently described that during CD, a high level of IL-2Rα expressed by mucosal T cells results in producing a notable amount of IL-2, which has also been reported to have a positive correlation with cytotoxicity of T cells in CD (de Souza et al., 2016). Along with IFN-γ produced by DC or macrophages, other pivotal cytokines like IL-12 and IL-18 can control Th1 T-cell differentiation ability and are up-regulated in CD (Fuss et al., 1996; Monteleone et al., 1997; Pizarro et al., 1999; Park et al., 2017). In addition to the Th1 cytokines, the Th2 signature cytokines, such as IL-5, IL-10, IL-13, IL-21 and IL-25, and transcription factors such as GATA-binding protein 3 (GATA3) were also increased during CD and UC (Fuss et al., 1996; Heller et al., 2005; Fuss et al., 2008). On the other hand, the reported decrease in production of IL-4 by mucosal T cells in both UC and CD, suggests a defect in exhibiting a full classical Th2 response in the pathogenesis of IBD (West et al., 1996; Park et al., 2017).

The IL-4 released by APC and specifically DC mediates the activation of the lineage-specific transcription factor GATA-3 and induces Th2 cells, which by secreting IL-4 and IL-10 participates in the B cells activation and targets the termination of extracellular bacteria (Becker et al., 2006; Annunziato et al., 2007; Kleinschek et al., 2009). The lineage induction of the other subset of T helper cells, Th17, occurs in the absence of IL-4 and IL-12 secretion, cytokines in charge of the polarization toward Th2 and Th1 respectively. Also, IL-6-mediates the activation of STAT3 by inducing RORγt transcription factor leading to the production of Th17 signature cytokines, such as IL-6, IL-17, IL-23 and TGF-β (Annunziato et al., 2007; Yang et al., 2008; Ivanov et al., 2009; Park et al., 2017). In IBD patients, high level of Th17 cell-associated cytokines, such as IL-17A and IL-17F, has been reported in response to the increase levels of IL-6 and IL-23 upon microbiome stimulation (Annunziato et al., 2007; Ivanov et al., 2009; Kleinschek et al., 2009) which leads to severe epithelial damages by recruiting neutrophils as well as increasing the levels of IL-1β, IL-6, IL-8 and TNF in CD (Siakavellas et al., 2012). In addition, several studies addressed the Th9 and Th22 cell subsets of Th17 cells implicated by describing higher levels of IL-9 produced by Th9 cell subset and reduction levels of Th22 subset in patients with UC (Gerlach et al., 2014; Leung et al., 2014). Th9 cells require IL-4 and TGF-β for maturation and they secret their signature pro-inflammatory cytokine, IL-9, and IL-10 in order to activate Th17 cells (Asseman et al., 1999; Becker et al., 2006; Park et al., 2017). CD4+ T Th22 cells in contrary to Th17, secret low level of RORγt, a transcription factor essential for TH17 development, and TGF-β inhibits IL-22 expression by Th22 cells. Th22 differentiation is promoted by IL-6 and TNF and due to the activation of Th22 cells results in the secretion of IL-22, IL-13, fibroblast growth factor, chemokines, and TNF. Several studies reported the protective role of Th22 cells against

bacterial, fungal or viral infection at the epithelial barrier of the gastrointestinal tract associated with accelerating the wound repair mechanism (Monteleone et al., 2001; Becker et al., 2006; Chaudhry et al., 2009).

1.2.1.4. Microbiome and IBD

1.2.1.4.1. Definition of Microbiome

For the first time the term "microbiome" was used by Joshua Lederberg (2001) and defined by Merriam Webster dictionary as the collective genomes of microorganisms including but not limited to bacteria, fungi, archaea and viruses inhabiting a particular environment and specifically the human body. There is a coexistence and co-evolution between human and microorganisms to keep a homeostatic body status while having a mutual symbiotic relationship benefiting both groups (Rapozo et al., 2017). The human microbiome contains approximately more than 10 times as many genes as the human genome and is entitled the "virtual organ" or "our forgotten organ" (Chung et al., 2016; Song et al. 2016). The estimation for a number of bacteria exist in a reference man (70 kg) is $3.8* 10^{13,}$ this is when the total count for human cells is $3.0*10^{13}$ equals to 0.2 kg of body weight (Sender et al., 2016). Around 5 million variable genes are estimated to be present in the human gut microbiota, which predominantly belong to Firmicutes, Bacteroidetes and Actinobacteria phyla, whereas, Proteobacteria, Fusobacteria, Cyanobacteria and Verrucomicrobia phyla are less represented (Qin et al., 2010; D'Argenio et al., 2015). Based on comparative varied cohort studies, biological factors such as genetic, age, diet, delivery method, lifestyle, and antibiotic usage can impact the composition of microbiome (Debelius et al., 2016; Dickson, 2017). Many ongoing studies tried to unravel the mechanism underlying the microbiome effect through the host/microbe interactions in different disease states compared to healthy

ones. Based on these studies several functions are associated with microbiota, consisting polysaccharide digestion, vitamin synthesis, fat storage, and angiogenesis regulation. Also microbiome profile contributes to shape and educate the local and systemic immune response, through inhibition of nutrients and adhesion sites for pathogenic and opportunistic bacteria with mediation of commensal bacteria. Any disturbances in the microbe—host interactions, such as a defect in the innate microbial sensing and clearance, loss of barrier functions, and failure to maintain intestinal epithelial cell homeostasis targeting Paneth cells, may lead to the chronic activation of immune mediators and ultimately dysbiosis (Becker et al., 2015; Nakamura et al., 2016).

To define a healthy state of microbiome within a specific site (Eubiosis), measurement of two aspects of "robustness" and "resilience" need to be determined to understand its contribution to the overall definition for the stability of a community (Backhed et al., 2012; Flores et al., 2014). In one hand, robustness refers to the capability of one community to withstand any changes undergoing in an ecological stress-related disturbance without having any effects on its functions (Backhed et al., 2012; Flores et al., 2014). Resilience measures the ability of a microbial community of how often or how strong it can recover and come back to an equilibrium state following a community perturbation (Backhed et al., 2012; Flores et al., 2014). In that context, dysbiosis refers to an imbalance in both robustness and the resilience of quasi-stable state of microbiome. This dysbiosis is due to overcome of pathogenic and opportunistic bacteria over commensals or simultaneously loss of beneficial microorganisms as well as disturbance in the richness or diversity of microbial communities that results in a disharmonic chronic alteration in the composition and functionality of the gut microbiome profile (Bien et al., 2013; Knights et al., 2013; Lepage et al., 2011; Willing et al., 2010; Frank

et al., 2007; Manichanh et al., 2006). Although the role of microbial dysbiosis in the pathogenesis of several diseases needs more explorations, recent large-scale studies demonstrated its considerable intervention in several immune-related pathologies both in human and animal models, including but not limited to allergic disorders, asthma, obesity, Type 1 diabetes, autism, colorectal cancer, rheumatoid arthritis and IBD. (Russell et al., 2012; Kostic et al., 2015; Butto et al., 2016; DeGruttola et al., 2016; Sokol et al., 2016)

1.2.1.4.2. Gut Microbial Dysbiosis and IBD

In recent years, the etiology of aberrant immune response to suboptimal gut microbiome attracted researchers and clinicians' attention in investigating the cause and consequence of microbiome alteration in the context of IBD. The considerable improvements in bioinfomatics analysis in association with in-vitro and in-vivo experimental models of CD and UC contributed to a better understanding about the role of the gut-microbial dysbiosis and microbiome/host interplays during the development of IBD and experimental colitis.

According to population cohort studies, an imbalance in intricate symbiotic correlation between host and gut microbiota is a dynamic characteristic for healthy populations (Backhed et al., 2012; Flores et al., 2014; Halfvarson et al., 2017). Although there are variations between microbiome of healthy individuals due to differences in their age, diet, ethnicity, etc., to study the long-term gut microbiome dynamics, these variations affect minor proportion of bacteria. This contrasts with IBD group, which traversed far from the healthy groups representing the notable volatility in the gut microbiome composition, diversity, and species richness in IBD patients (Halfvarson et al., 2017). These studies define the dysbiosis associated with IBD pathogenesis through alteration in both disrupted physiological functions and compositional perturbation.

In the context of IBD, resident and transient microbiota as well as sample types and sites (e.g. remission or inflamed conditions, surgical samples, location) revealed differences in both abundance and composition of bacteria between various counterparts of the digestive system (lumina and mucosa) (Seksik et al., 2003; Peterson et al., 2008; Gu et al., 2013; Miyoshi et al., 2017). Previous research revealed an increase in the abundance of Enterobacteriaceae in fecal samples of CD patients (Seksik et al., 2003) and a decrease in the proportion of butyrate-producing *Roseburia hominis* and *Faecalibacterium prausnitzii*, with anticipated anti-inflammatory role, in fecal samples of UC patients (Sokol et al., 2016(Ott et al., 2004; Machiels et al., 2014; Sokol et al., 2016). Such declines were also observed in resected ileal samples of CD patients associated with endoscopic recurrence after surgery. Gophna et al. (2006) demonstrated an increased in Proteobacteria and Bacteroidetes and a down-regulation in Clostridia in biopsy samples from CD patients (Gophna et al., 2006). Additionally, Mylonaki et al. (2005) observed less Bifidobacteria and more Escherichia coli in IBD biopsies (Mylonaki et al., 2005). Another study targeting newly diagnosed pediatric CD patients showed an increase in abundance of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, and Fusobacteriaceae, and a decrease in the population of Erysipelotrichales, Bacteroidales, and Clostridiales in the mucosal samples from ileal and rectal biopsies (Gevers et al., 2014). Additionally, a depleted portion of abundance of Bacteroidetes and Lachnospiraceae in tissue samples from resected GI tract of CD and UC patients were reported by Frank et al. (2007). Recent studies claim for a direct effect of fungi and archaea dysbiosis in the pathogenesis of IBD (Sokol et al., 2016) which to wrap up reveals a significant dysbalance in both CD and UC disease.

1.2.1.5. Treatments for IBD

Although there is still no cure for IBD several treatments are suggested. These treatments mostly target the over-activated immune response, which result in suppressing the active flare stage of IBD while leading to remission state, with the hope of long-term healing. Such treatments are prescribed with different dosages and different injection methods, mainly subcategorized into two groups: *Immunomodulatory drugs* as well as *antibiotics*. Immune-targeting drugs impact inflammatory pathways with suppressive, anti-inflammatory characteristics. The mostly used anti-inflammatory drugs are Aminosalicylates and Corticosteroids (Brown et al., 1952; Modigliani et al., 1990; Olaison et al., 1990). With regard to immune system suppressors Azathioprine, Mercaptopurine, Cyclosporine, Infliximab, Adalimumab, Golimumab, Methotrexate, Natalizumab, Vedolizumab, and Ustekinumab are mainly used (Dulai et al., 2016; Atreya et al., 2017; Neurath, 2017). The second group as antibiotics (Metronidazole, Ciprofloxacin) target pathogenic bacteria and other microorganisms, which carry the drawback of bacterial robustness after continuous usage (Sartor, 2004; de Souza et al., 2016). Antibiotics also increase the commensal bacteria activity to recover them faster from dysbiosis stage toward normal equilibrium. In several UC cases, there is recommendation for surgery that eliminates the signs and symptoms of IBD (Seifarth et al., 2017). It is crucial to know that each of the above treatments is recognized with several side effects. According to clinical therapeutic reviews in 2017 (Atreya et al., 2017; Neurath, 2017), the effects of each treatment (on both CD and UC patients) on mucosal healing symptoms are investigated and the authors confirmed the induction of mucosal healing for the indicated treatments. Also, Neurath (2017) in his comprehensive overview of current and future therapeutic approaches for IBD mentioned the role of several cytokine inhibitors in

addition to TNF blockers. These drugs are interacting with pro-inflammatory cytokines, such as IL-6, IL-12, IL-23, and can also modulate several cytokine-signaling pathways, such as JAK inhibitors and SMAD7 blockers, or transcription factors inhibitors, such as GATA3 and RORγt ((Sandborn et al., 2012; Neurath, 2017).

1.2.2. The Role of Receptor Activator of Nuclear Factor Kappa-B in IBD

1.2.2.1. Denosumab, Monoclonal Antibody Against RANKL

Denosumab, with two brand names as Prolia™ and Xgeva™, is a humanized monoclonal antibody against RANKL (Fouque-Aubert et al., 2008). Denosumab has been developed as an effective treatment for postmenopausal osteoporosis (PMO) with high fracture risk (Dempster et al., 2012). Denosumab is considered as an appropriate first-line pharmacologic option for PMO management due to its efficacy, safety, and potential to improve adherence rates (Anastasilakis et al., 2009; Miller, 2011; Josse et al., 2013). The discovery of antibodies against RANKL was the culmination of clarification about internal bone microenvironment regulation of bone remodeling: the process of old bone resorption and new bone formation by osteoclasts and osteoblasts (Moschen et al., 2005). This regulation is under the control of three main molecules including receptor activator of nuclear factor-κB (RANK, TNFRSF11A), RANK ligand (RANKL, TNFSF11) and osteoprotegerin (OPG, TNFRSF11B) (Moschen et al., 2005; Boyce et al., 2007). RANK is a type I transmembrane protein that can be found on osteoclasts, dendritic cells, fibroblasts, B and T cell lines (Fouque-Aubert et al., 2008; Cankaya et al., 2013). The high-affinity binding between RANKL and RANK expressed by osteoclasts and their precursors results in the fusion, differentiation, and activation of osteoclasts (Anderson et al., 1997; Lacey et al., 1998; Hsu et al., 1999), which leads to the activation of several transcription factors including activator

protein-1 (AP1) and NFκB (Jimi et al., 1999; Fouque-Aubert et al., 2008). Osteoprotegerin, a secreted glycoprotein with homology to members of the TNF receptor family (Jones et al., 2002; Fouque-Aubert et al., 2008), is a soluble decoy receptor for RANKL, which is expressed by osteoblasts in bone and lymphoid cells and DCs in the immune system (Lacey et al., 1998; Yun et al., 2001; Nahidi et al., 2011). OPG through its binding to the receptor RANKL or by inhibiting the RANKL/RANK ligation plays a major role as an inhibitory factor for osteoclastogenesis (Lacey et al., 1998; Nahidi et al., 2011). Up-regulation of RANKL simultaneously with OPG down-regulation is due to an estrogen deficiency in the context of the postmenopausal state and is known as the major mechanism for bone loss and osteoporotic fractures in such patients (Young-Yun Kong et al., 1999; Yun et al., 2001; Boyce et al., 2007; Nahidi et al., 2011; Dempster et al., 2012; Cook et al., 2014; De Voogd et al., 2016). Denosumab, through its binding to RANK Ligand, can compensate the lack of OPG level and reduce bone resorption by reducing the activation of osteoclasts (Miller, 2011; Dempster et al., 2012). Phase III clinical trial studies showed an effective role of a monoclonal antibody against RANKL on both men and women and potentially effective in glucocorticoid-induced osteoporosis (Buehring et al., 2013).

1.2.2.2. Rational for the Use of a Monoclonal Antibody Against RANKL in the Treatment of IBD

2 main concepts showed promises for the possible therapeutic effect of a monoclonal antibody against RANKL on CD patients including:

1- The symptoms of mutation at the locus, which encodes RANKL in CD patients.

2- The increased risk of osteoporosis associated with CD patients.

1.2.2.2.1. Mutation at RANKL Locus

A GWAS meta-analysis for CD demonstrated the presence of a variant near the gene TNFSF11 encoding for RANKL that together with TNF-α activates the NF-κB pathway (Dixon et al., 2007; Tremelling et al., 2007; Franke et al., 2010). As discussed above, this pathway is critical in the regulation of intestinal inflammatory response. Sanseu et al. (2012) showed a strong implication of CD risk allele at TNFSF11 gene in overexpression of the TNFSF11 transcript in all cell lines and in CEU B-lymphoblasts and osteoblasts. In addition, the strong association of such risk allele with increased expression of RANKL was reported in a publicly available database (LOD = 5.9) (Dixon et al., 2007). Hence, these data provide several evidences about genetic mutations that are more common in CD patients with ultimate overexpression of RANKL.

1.2.2.2.2. Osteoporosis During IBD

Population-based studies showed the rise in fracture risk in IBD patients approximately by 40%-60% compared to match controls (Bernstein et al., 2000; Card et al., 2004). According to a comprehensive bone mineral density (BMD) study, there is a significant decrease in BMD of IBD patients (Schule et al. 2016). Authors indicated the severity of gut inflammation, perianal disease including fistulas, systemic steroid usage, low BMI, age, and intestinal malabsorption leading to calcium and vitamin D deficiency as risk factors for low BMD (Bernstein et al., 2000; Card et al., 2004; Jahnsen et al., 2004; Ali et al., 2009; Azzopardi et al., 2013; Krela-Kazmierczak et al., 2016; Schule et al., 2016). Also, IBD patients under long-term corticosteroid therapy developed osteoporosis (Reinshagen, 2008). It decreased the level of calcium, which absorbed by intestine as an essential bone-remodeling mineral. Simultaneously, the decrease in estrogen secretion level contributes to high

osteoclastogenesis through reduction in OPG level (Krela-Kazmierczak et al., 2016; Schule et al., 2016; Narula et al., 2012; Reinshagen, 2008).

Local or generalized inflammation during IBD attracts systemic T cell activation through DCs/Th1 cell interaction; which leads RANKL induction (Ashcroft et al., 2003; Moschen et al., 2005; Boyce et al., 2007; Fouque-Aubert et al., 2008; Narula et al., 2012). This RANKL production not only triggers osteoclast activation, but also is in charge of activation of inflammatory pathways in the gut immune system (Narula et al., 2012; Moschen et al., 2005; Bernstein et al., 2003). Several animal models studies demonstrated compelling evidence for the importance of RANKL paradigm in bone hemostasis maintenance during IBD, suggesting a therapeutic role for OPG as a blocker for RANKL. In the context of experimental colitis, Ashcroft et al. (2003) used IL-2 deficient mouse model of colitis, model known to develop both osteopenia and colitis and demonstrated that animals had significant increase in bone marrow mononuclear cell expression of soluble RANKL (sRANKL) and OPG mRNA as well as serum sRANKL and OPG in comparison to control group. Osteopenia was not evident in IL-2 deficient mice crossbred to be T cell deficient, and osteopenia could be induced in T cell deficient mice by adoptive transfer of T cells from IL-2 deficient mice (Ashcroft et al., 2003; Boyce et al., 2007; Bernstein, 2005). Importantly the administration of exogenous OPG reversed both the osteopenia and the colitis as shown by colitis abrogation through a significant reduction in colonic dendritic cells, while circulating inflammatory cytokines were unaffected by exogenous OPG (Bernstein, 2006). These data illustrate the importance of OPG and RANKL in osteopenia and colitis in IL-2 deficient mice and the importance of activated T cells in mediating these conditions. Hence, RANKL plays a critical

role in association of mucosal inflammation with deregulated bone metabolism and, consequently, osteoporosis.

1.2.2.3. Monoclonal Antibody Against RANKL Safety

The safety of Denosumab in the treatment of osteoporosis for both genders has been assessed in phase III clinical trials in over 3876 multinational women and 1000 men aged from 60 to 91 years (Jean-Jacques Body, 2006; Anastasilakis et al., 2009; Fizazi et al., 2011; Miller, 2011; Saad et al., 2012; Smith et al., 2012). All patients received a single 60 mg dose every 6 months administered subcutaneously and all were instructed to take calcium (≥500 mg) and vitamin D (≥400 IU) supplementation per day. Adverse reactions including hypocalcemia, serious infections, osteonecrosis of the jaw and some dermatological reactions were reported in more than 2% of postmenopausal women and more than 5% of men with osteoporosis and more frequently in the Prolia-treated patients when compared with the placebo-treated group. Hypocalcaemia, which represents the serum calcium level reduction to less than 8.5 mg/dL at any visit, was reported 13% more in woman receiving Prolia (Saad et al., 2012). Osteonecrosis of the jaw, which is associated with tooth extraction or local infection, was reported in less than 1% of women with postmenopausal osteoporosis receiving Prolia (Neuprez et al., 2014; Olate et al., 2014). Epidermal and dermal adverse effects such as dermatitis, eczema, and rash were reported 2.6% more in Prolia-treated patients (Fizazi et al., 2011). Additionally, blocking the RANKL receptor on activated T- and B-lymphocytes increase the risk of infection, although there were no reports related to serious infections.

1.2.3. Animal Models to Study IBD

For research purposes, studies using samples from affected patients provide the most reliable data; however, due to difficulties in human tissues sample collection, few and small

sample size, animal models are used (Coors et al., 2010). Since animal model studies provide large sample sizes, fewer limitations exist for invasive sampling, and existence of genetically homogeneous animals (i.e. genetically engineered rodent models).

1.2.3.1. Characteristics of a Suitable Animal Model of IBD

To select an ideal animal model, several characteristics need to be considered including: availability of animals, being relatively inexpensive, being fast reproducible for more off springs per litter, and yielding information that could be easily translated to humans. Also, it should exhibit human UC/CD disease symptoms and should respond to treatment or therapeutic measures the same way the IBD patients would respond. Since all models do not have all IBD characteristics simultaneously, models that possess some of these characteristics are utilized (Mizoguchi et al., 2008)

1.2.3.2. Common Animals Used in IBD Research

A number of animal species are used to mimic IBD pathogenesis including: the non-human primates (e.g. monkeys, cotton-top tamarins), zebra fish, rodents (e.g. mice, rat, guinea pigs), and domestic pigs (Heinritz et al., 2013). While non-human primates provide the best comparable data to people, they are not widely used due to their high costs, ethical purposes, and the potential hazards of carrying highly virulent zoonotic agents (Coors et al., 2010; Ideland, 2009). Zebra fish in IBD are used to study both innate and adaptive immune responses (Lam et al., 2004; Oehlers et al., 2011). Rat's models are larger thus allowing larger sample acquisition. Also rodent's fermentation happens in their large cecum (Houpt et al., 1979) and have faster digestive passage rates which defines as requiring more feed per unit body weight, in addition to a lower comparable capacity for fiber digestion compared to humans (Heinritz et al., 2013). Mice are good animal models because of shared characteristics

in their intestinal genes with humans (Waterston et al., 2002; Bryda, 2013). In addition, human and murine intestinal gut microbiota exhibit comparable diversity of species within the Firmicutes and Bacteroidetes phyla (Dethlefsen et al., 2008; Heinritz et al., 2013). Mice with the presence of similar populations of B cells, T cells, and isotype antibodies exhibit adaptive immune response features same as IgG and IgM (Mestas et al., 2004). Also, their small size, low breeding costs, relatively short estrous cycles and gestation period, and large litter sizes (Nguyen et al., 2008) lead them to be selected as an advantageous model to study immune related diseases.

1.2.3.3. Types of Animal Models of IBD

Despite existence of several IBD models, none completely photocopies human IBD. These models in overall categorized in 5 types, antigen-induced colitis or colitis induced by microbiota (Iqbal et al., 2002), chemical inducible forms of colitis (Cooper et al., 1993), genetic or transgenic colitis models (Kuhn et al., 1993), adoptive transfer of immune cell models (German et al., 2000; Mansfield et al., 2001), and spontaneous colitis models (Kobayashi et al., 2014). Between such models, only few can mimic CD, which is the target of this thesis including: TNBS or DNBS models. The effectiveness of these chemicals for mimicking tissue injuries similar to CD are affected by the molecular weight of the chemical, its concentration and manufacturer (Perse et al., 2012) as well as the species, gender (Mizoguchi et al., 2008), and the genetic background of the animal model (Wirtz et al., 2007; Lakhan et al., 2010; Wirtz et al., 2017). More importantly, the administration method affects the severity of disease, as some chemicals work well to induce post-ingestion inflammation (Mizoguchi, 2012), while others need to be applied directly to the infection site to be effective (e.g. intrarectal administration) (Elson et al., 1995). Furthermore, the differences in intestinal

microbiome profile can influence the effectiveness of chemicals in tissue injury induction (Kitajima et al., 2001).

1.2.3.3.1. Trinitrobenzene Sulfonic Acid (TNBS)

Trinitrobenzene sulfonic acid is primarily used to induce acute intestinal inflammation in animal models, but can also be employed to induce chronic inflammation in rodents (Fitzpatrick et al., 2010; Mariman et al., 2012), pigs (Pouillart et al., 2010), rabbits (Mizoguchi, 2012), guinea pigs (Robinson et al., 2014), and non-human primates (Kim et al., 1992). TNBS is a hapten that when it binds to a high molecular tissue protein gets turned into an antigen. TNBS needs to be dissolved in Ethanol to be activated. TNBS by altering host proteins through covalent bonds formation with trinitrophenyl haptens of TNBS (Elson et al., 1995) stimulates an immune-inflammatory response. Ethanol, by contributing to disruption of epithelial barrier, helps TNBS to colon shortening, epithelial necrosis causing crypt architecture destruction, and transmural inflammation in parallel with increased colonic Th1 immune response (Neurath et al., 2000; Kawada et al., 2007; Kremer et al., 2012). Neurath et al. (2000) indicated mice as the best models for investigating TNBS-Ethanol induced colitis due their genetic background and phenotypic profile of the mouse. For example, C57BL/6 and DBA/2 strains showed robustness to TNBS treatment, whereas SJL/J, C3HeJ and BALB/c mice produce significant tissue ulceration associated with TNBS-induced colitis (Elson et al., 1995; Kawada et al., 2007)

1.2.3.3.2. Dinitrobenzene sulfonic Acid (DNBS)

DNBS is also a hapten used to induce colonic inflammation, with similar features to TNBS model although TNBS due to having an additional active nitro group has higher affinity binding to proteins and enzymes. On the other hand, DNBS more selectively binds

only to the e—amino group of lysine (Hawkins et al., 1997). Previously, DNBS dissolved in 50% Ethanol, was administered as an enema to induce CD, but the method was modified where colitis is induced in lightly anesthetized mice by an intrarectal injection, delivered 3 cm into the colon via a polyethylene catheter (Cuzzocrea et al., 2001). The physical, histologic features, and inflammatory responses in the DNBS model, are comparable to observations made in the TNBS models (KoCho, 2005; Ko, Lam, et al., 2005; Joshi et al., 2011).

Chapter Two

Rational, Hypothesis and Aims

2.1. Rational

In IBD, CD and UC are characterized by chronic intestinal inflammation. Genetic susceptibility, environmental triggers, deregulated immune system as well as imbalanced host/gut microbiome interactions are recognized as prominent factors contributing to the development of IBD (Abraham et al., 2011; Khor et al., 2011; Maloy et al., 2011; Manichanh et al., 2012). RANKL plays a major role in the development of rheumatoid arthritis (Jones et al., 2002) and genome-wide association meta-analysis for CD identified a variant near the TNFSF11 gene that encodes RANKL. RANKL is expressed in immune cell and cell lines that contributes to the CD pathogenesis (Dixon et al., 2007; Franke et al., 2010). Despite of RANKL role in rheumatoid arthritis (RA), its implication in the pathogenesis of IBD and CD is unknown. This study aims to investigate if the RANKL inhibitor (Prolia) can modify the gut microbiota dysregulation under experimental colitic conditions and how ultimately this treatment can be used to decrease the colitic condition in DNBS model of colitis and therefore serve as a potential therapeutic target in IBD.

2.2. Hypothesis

RANKL inhibitor treatment (Prolia^{TM}) decreases colitic condition in an experimental model of CD through a decrease of colonic mucosal proinflammatory cytokines and a modification of the intestinal microbiome.

2.3. Aims

1. To investigate the effect of DNBS/Ethanol-induced colitis on gut microbiome composition.
2. To demonstrate the effect of RANKL inhibitor in suppression the DNBS/Ethanol-induced inflammatory response.
3. To investigate the role of RANKL inhibitor on microbiome dysbiosis induced by DNBS/Ethanol-induced colitis.

Chapter Three

Material and Methods

3.1. Animals and Experimental Design

Forty-eight, 7-week-old male C57Bl/6 mice were received from Charles Rivers, (Winnipeg, MB, Canada) and maintained under co-housed pathogen-free conditions in the animal care facility at the Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada. Mice were treated with daily intraperitoneal (*i.p.*) injection of PBS 1% (Vehicle; n=24) or Denosumab (Prolia™, n=24) at 10 mg/kg/d for four days. On day two of the experiment, mice were divided into four subgroups (n=6/subgroup) and subjected to different colitis induction models: a) **PBS 1%**, b) **Ethanol 30%**, c) DNBS (4mg/kg) dissolved in PBS 1% (**DNBS/PBS**), and d) DNBS (4mg/kg) dissolved in 30% Ethanol (**DNBS/Ethanol**) (Fig 1). Injections were done intrarectally using a PE-90 tubing (10 cm long; ClayAdam, Parisppany, NJ, USA) inserted 3.5 cm into their colons and attached to a tuberculin syringe (BD, Mississauga, ON, Canada). All mice were received a similar standard chow diet. The experimental protocol (–010) was approved by the University of Manitoba Animal Ethics Committee and conducted under the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

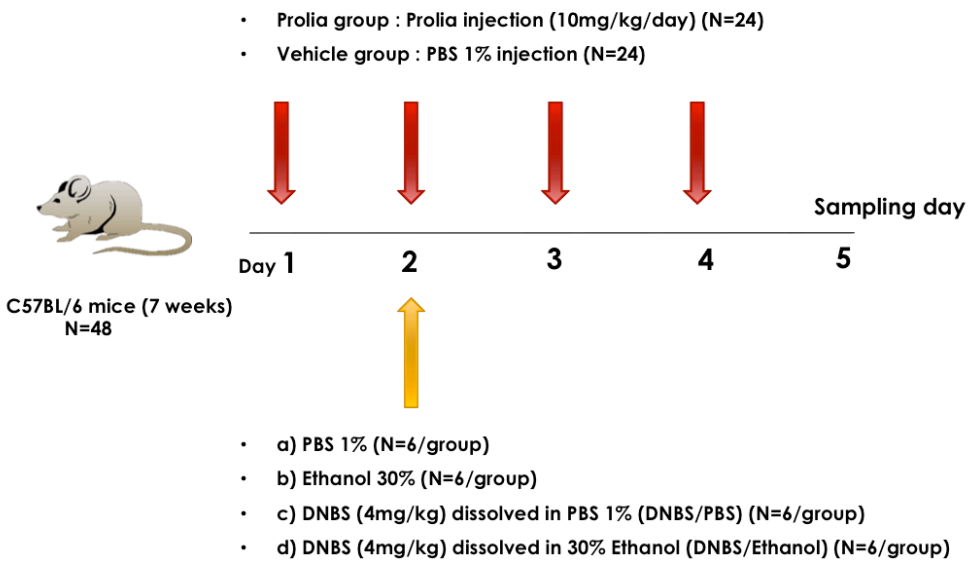


Figure 1. Experimental design. Mice were treated with daily injection (i.p.) of PBS 1% (Vehicle; n=24) or Prolia (n=24) at 10 mg/kg/d for four days. On day two of the experiment, mice were divided into four subgroups (n=6/subgroup) and subjected to different colitis induction models: a) PBS 1%, b) Ethanol 30%, c) DNBS (4mg/kg) dissolved in PBS 1% (DNBS/PBS), and d) DNBS (4mg/kg) dissolved in Ethanol 30% (DNBS/Ethanol). On day 5, all mice were sacrificed and colon tissue/mucosa and feces samples were collected.

3.2. Evaluation of Inflammation

Disease activity index (DAI) is a composite index taking into consideration the percentage of weight loss, stool consistency and fecal blood scores. DAI was assessed from day 1 to day 4 over the period of DNBS/Ethanol-induced colitis treatment. The DAI scoring system was defined as follows: weight: 0, no loss; 1, 5–10%; 2, 10–15%; 3, 15–20%; and 4, >20%; stool: 0, normal; 2, loose stool; and 4, diarrhea; and bleeding: 0, no blood; 2, presence of blood; and 4, gross blood. Presence of blood in the stool was assessed using the Hemoccult II test (Beckman coulter, Oakville, ON, Canada).

On the sacrifice day, to evaluate the colonic macroscopic damage, colon was opened longitudinally and macroscopic score was assessed immediately using previously established scoring system (Cooper et al., 1993; Khan et al., 2002). Macroscopic score was evaluated based on four parameters including rectal bleeding, rectal prolapse, diarrhea, and colonic bleeding. Histology analysis was assessed using fixed colonic segments that were paraffin (Sigma, Mississauga, ON, Canada)—embedded and then stained (10 μm sections) using hematoxylin-eosin (H&E) (Sigma, Mississauga, ON, Canada). Architectural derangements, goblet cell depletion, edema/ulceration and degree of inflammatory cells infiltrate were considered as evaluating the inflammatory response (Ghia, Blennerhassett, et al., 2009). Serum CRP was measured through collecting the blood by intracardiac puncture under isoflurane (Abbot, Mississauga, ON, Canada) anesthesia. Colonic inflammatory cytokines were assessed after colon samples homogenization in 700 μL Tris-HCl buffer containing protease inhibitors (Sigma, Mississauga, ON, Canada), centrifuged at 13000 × g for 20 min at 4 °C. The supernatant was frozen at -80 °C until assay. Serum CRP, MPO activity (Hycult Biotech, PA, USA) level and colonic cytokine levels (IL-6, IL-1β, TNF-α) were quantified using ELISA (R&D Systems, Inc., Minneapolis, MN, USA) according the manufacturer instructions.

3.3. Enzyme-Linked Immunosorbent Assays (ELISA)—Sandwich Type

The sandwich ELISAs with using pair of antibodies, as capture and detector, targets two or more distinct epitopes of antigens to specify antigen detection (Stanker et al., 2015). In brief, the antigen specific capture antibodies are first coated on the microtiter plate, then a series of sample dilutions consisting target antigen and standard antigen are added. The antigen are captured by capture antibody and as the next step, the bound antigens are

subsequently detected by adding a specific amount of detector antibodies; therefore, the antigens get trapped and—sandwiched in between the capture and detector antibodies. To remove the excess or unbound proteins, multiple washing steps were performed in between each step. The bound antigen-antibodies complexes are detected by the addition of the enzyme-conjugated secondary antibodies (second antibody which will bind specifically to the detector antibody), followed by incubation of the enzyme substrate. As a result, the colorimetric signal produced during the enzymatic reaction is proportional to the amount of enzyme-conjugate bound to the plate as measured with the ELISA plate reader. A direct relationship exists between the concentration of the antigen-antibody and the intensity of the signal (or color). As the concentration of antigen in the sample increases, the color becomes more intense. The sandwich ELISA was used to measure the level of MPO, CRP, pro-inflammatory cytokines, and total protein concentrations as previously described in our group (Ghia et al., 2008; Ghia, Li, et al., 2009).

3.4. RNA Extraction, cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For RNA extraction using TRIzol (Gibco BRL, Life Technologies, NY, USA), approximately 30–40 mg of colon tissue was used. Quality and quantity of RNA were determined by measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples had an absorption ratio A260/A280 greater than 1.8 RNA (1 μ g). Reverse transcription was performed using SuperScript VILO cDNA Synthesis Master Mix (Invitrogen, Grand Island, NY, USA) in an Eppendorf Thermo cycler at 25 °C for 10 min, followed by 42 °C for 60 min, and 85°C for 5 min according to the manufacturer's instructions. Samples were stored at -20

°C for RT-qPCR analysis. Real-time PCR (RT-PCR) reactions were performed in a Roch light Cycler 96 Real-Time System using Power SYBR green master mix (Life Technologies) in a final volume of 20 μL reactions. The PCR conditions were as follows: 95 ° C for 10 min, followed by 40 cycles at 95 ° C for 15 s and at 60 ° C for 60 s. As the reference gene, the TATA Box Binding Protein (*tbp*) primer (forward ACCGTGAATCTTGGCTGTAAAC, reverse GCAGCAAATCGCTTGGGATTA), *il1b* (forward, GCAACTGTTCCTGAACTCAACT reverse ATCTTTTGGGGTCCGTCAACT), *il6* (forward, TAGTCCTTCCTACCCCAATTTCC reverse TTGGTCCTTAGCCACTCCTTC) and *tnfa* (forward CCCTCACACTCAGATCATCTTCT, reverse GCTACGACGTGGGCTACAG) were used, designed from nucleotide sequences identified using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All RT-qPCRs were run in duplicate, the average standard deviation within duplicates of all samples studied was 0.25 cycles.

3.5. DNA Extraction and Quality Control

Colon mucosa and fecal samples were homogenized at room temperature and their DNA were extracted using ZR Tissue and Insect DNA extraction Kit (Zymo Research Corp., Orange, CA, USA) and ZR fecal DNA extraction kit (Zymo Research Corp., Orange, CA), respectively. Bead-beating step for the mechanical lysis of the microbial cells was included in both kits. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA), and the DNA quality was evaluated by PCR amplification of the 16 S rRNA gene using universal primers 27F (5′ -GAAGAGTTTGAT CATGGCTCAG-3′) and 342R (5′ -CTGCTG CCTCCCGTAG-3′), as previously described (Khafipour et al., 2009). Amplicons were verified using agarose gel electrophoresis.

3.6. Library Construction and Illumina Sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. (2016). Briefly, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al., 2012), as previously described (Khafipour et al., 2009; Derakhshani, De Buck, et al., 2016). Briefly, the reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. The PCR reaction for each sample was performed in duplicate and contained 1.0 μL of pre-normalized DNA (20ng/μL), 1.0 μL of each forward and reverse primers (10 μM), 12 μL HPLC grade water (Fisher Scientific, Ottawa, ON, Canada), and 10 μL 5 Prime Hot MasterMix (5 Prime, Inc., Gaithersburg, MD, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec, and an extension step at 72°C for 10 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA) to remove primers, dNTPs, and reaction components. The V4 library was then generated by pooling 200 ng of each sample and quantified using Picogreen (Invitrogen, Burlington, NY, USA). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1; Illumina, San Diego, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, measured by Qubit2.0 Fluorometer (Life technologies, Burlington, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTG CCAGCMGCCGCGGTAA-3′), read2 (5′-AGT

CAGTCAGCCGGACTACHVGGGTWTCTA AT-3′), and index read (5′-ATTAGAWACCCBDGT AGTCCGGCTGACTGACT-3′; Integrated DNA Technologies, Coralville, IA, USA) were added to the MiSeq Reagent V2 Kit (300-cycle; Illumina, San Diego, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories (Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada).

3.7. Bioinformatics Analyses

Bioinformatics analyses were performed as described previously (Derakhshani et al., 2016). Briefly, the PANDAseq assembler (Masella et al., 2012) was used to merge overlapping paired-end Illumina fastq files. The output fastq file was then analyzed using downstream computational pipelines in the open source software package QIIME (Caporaso et al., 2010). Chimeric reads were filtered using UCHIME (Edgar et al., 2011) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (Edgar, 2010) at 97% pairwise identity threshold using an open reference OTU picking process (Rideout et al., 2014). Taxonomies were assigned to the representative sequence of each OTU using an RDP classifier (Wang et al., 2007) and aligned with the Greengenes (v. 13.5) core reference database (DeSantis et al., 2006) using PyNAST algorithms (Caporaso et al., 2010). In order to compare microbial communities, the phylogenetic tree was built with FastTree 2.1.3. (Price et al., 2010).

3.8. Alpha—and Beta—Diversity

Within-community diversity (α-diversity) was calculated by different indices of species richness and evenness including Chao1 and Shannon using the open source bioinformatics package QIIME (Caporaso et al., 2010) and Phyloseq R package (3.1.0) (McMurdie et al., 2013). The p-values were calculated using the MIXED procedure of SAS (SAS 9.3) using a randomized factorial design where the effects of treatment (Vehicle vs. Prolia), induction model (PBS 1%, Ethanol 30%, DNBS/PBS, DNBS/Ethanol), and their interaction were considered as fixed factors and the effect of mice as a random factor. An even depth of 1500 and 25000 sequences per sample was used to calculate the richness and diversity indices for the colon mucosa and feces, respectively. To assess the beta-diversity (β-diversity) differences among bacterial communities from different treatments within each induction model, non-metric multidimensional scaling (nMDS) ordination plots were generated using R software (3.1.1) by employing Bray-Curtis similarity matrices with a conventional cut-off of <0.2 for the stress value (Munyaka et al., 2016). The resulting minimum stress solution was used to produce the nMDS plots, in which each data point represents one sample. The spatial distance between points in the plot was interpreted as the relative difference in the bacterial community composition; thus, points that were closer were more similar than points that were more distant. To assess the statistical differences in β-diversity of bacterial communities among treatment groups, permutation multivariate analysis of variance (Anderson, 2005) was performed using the above-mentioned statistical model and p-values were calculated.

3.9. Clustering Analysis

To illustrate the distinct clustering pattern within colonic vehicle and Prolia groups,

the relative abundance of the OTUs were binned into genus-level taxonomic groups and filtered to keep the most abundant genera found across all samples (cutoff value of> 0.1% of the community) (Derakhshani, Tun, et al., 2016). The resulting relative abundance table was normalized to correct for compositionality and also assist heat map-visualization of differentially abundant genera. The dissimilarity of samples were calculated based on Bray–Curtis measures using R "vegan" package (Oksanen, 2007) and the resulting matrix was subjected to unsupervised hierarchical clustering using R "dendextend" package (Galili, 2015) and visualized over the heat map of abundance matrix using R "complexheatmap" package (Gu et al., 2016). Genera were also clustered based on their Spearman's correlation coefficient using R "complexheatmap" package.

3.10. Correlation Coefficients

Associations between bacterial taxa with an abundance ≥0.5% of the community in the colon mucosa, inflammatory markers (IL-1β, IL-6, TNF-α, CRP, MPO) and tight junction proteins (F.actin and OCl) and alpha-diversity indices were explored using non-parametric Spearman's rank correlation implemented in PAST software (Hammer, 2001). For each correlation, correlation coefficient (Spearman's Rho) and p-value were obtained (Wei, 2016) and the resulting correlation matrix was visualized in a heatmap format generated by the corrplot package of R (Corrplot: visualization of a correlation matrix. R package ver. 02-0.2010; http://CRAN). The correlation coefficient values ranged from −1 to +1 with larger absolute values indicating stronger relationship while positive and negative values indicating the direction of association. Alpha value for the correlation confidence intervals was set up as 0.05.

3.11. Prediction of Functional Metagenomics

The open source software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; v. 1.0.0-dev) was used to predict the functional capacity of microbiome using 16S rRNA gene sequencing data and Greengenes (v. 13.5) reference database (DeSantis et al., 2006). To make our open-reference picked OTUs compatible with PICRUSt, all de-novo OTUs were removed and only those that had matching Greengenes identifications were retained. The new OTU table was then used to generate metagenomic data after normalizing the data by copy numbers, and to derive relative Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundance (Langille et al., 2013). The KEGG data was analyzed using STAMP (STatistical Analysis of Metagenomic Profiles) (Parks et al., 2014).

3.12. Other Statistical Analysis

To test the normality of residuals for alpha biodiversity, ELISA and qRT-PCR data the SAS UNIVARIATE procedure (SAS 9.3, 2012) was used and non-normal distributed data were logged transformed. Transformed data were further analyzed using SAS MIXED procedure with the effects of treatment (Vehicle vs. Prolia), induction model (PBS 1%, Ethanol 30%, DNBS/PBS, DNBS/Ethanol), and their interaction were considered as fixed factors and the effect of mice as a random factor. Tukey studentized range adjustment was used for all pairwise comparisons among the groups. GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA) was used for ELISA and qRT-PCR results graph plotting using the multiple comparisons of two-way analysis of variance (ANOVA). Differences were reported as statistically significant when $P<0.05$ while trends were discussed at $0.05 < P<0.1$.

Chapter Four

Results

4.1. Effect of Prolia Treatment on the Disease Activity Index

DNBS/Ethanol induced a colitis that was characterized by increased DAI (p=0.02), weight loss (p=0.01), decreased stool consistency and increased blood presence in the feces, which was evident on day 2 post colitis induction (day 4 of the study) in the vehicle group (Fig 2a,b). Within the vehicle group, on day 4, the DAI increased (p=0.03) by 34.5 fold in DNBS/Ethanol compared to Ethanol. PBS 1%, Ethanol 30% or DNBS/PBS treatments did not affect the development of colitis across treatments. Prolia treatments did not modify the DAI in Prolia group across different induction models. In non-colitic condition (PBS 1%, Ethanol 30%, DNBS/PBS), Prolia did not affect the weight loss (Fig 2a,b), stool consistency and presence of blood in the feces.

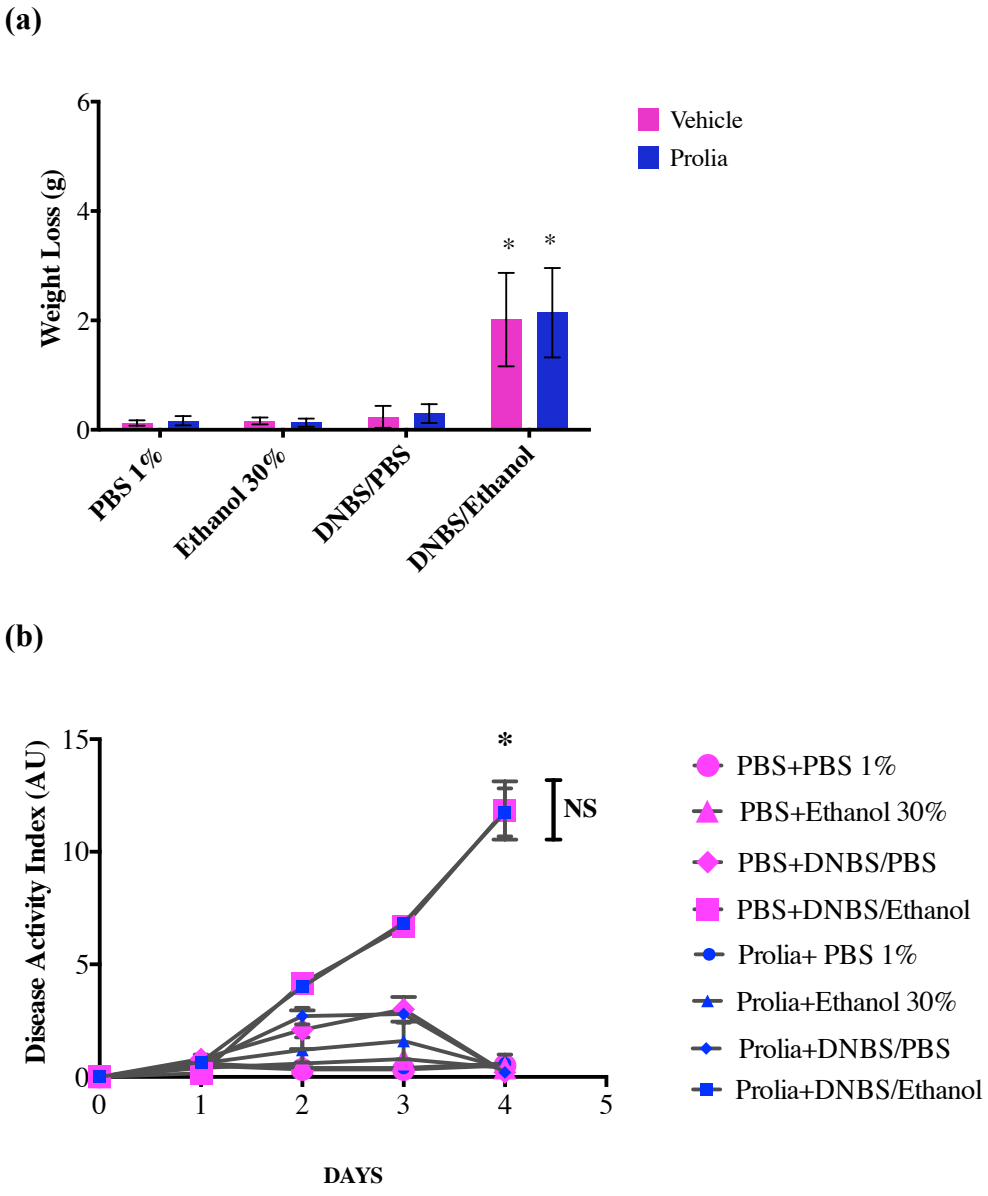


Figure 2: The effects of DNBS/Ethanol-induced colitis and Prolia treatment on a. weight loss and b. disease activity index. Vehicle=PBS 1%. Error bars are shown as SEM.*: P<0.001 when compared with other induction models within the Prolia or vehicle group calculated using SAS UNIVARIATE procedure and illustrated by Prism using two-way ANOVA. n=6 mice per group.

4.2. Effect of Prolia Treatment on the Macroscopic and Microscopic Scores

On day 3 post DNBS induction (day 5 of the study), mice were euthanized and external overall appearance of the colon was scored. Rectal bleeding, rectal prolapse, diarrhea, and colonic bleeding inside the colon of the mice were quantified. Results confirmed the DAI score. Within the Vehicle group, compared to Ethanol 30%, DNBS/Ethanol increased ($p=0.01$) the macroscopic score by 6-fold (Fig 3). PBS 1%, Ethanol 30% or DNBS/PBS groups did not show any effect on the macroscopic index as no differences were detected in rectal bleeding, rectal prolapse, diarrhea and colonic bleeding. In DNBS/Ethanol colitic conditions, Prolia treatment did not decrease the macroscopic score (Fig 2). Similarly, in non-colitic conditions (PBS 1%, Ethanol 30% or DNBS/PBS), Prolia did not have any effect on all the markers studied.

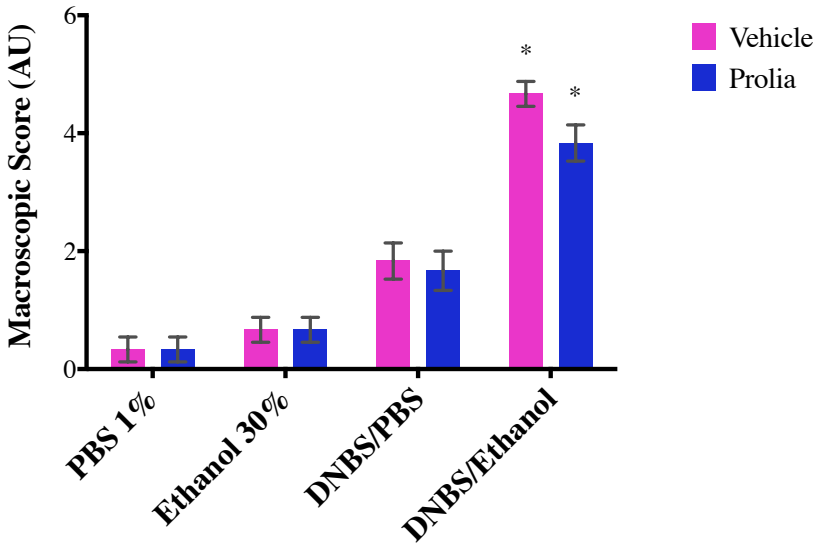


Figure 3. The effects of DNBS/Ethanol-induced colitis and Prolia treatment on colonic macroscopic score. Error bars are shown as SEM Error bars are shown as SEM.*: $P<0.001$ when compared with other induction models within the Prolia or vehicle group calculated using SAS UNIVARIATE procedure and illustrated by Prism using two-way ANOVA. n=6 mice per group.

In parallel, based on the H&E staining, across treatments DNBS/Ethanol mice experienced a notable increase in epithelial integrity disruption level as well as in necrosis and transmural infiltration of immune cells (Fig 4a, as presented by black dots). When compared to vehicle, DNBS/Ethanol treatment increased ($p=0.03$) the histologic score by 3.5-fold. Across all indcution models, Prolia treatment did not improve the histological score (Fig 4).

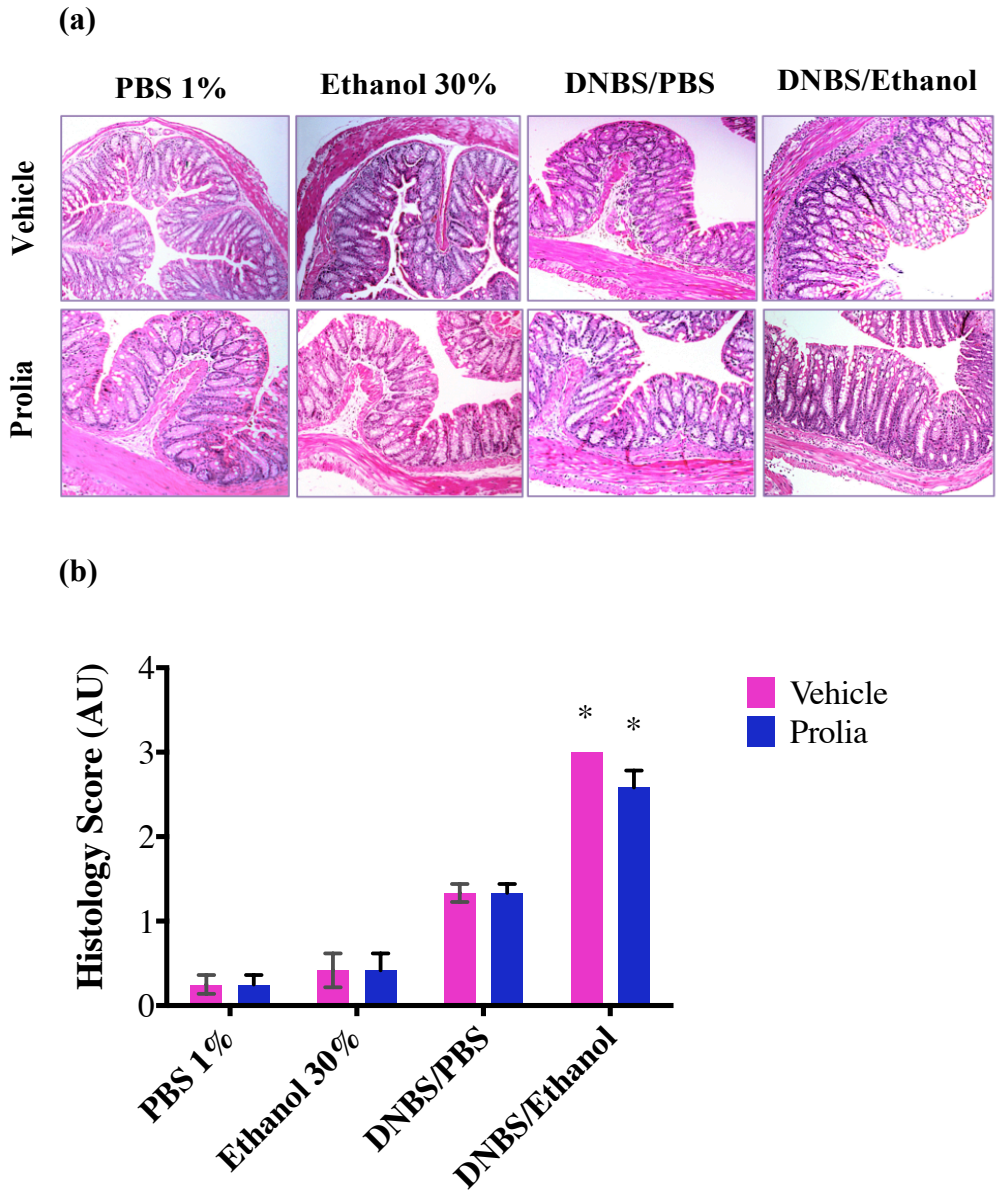


Figure 4. The effects of DNBS/Ethanol-induced colitis and Prolia treatment on representative hematoxylin and eosin (H&E) stained colon mucosa and histologic score. The H&E stained colon mucosa section showed a significant disruption of epithelial integrity, necrosis and transmural infiltration of immune cells. DNBS/Ethanol group increased ($p=0.03$) the histological score while Prolia did not alleviate colitis. b. Histological scores. AU: arbitrary unit. Error bars are shown as SEM.*: $P<0.001$ when compared with other induction models within the Prolia or vehicle group calculated using SAS UNIVARIATE procedure and illustrated by Prism using two-way ANOVA. n=6 mice per group.

4.3. Effect of Prolia on Colonic and Serum Acute Inflammatory Markers

To demonstrate if the treatment with Prolia would have a systemic and more specific effect on granulocytic activation, colonic MPO activity and serum CRP were quantified. Our data indicated that under DNBS/Ethanol colitic conditions, DNBS/Ethanol increased (p=0.02) colonic activity of MPO (3.2-fold) and serum concentration of CRP (4.74-fold) compared to other induction model within vehicle group, however, Prolia treatment did not reduce the level of these inflammatory markers (Fig 5).

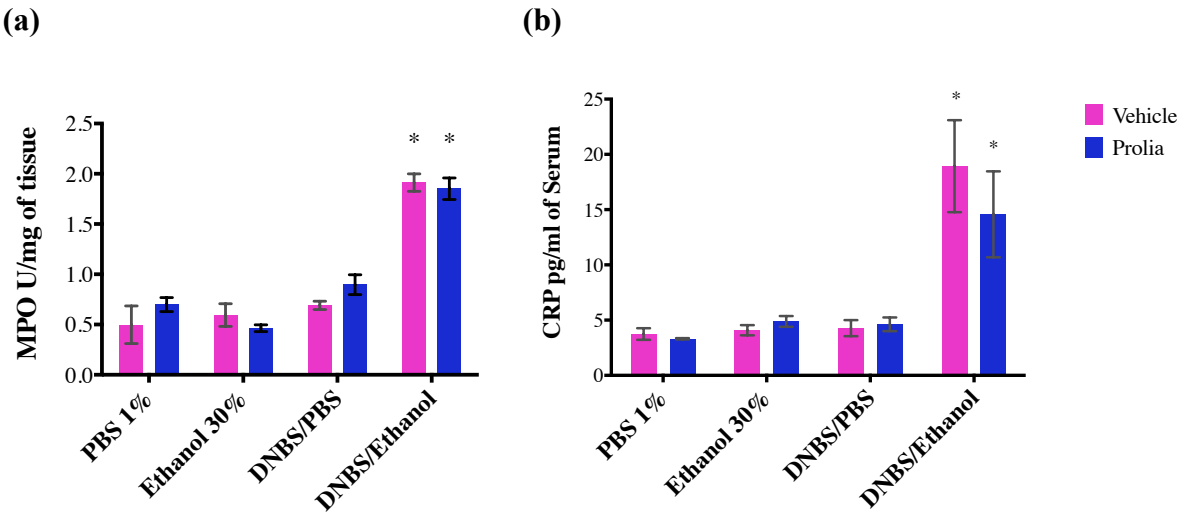


Figure 5. The effects of DNBS/Ethanol-induced colitis and Prolia treatment on regulating the colonic MPO activity and serum CRP. DNBS/Ethanol significantly increased a. colonic MPO activity and b. serum CRP. Prolia did not ameliorate the negative effects of DNBS/Ethanol-induced colitic conditions. Vehicle=PBS 1%. Error bars are shown as SEM.*: P<0.001 when compared with other induction models within the Prolia or vehicle group, is calculated using SAS UNIVARIATE procedure and illustrated by Prism using two-way ANOVA. n=6 mice per group.

4.4. Effect of Prolia on Colonic Pro-inflammatory Cytokines

As macroscopic and microscopic scores do not have high precision as inflammatory indicators, we evaluated the effect of DNBS/Ethanol and Prolia treatment on colonic pro-inflammatory cytokines expression and their concentrations.

DNBS/Ethanol colitis induction increased (p=0.03) colonic mRNA levels of *Il1β*, *Il6* and *Tnfα* and, Prolia treatment decreased (p=0.01) their relative expression level from 1.26 ± 0.44 to 0.31 ± 0.15 for *Il6*, from 9.40 ± 5.90 to 0.95 ± 1.24 for *Il1β*, from 3.99 ± 2.16 to 0.62 ± 0.77 for *Tnfα* (Fig 6a-c). PBS 1%, Ethanol 30% or DNBS/PBS with or without Prolia did not have any effect on the mRNA expression of the studied markers.

To confirm these results at the protein level, colonic concentration of TNF-α, IL-1β, and IL-6 were quantified. Prolia treatment decreased the colonic concentration of TNF-α, IL-1β, and IL-6 significantly from 234.36 ± 59 to 135.23 ± 64.63 pg/mg for IL-6 ($P<0.05$), from 278.62 ± 130.8 to 68.84 ± 58.98 pg/mg for IL-1β ($P<0.03$), from 70.86 ± 11.8 to 31.11 ± 14.86 pg/mg for TNF-α ($P<0.03$) in DNBS/Ethanol-induced colitis (Fig 6d-f). PBS 1%, Ethanol 30% or DNBS/PBS with or without Prolia did not have any effect on the colonic concentration of the three markers studied.

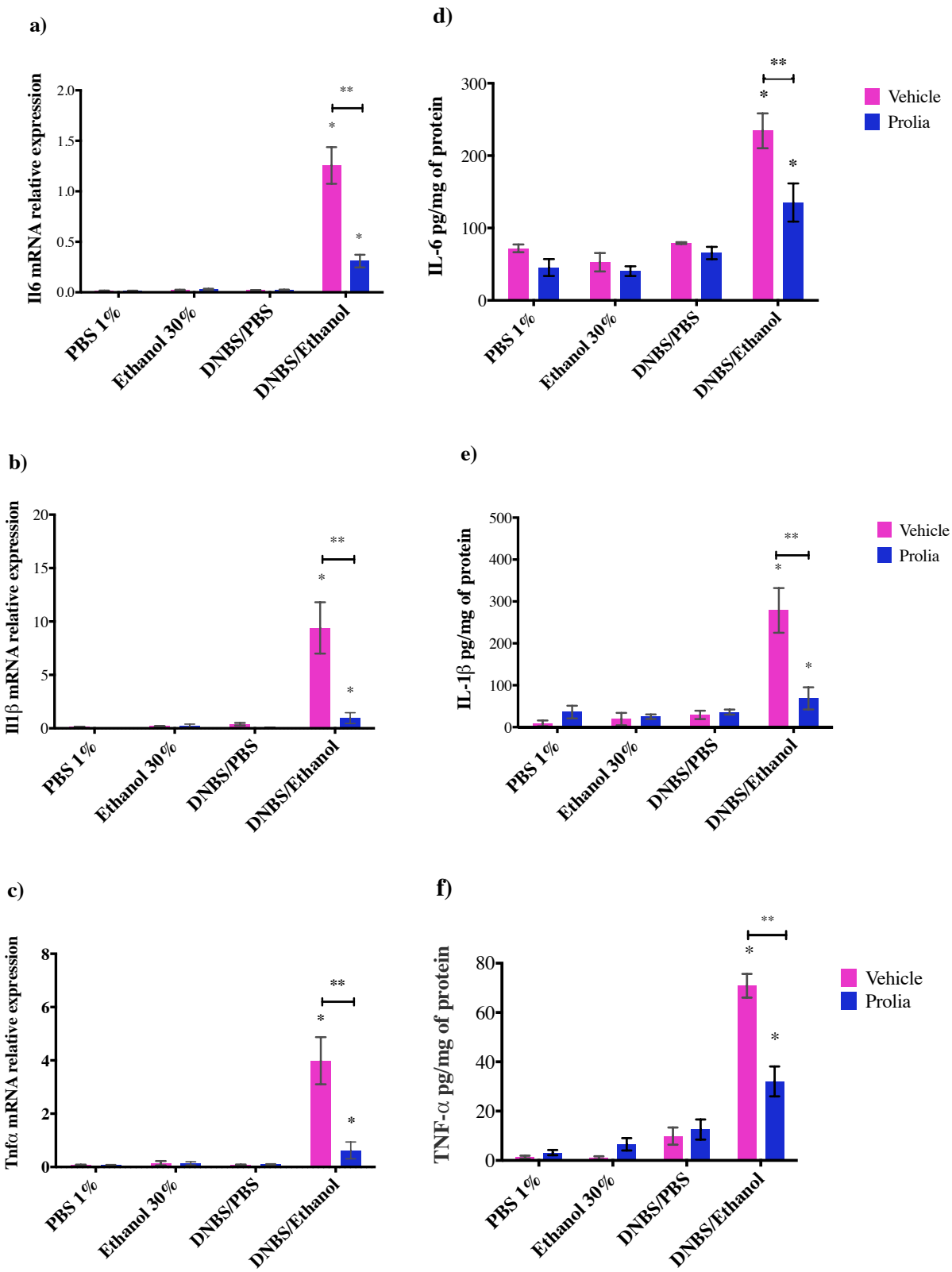

a)
Il6 mRNA relative expression
2.0
1.5
1.0
0.5
0.0
PBS 1%
Ethanol 30%
DNBS/PBS
DNBS/Ethanol
d)
IL-6 pg/mg of protein
300
200
100
0
Vehicle
Prolia
b)
Il1β mRNA relative expression
20
15
10
5
0
e)
IL-1β pg/mg of protein
500
400
300
200
100
0
c)
Tnfα mRNA relative expression
8
6
4
2
0
f)
TNF-α pg/mg of protein
80
60
40
20
0

Figure 6. The effects of DNBS/Ethanol-induced colitis and Prolia treatment on the colonic IL1β, IL-6, and TNF-α pro-inflammatory cytokines. DNBS/Ethanol increased ($P<0.05$) colonic pro-inflammatory cytokines (a-c). The cytokine expression levels were confirmed using qRT-PCR analysis (d-e). TATA Box Binding Protein (TBP) is used as housekeeping genes for qRT-PCR. Error bars are shown as SEM.*: $P<0.001$ when compared with other induction models within the Prolia or vehicle group calculated using SAS UNIVARIATE procedure and illustrated by Prism using two-way ANOVA. n=6 mice per group.

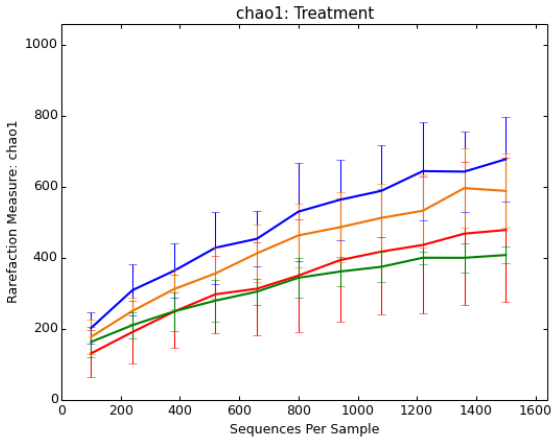
4.5. Microbiome Analyses

4.5.1. Colonic and Fecal Alpha-Diversity

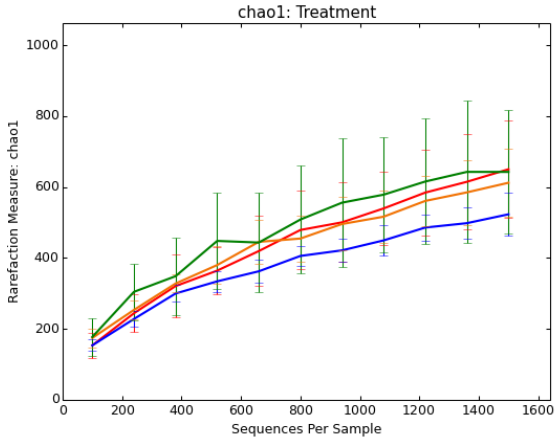
We firstly investigated the impact of treatments on "within communities' species richness" and "diversity" using Chao1 and Shannon-index, respectively. In Chao1 rarefaction plots, X-axis indicates the rarified 1600 and 25000 sequences per sample for colon and fecal samples, respectively, while Y-axis represents the Chao1-index of species richness. SAS MIXED procedure was used to calculate p-value and R software for plotting of the alpha diversity graphs (Fig 7, 8). As the Chao1 rarefaction graphs show, we looked at both resident colon microbiota and the transient fecal microbiota. Data indicates that in the colon samples, within the vehicle group, all induction models increased the chao1-index compared to PBS 1%, while DNBS/PBS resulted in the most significant increase in the species richness ($p=0.021$) (Fig 7a). In the case of fecal samples, in the absence of Prolia, all induction models reduced ($p=0.03$) chao1-index notably. In the DNBS/Ethanol colitic condition, there was a decrease ($p=0.03$) from PBS 1% in the average chao1-index from 3560 to 2620 (Fig 7c). After 4 days continuous i.p. injection of Prolia, chao1 index of alpha-diversity of all induction models stayed at the approximately similar level ($p=0.2$) which indicating Prolia could alleviate the dysbiosis made by colitic inducers (Fig 7d). Therefore, our alpha-diversity data

confirmed the existence of dysbiosis within both resident and transient microbiota in vehicle group, and also the alleviating effect of Prolia in these samples.

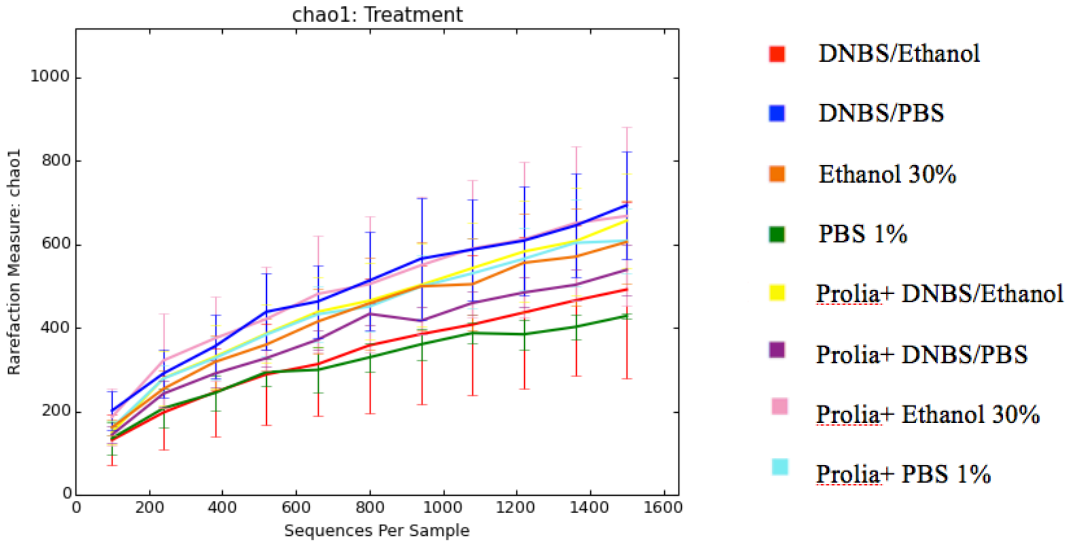
a) Colon, Vehicle



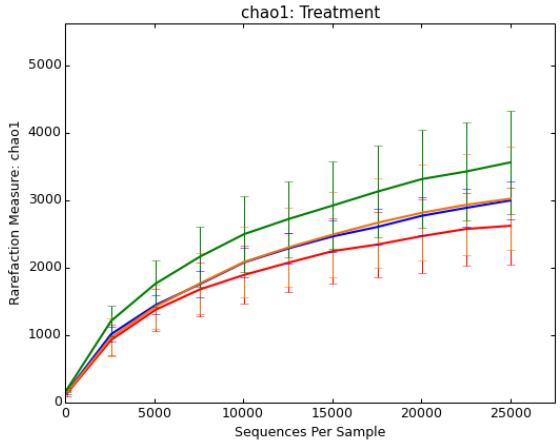
b) Colon, Prolia



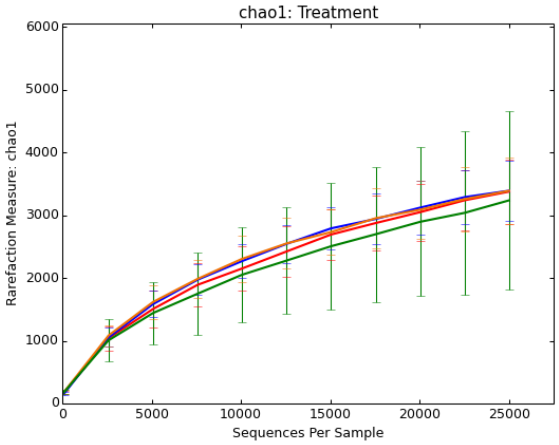
c) Colon, Vehicle vs. Prolia



d) Feces, Vehicle



e) Feces, Prolia



f) Feces, Vehicle vs. Prolia

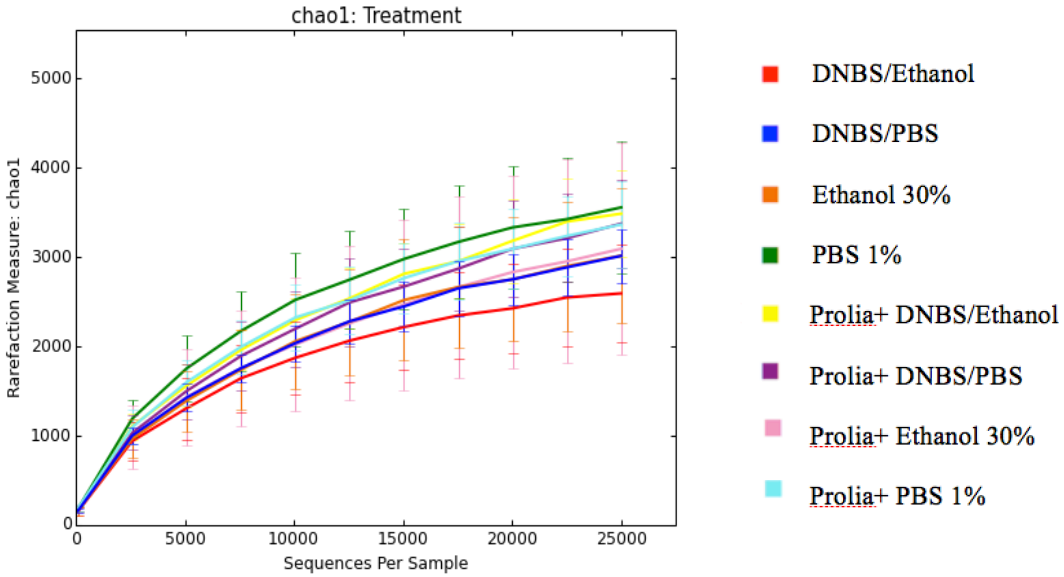
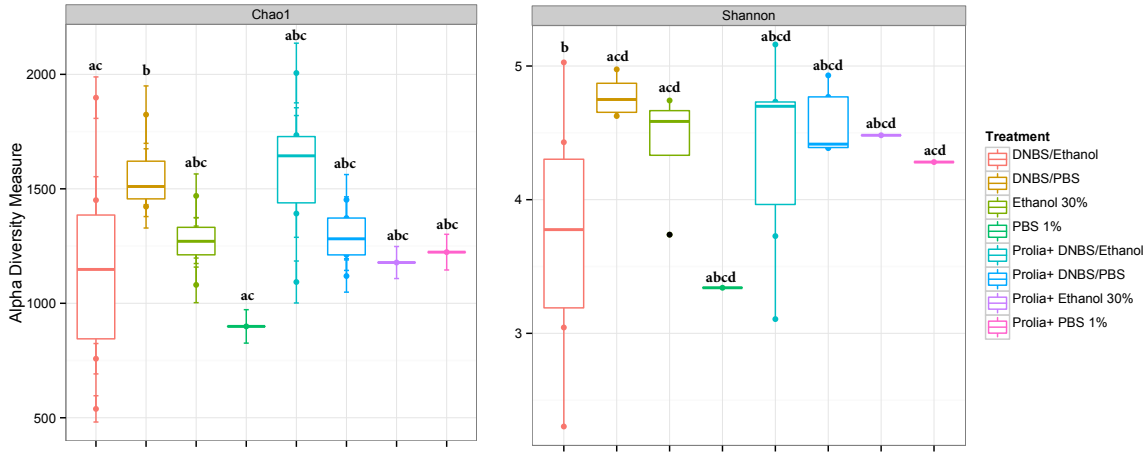


Figure 7. Alpha-diversity of colonic and fecal microbiota of DNBS/Ethanol-induced colitis and Prolia treated mice. (a-f) Measure of species richness based on operational taxonomic unit (OTU) for colon (a) and fecal (d) vehicle group (i.r. administration of PBS 1%) and colon (b) and feces (e) of Prolia groups in samples collected after induction of colitis with DNBS/Ethanol and its controls: PBS 1%, Ethanol 30%, and DNBS/PBS. (c,f) the comparison of Chao1 index of species richness in vehicle (PBS 1%) and Prolia groups interactions, within colonic and fecal samples.

a) Colon, Vehicle vs. Prolia



b) Feces, Vehicle vs. Prolia

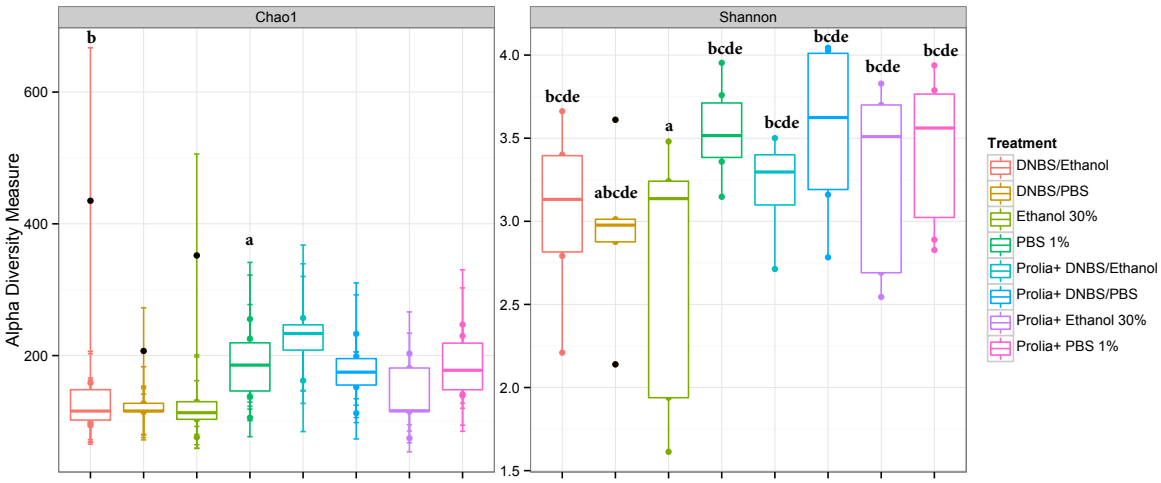


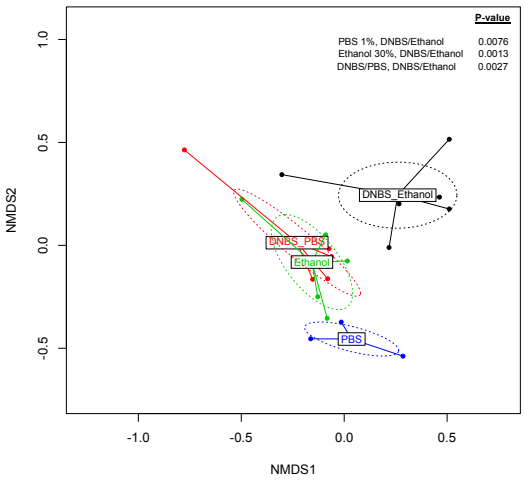
Figure 8. Summary of alpha-diversity indices of vehicle- and Prolia-treated colon and vehicle samples. (a-b) Comparison of Chao1 and Shannon indices of colonic microbiota between Prolia-treated vs. vehicle animals with or without induction of DNBS/Ethanol colitis. Statistical analyses were conducted using mixed procedure of SAS. Alphabetical letters on each graph shows the significant differences ($P<0.05$) among treatments.

4.5.2. Colonic and Fecal Beta-Diversity

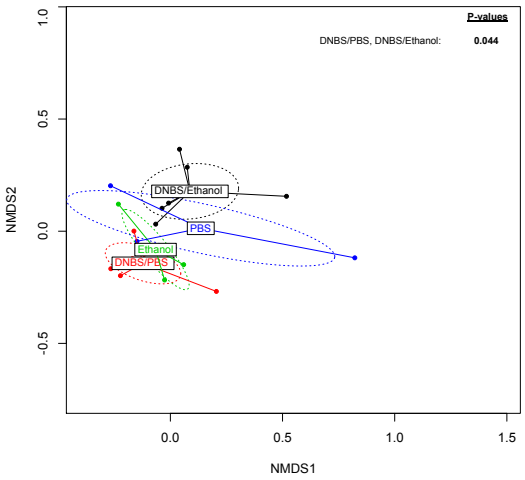
To compare diversity between different induction models within Prolia and vehicle groups, nMDS plots based on Bray-Curtis dissimilarity matrices were generated. Colonic mucosa-associated microbiota clustered separately (p=0.002) in DNBS/Ethanol compared to other induction models in vehicle group. The same pattern was observed in fecal samples of vehicle group. Additionally, the fecal microbiota of PBS 1% and Ethanol 30% were clustered separate (p=0.02) from each other. This confirms the role of Ethanol in accelerating the dysbiosis within both resident and transient microbiota. In colon mucosa, there was a clear evidence of alleviating the dysbiotic effects of DNBS/Ethanol in Prolia-treated animals according to bray-curtis clustering patterns. This pattern was similarly observed in fecal samples (Fig 9) and Prolia could avoid dysbiosis in the transient microbiota within PBS 1% and Ethanol 30% treated controls.

Figure 9 (c, f) compares DNBS/Ethanol and Ethanol treatments between vehicle and Prolia treatments. Results showed that DNBS/Ethanol with Prolia-treatment has a trend toward the Ethanol 30% in both vehicle and Prolia groups (p=0.1). In addition there was a significant difference between DNBS/Ethanol of vehicle group and both Ethanol 30% of vehicle and Prolia groups. Taken together, DNBS/Ethanol altered (p=0.05) beta-diversity of colon and fecal microbiota compared to its control treatments but its negative effects on colon microbiota were reduced following administration of Prolia.

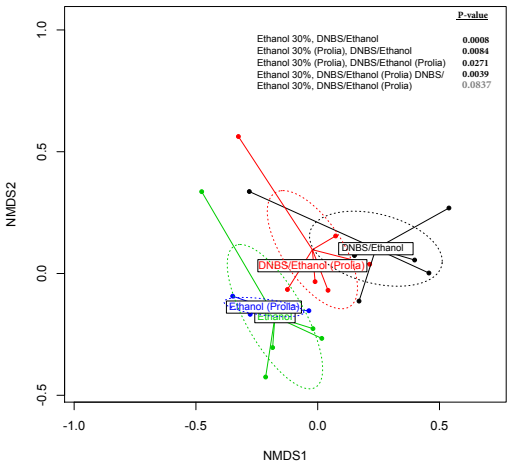
(a) Colon: Vehicle group



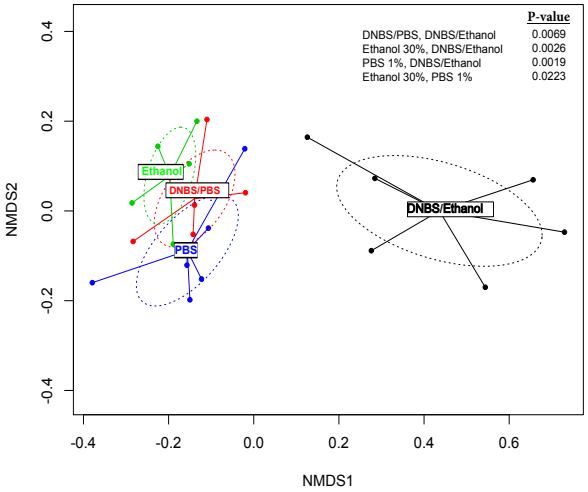
(b) Colon: Prolia group



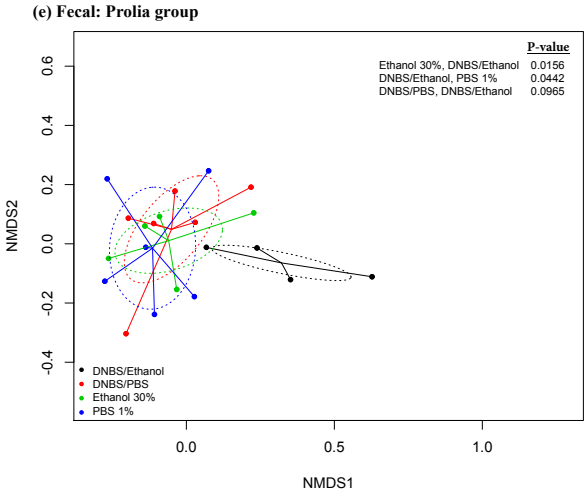
(c) Colon: Vehicle vs. Prolia



(d) Fecal: Vehicle group



(e) Fecal: Prolia group



(f) Fecal: Vehicle vs. Prolia

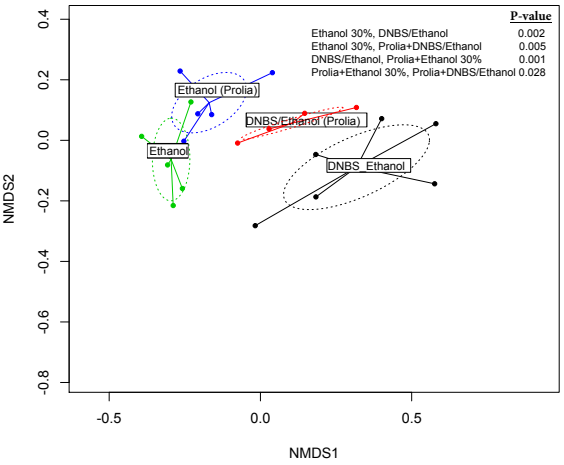


Figure 9. Non-metric multidimensional scaling (nMDS) ordination plot, a measure of relative dissimilarities in the bacterial community composition in the colon mucosa and feces of (a, d) vehicle and (b,e) Prolia treated mice; (c, f) comparison between two groups of Prolia and vehicle in DNBS/Ethanol vs. Ethanol treatments. The colored points are shaded according to different treatment groups. The p-values were calculated using PERMANOVA. For the multiple-comparison tests, only the significant p-values were included. Trends were discussed at $P<0.1$.

Table 1. Summary of p-value for treatment groups across vehicle, Prolia, and the interaction between vehicle vs. Prolia treated groups in colon and fecal samples. The p-values were calculated using PERMANOVA.

Treatment	p-value	
	Colon	Feces
PBS 1%	0.33	0.31
Ethanol 30%	0.26	0.04
DNBS/PBS	0.25	0.28
DNBS/Ethanol	0.05	0.39
Group*Treatment	0.03	0.15

4.5.3. Clustering of Colonic and Fecal of Microbiota

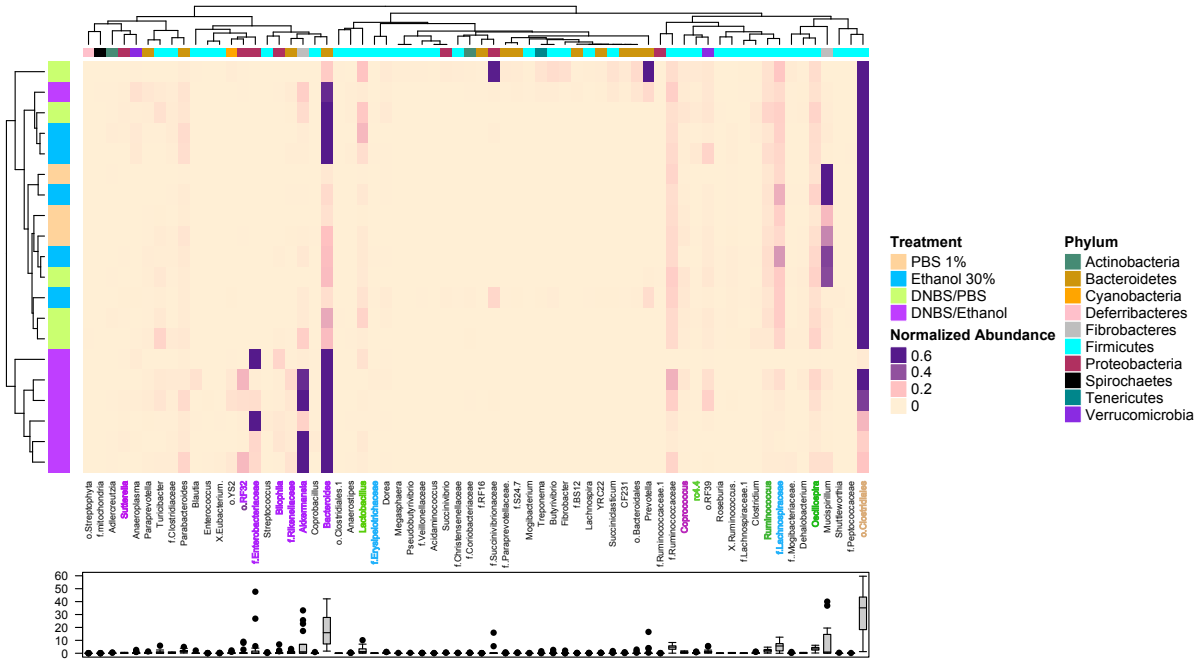
Next, we investigated which phyla and genera were responsible for the gut dysbiosis and how is the clustering pattern of microbiota at the genus level changed following DNBS/Ethanol colitis induction and Prolia administration. For this purpose, a clustering analysis based on Bray-Curtis dissimilarity was employed in R (Gu et al., 2016). The X-axis on the graphs represents the composition of bacterial communities at the genus level while the phylum they belong to is color coded at the top. The Y-axis represents the color-coded

induction models (PBS 1%, Ethanol 30%, DNBS/PBS, DNBS/Ethanol) within vehicle (a, c) or Prolia groups (b, d) in the colon mucosa (a, b) or feces (c, d).

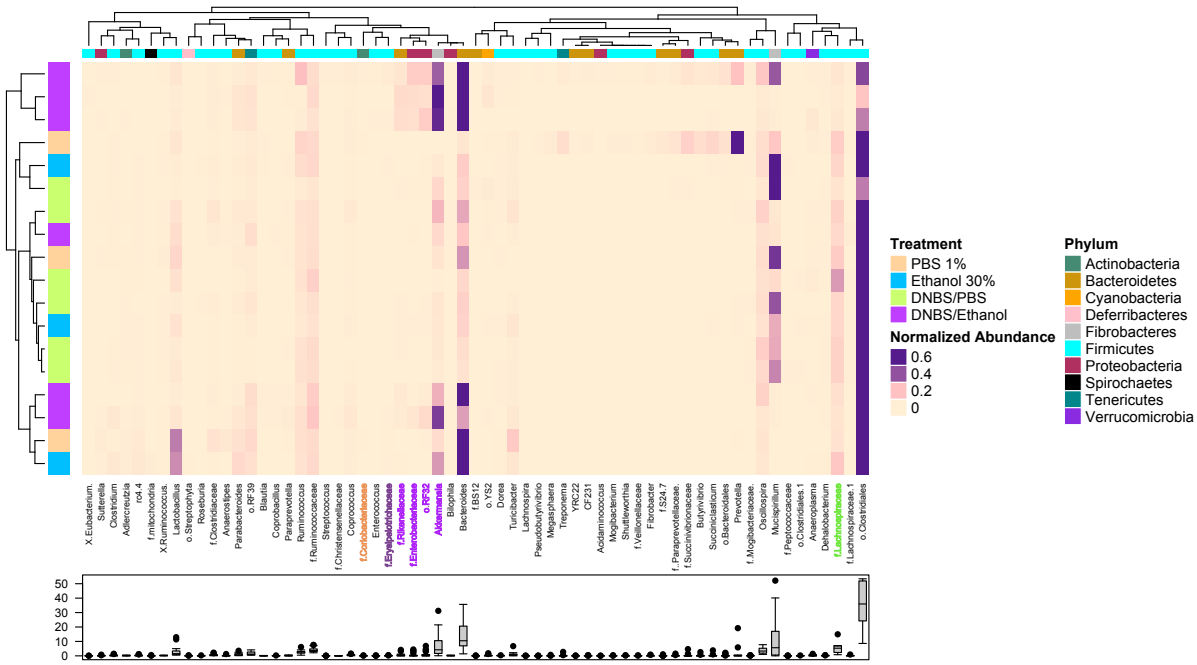
As these graphs reveal within vehicle group of both colon mucosa and feces (Y-axis of Fig 10a, c), the DNBS/Ethanol induction model clustered separately ($p=0.03$) from its control groups while DNBS/PBS, Ethanol 30%, and PBS 1% didn't cluster separately. Bacteroidetes, Fibrobacteres, and Proteobacteria were the phyla with relative abundances highly associated ($p=0.01$) with DNBS/Ethanol both in the colon mucosa and feces of vehicle mice. In contrast, phylum Firmicutes was highly associated with colon mucosa in the vehicle group (Fig 10a). In the feces, Bacteroidetes, Verruocomicrobia and several members of Firmicutes were significantly associated with vehicle group (Fig 10c).

The clustering analysis of Prolia-treated mice showed a clear alteration in clustering pattern in both colon mucosa and feces microbiota (Fig 10b, d). There was no significant difference between clustering of DNBS/Ethanol group vs. its controls. These data also confirm the colitic effect of DNBS/Ethanol in colon and fecal microbiome dysbiosis and also the alleviating role of Prolia treatment on colitic situation.

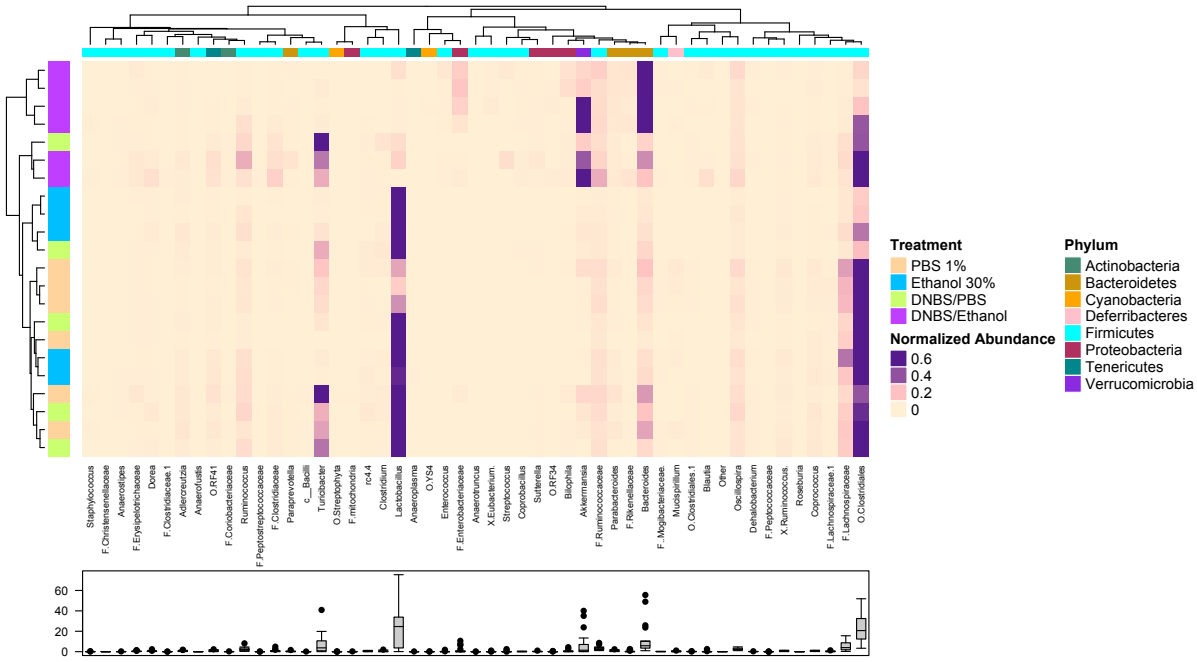
a) Colon: Vehicle group



b) Colon: Prolia group



c) Fecal: Vehicle group



d) Fecal: Prolia group

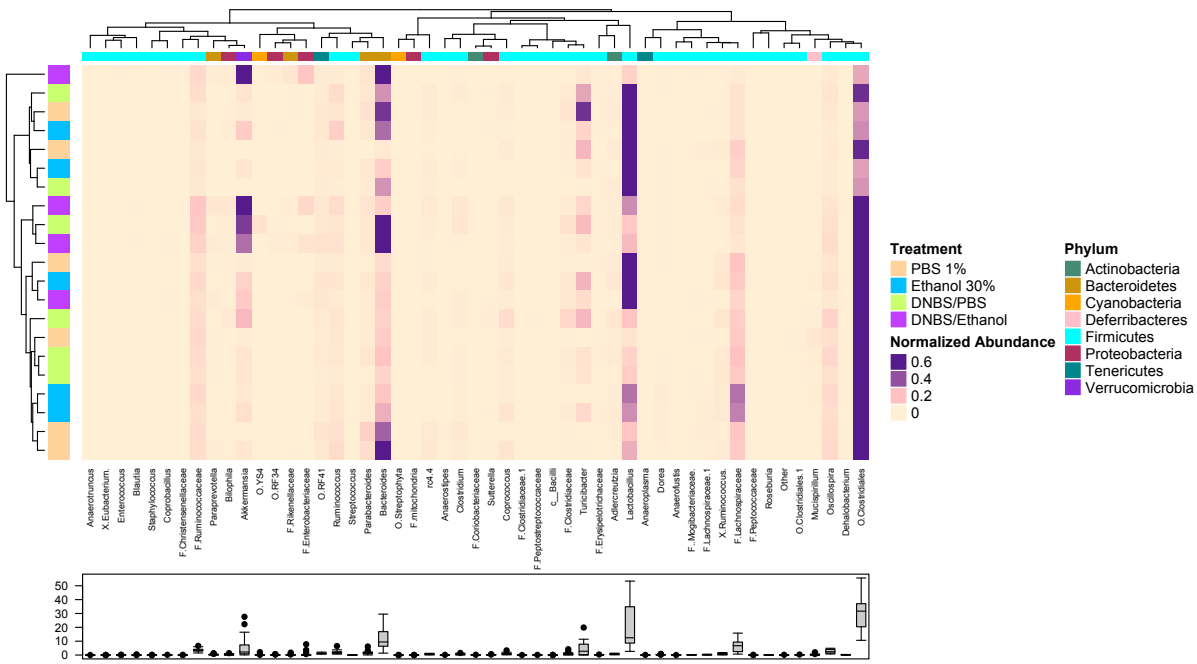
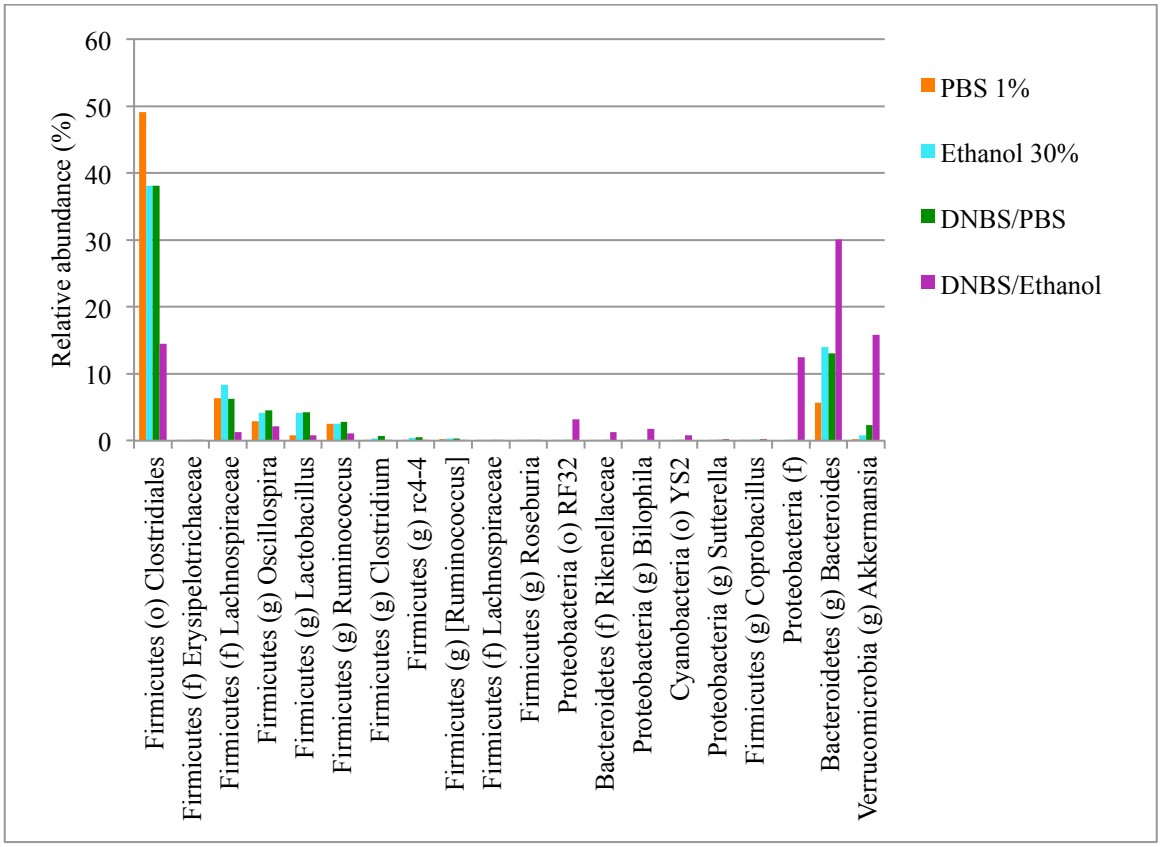


Figure 10. The Clustering pattern of colonic and fecal microbial communities of vehicle and Prolia treated samples. Rows correspond to samples and columns correspond to abundant genera (> 0.1% of community). The "Normalized Abundance" key relates colors to the

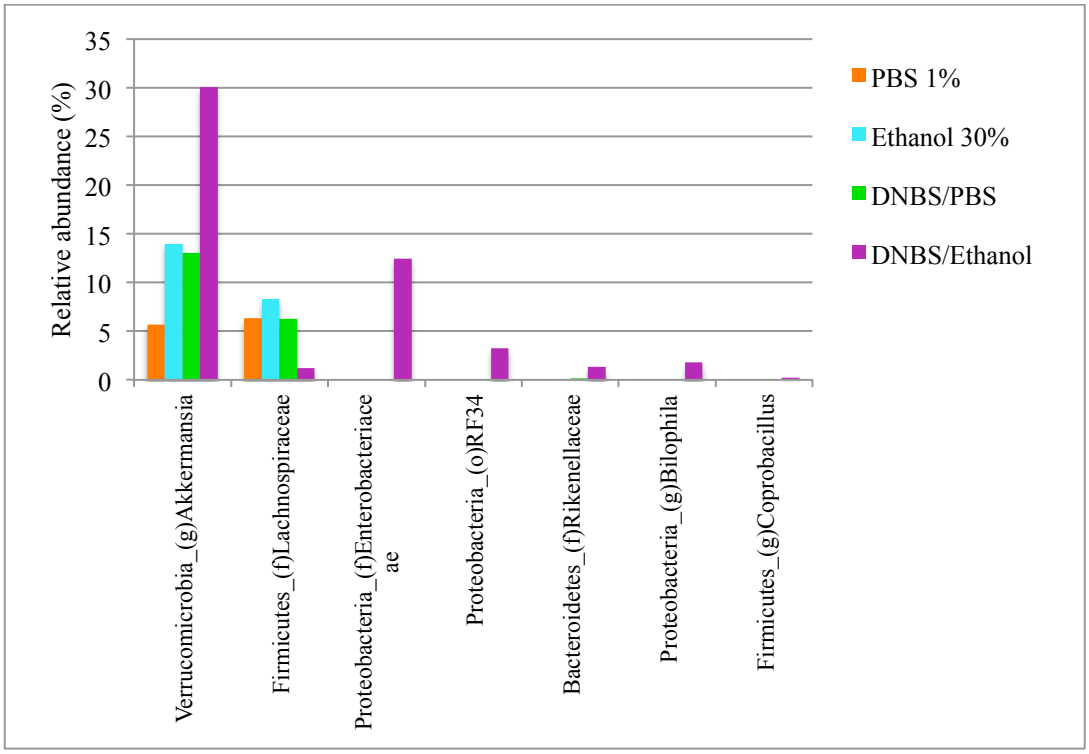
normalized proportions of genera (relative abundance of each genus divided by the Euclidean length of the column vector). The left dendogram shows how samples are clustered based on their Bray–Curtis dissimilarities (using unweighted pair group method with arithmetic averaging UPGMA). The significance of clustering patterns has been calculated based on 9999 permutations and p-values calculated based on PERMANOVA. The top dendogram shows how genera correlate (co-occur) with each other based on their Spearman's correlation coefficient. The "Phylum" key relates the top annotations to the corresponding phylum of each genus. The "Treatment", key relates samples to the treatments group (PBS 1%, Ethanol 30%, DNBS/PBS, DNBS/Ethanol). The bottom box-plot shows the distributions of the non-normalized relative abundances of genera in Vehicle group (a, c) and Prolia group (b, d). Color codes have been also used to highlight bacterial genera that were significantly associated with treatment groups (colors are in accordance with the colors of treatment groups; $P<0.05$).

Following up on the previous analyses, using LEfSe and MIXED procedure of SAS, the most significant increases in the relative abundance of bacterial taxa were identified. Bacterial taxa that were significantly increased in the colon mucosa of the vehicle and Prolia groups are presented in Fig 11 (a) and (b), while Fig 11 (d) and (e) show taxa which their relative abundances significantly changed under DNBS/Ethanol condition compared to other induction model in vehicle group. DNBS/Ethanol decreased Lachnospiraceae and Clostridials from Firmicutes phylum (Fig 11c, $p=0.02$) and increased Bacteroidaceae and *Akkermansia* from Bacteriodetes and Veruccomicrobia (Fig 11.d, $p=0.01$), respectively. Prolia treatment tended ($p=0.1$) to increase the Lachnospiraceae and Clostridials compensating their low abundance in the colon mucosa of DNBS/Ethanol and DNBS/PBS induction models. Prolia treatment also decreased ($p=0.02$) the relative abundance of Bacterioidaceae in the abovementioned groups.

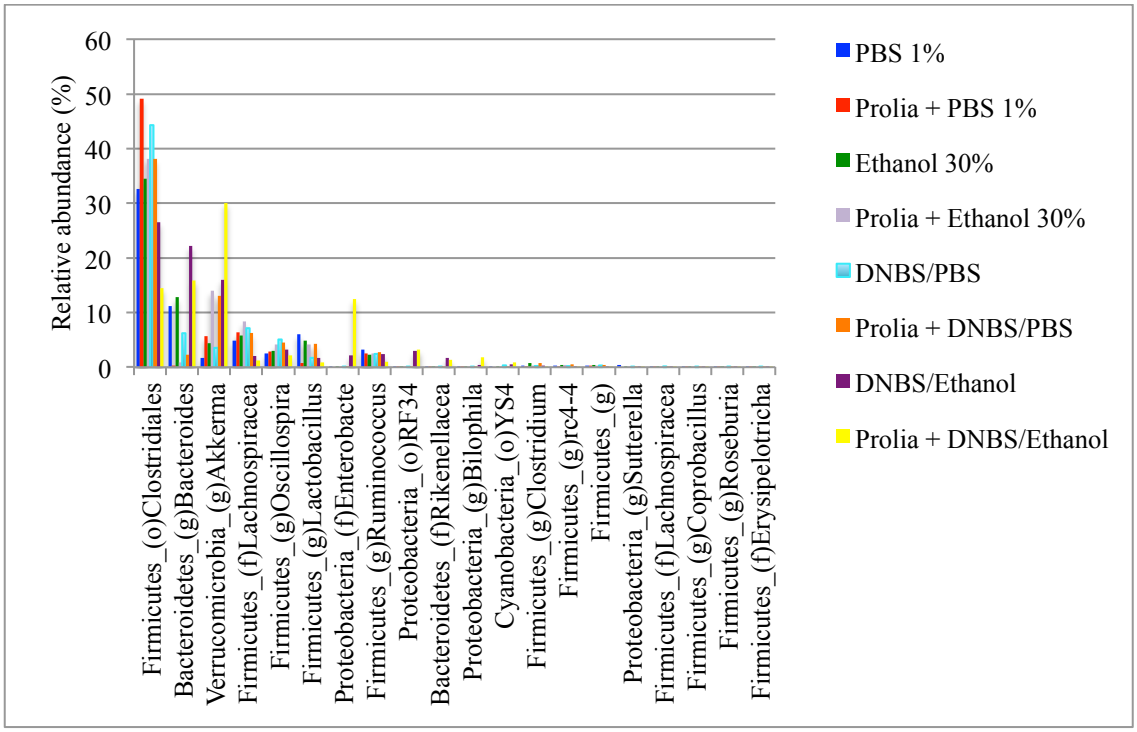
a) Colon: Vehicle group



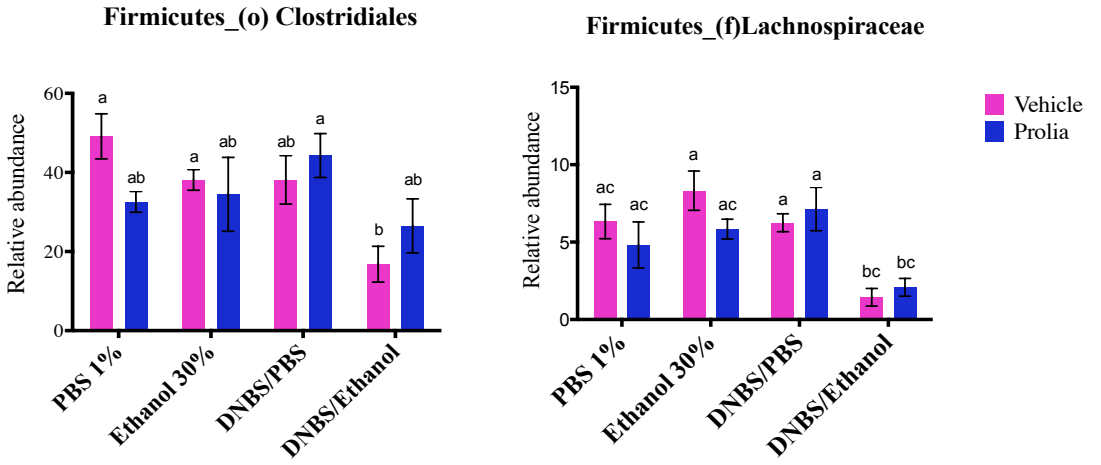
b) Colon: Prolia group



c) Colon: Vehicle vs. Prolia



d)



e)

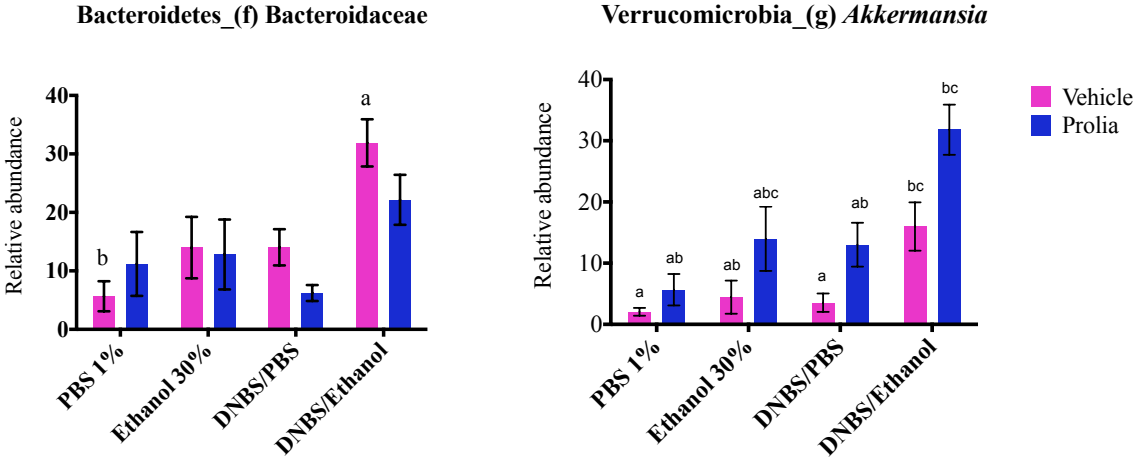
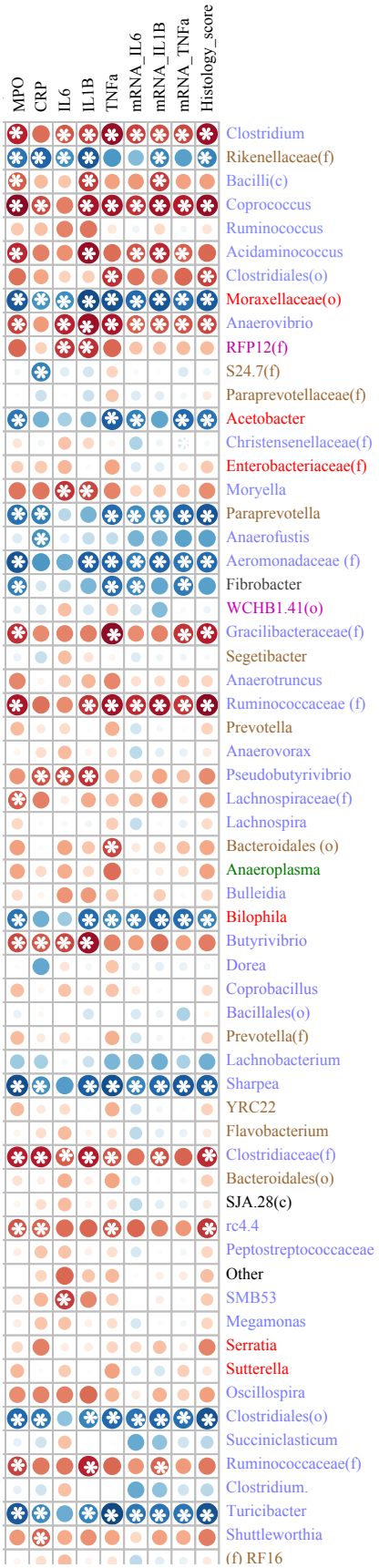


Figure 11. Colonic mucosa-associated bacterial taxa with the significant relative abundance increase in (a) vehicle (i.p. administration of 1% PBS) or (b) Prolia treated mice. The legend shows four different induction models (PBS 1%, Ethanol 30%, DNBS/PBS, DNBS/Ethanol) within vehicle and Prolia treatments. (c) Bacteria which showed significant changes in both vehicle and Prolia treatments. (d) Bacteria that their relative abundances were significantly decreased in colon mucosa-associated microbiota under DNBS/Ethanol colitic conditions. (e) The significant increased colon-associated genera of bacteria in colotic conditions. *, a, b, c, d indicate significant difference ($P<0.05$) among treatment groups. The letters (f) and (g) in the graphs are representing family and genus of bacteria, respectively.

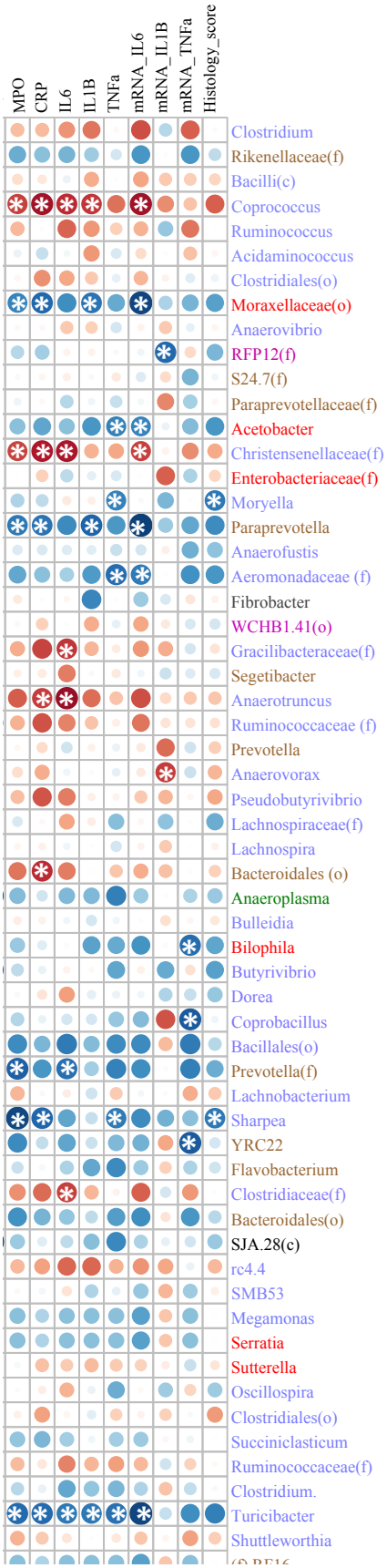
4.5.4. Correlation Analysis

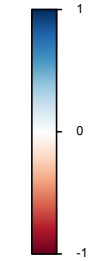
The non-parametric Spearman's rank correlation analysis showed associations between several bacterial taxa with an abundance ≥ 0.5% of community in the colon mucosa and inflammatory markers (IL-1β, IL-6, TNF-α at both protein and gene level), histologic score, CRP, and MPO. According to figure 12 (a), the i.r. administration of DNBS/Ethanol compared to its control Ethanol 30% resulted in a notable dysbiosis as shown by white asterisk, while some genera were associated with a negative or positive correlation ($p \leq 0.05$). In the presence of Prolia (Fig 12b), less significant number of correlations were observed demonstrating the effective role of Prolia in alleviating the dysbiotic effect induced by DNBS/Ethanol.

a) Vehicle group



b) Prolia group





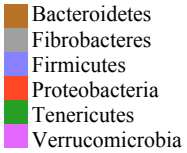


Figure 12. Correlation coefficient between the proportion of abundant colonic bacteria taxa (≥0.5% of community) and immunological factors (inflammatory markers), in both vehicle and Prolia-treated mice. The non-parametric Spearman's rank correlation implemented in PAST software was used. For each correlation, correlation coefficient (Spearman's Rho) and p-value were obtained and the resulting correlation matrix was visualized in a heatmap format generated by the corrplot package of R ver. 02-0.2010. The correlation coefficient values ranged from −1 (red) to +1 (blue) with larger absolute values indicating stronger relationship while positive and negative values between DNBS/Ethanol vs. Ethanol 30% and immunological factors indicating the direction of association. Alpha value for the correlation confidence intervals was set up as 0.05. *: $P<0.05$.

4.5.5. Prediction of Functional Capacity of Microbiota

To assess the functional capacity of microbiome under each treatment and induction model, PICRUSt was used. Several metabolic pathways were associated with DNBS/Ethanol-induced colitis in vehicle group. Prolia administration altered several KEEG pathways predicted by PICRUSt. Using LEfSe (Segata et al., 2011), several metabolic pathways that increased in association with each treatment, were highlighted (Fig 13). Each color was assigned to one treatment. DNBS/Ethanol administration in vehicle group increased several pathways including amino acid uptake, carbohydrate synthesis, lipid uptake, and mucin overproduction metabolism while Prolia treatment reduced such pathways in DNBS/Ethanol colitic mice.

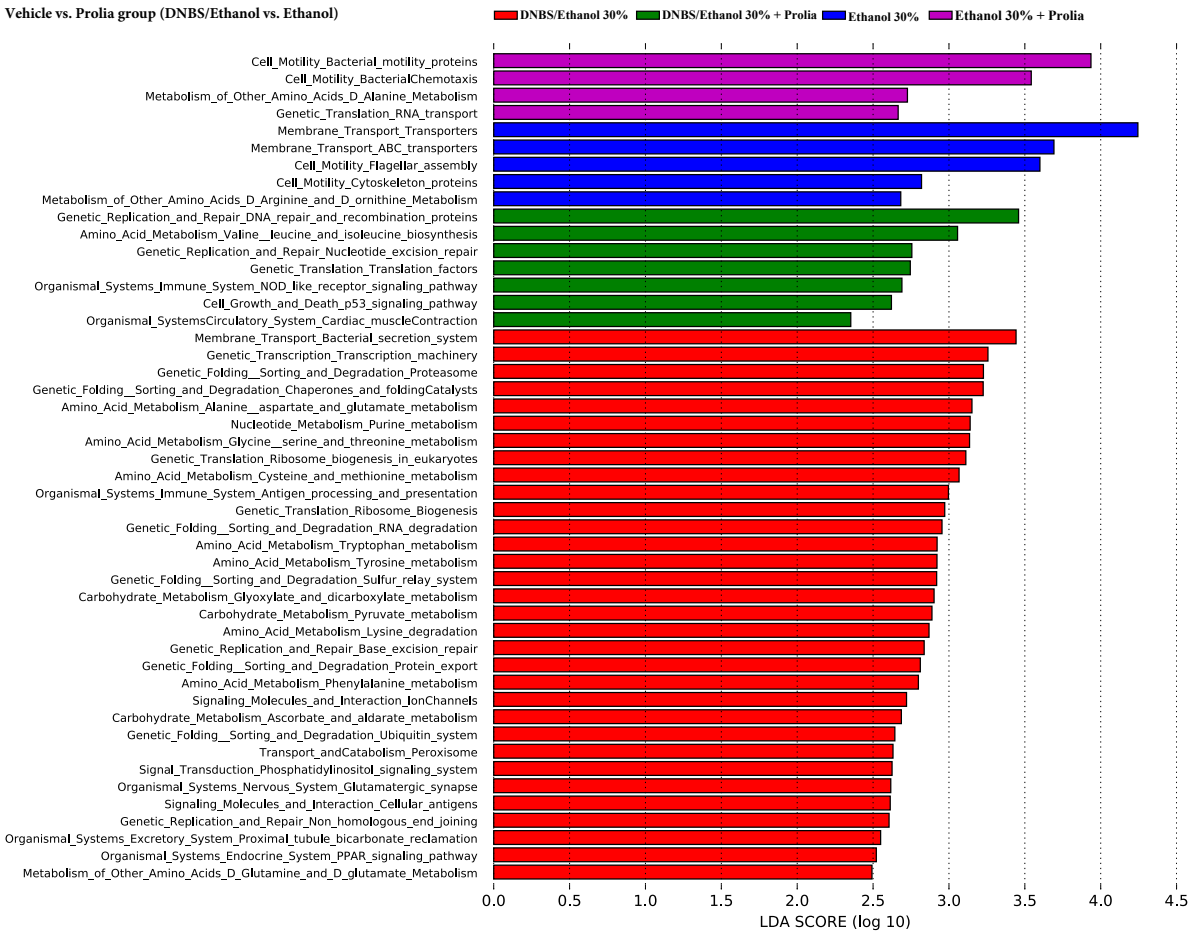


Figure 13. Prediction of functional capacity of colon-associated micobiota in vehicle vs. Prolia-treated mice. A statistical differences between KEGG pathways (explored at Levels 1–3, indicated as L1–L3) of predicted colonic mucosa metagenomes were evaluated by LEfSe, a metagenome analysis approach which performed the linear discriminant analysis following the Wilcoxon Mann–Whitney test to assess effect size of each differentially abundant variable. The length of the horizontal bars indicated log-fold changes for each variable. Color code represents the class of treatments; red and green represent colitic condition of DNBS/Ethanol, while blue and purple are showing the Ethanol 30% condition in the absence and presence of Prolia, respectively.

Chapter Five

Discussion

As highlighted in the literature review of this thesis, IBD is a gastrointestinal idiopathic inflammatory condition that manifests in two main subtypes, CD and UC (Strober et al., 2007; Manichanh et al., 2012). The etiopathology suggested encompasses environmental triggers, susceptible genetic variants and deregulated immune response toward a potentially dysbiotic gut microbiota of IBD patients (Strober et al., 2002; Nagalingam et al., 2011; Manichanh et al., 2012; Molodecky et al., 2012). Despite extensive research, identification of contributory candidate for IBD pathogenesis is still not completely understood. This lack of understanding hinders the availability of the optimal treatments. This calls for more research to further investigate the role of perceived triggers in initiation or development of IBD, also exploring alternative therapeutic candidates that add values to the existing treatments.

Therefore, the overall objectives of this research were to investigate the effective role of Prolia, an inhibitor of pro-inflammatory receptor activator of nuclear factor kappa-b ligand, on the inflammatory immune response and the gut microbiome dysbiosis in a DNBS/Ethanol-induced experimental model of CD. In a second intent, this thesis also addressed whether or not DNBS/Ethanol-induced colitis can recapitulate the IBD-associated dysfunctional immune response and the gut microbiome dysbiosis seen in human studies.

We investigated the role of RANKL inhibitor on immune response and also the gut microbiota. RANKL plays a prominent role in development of rheumatoid arthritis and osteoporosis (Fouque-Aubert et al., 2008; De Voogd et al., 2016), but there is no study

demonstrating its role in IBD pathogenesis. Blocking osteoclast-membrane RANK/soluble osteoblasts secreted RANKL pathway with a soluble decoy receptor for RANKL called osteoprotegerin (OPG), results in balanced osteoclasts activation (Moschen et al., 2005; Nahidi et al., 2011; De Voogd et al., 2016). Several studies reported low bone density minerals (BMD) in teenage IBD patients, the year that bone density is supposed to be at its peak, reflecting the potential aberrant osteoclastogenesis in such patients (Compston, 2003; Roshandel et al., 2010; Pichler et al., 2014; Krela-Kazmierczak et al., 2016; Schule et al., 2016). The reduction in vitamin D, vitamin K, calcium, and estrogen level, as well as mutation in their intestinal receptors, result in a dysfunctional absorption of such minerals. These deficiencies may contribute to the impaired osteoblast differentiation and excessive osteoclastogenesis during IBD (Saleiro et al., 2012; Cook et al., 2014; Wu et al., 2014; Fletcher, 2016). Regardless of decreased bone density in IBD patients, the other rational would be the existence of the RANK/RANKL/OPG pathway inside immune system (Moschen et al., 2005; Boyce et al., 2007; Fouque-Aubert et al., 2008). B- and T-lymphocytes, macrophages, and dendritic cells express such pathway, which through the innate or the adaptive immune response, toward invasive bacteria, result in inflammatory cascades activation and gut inflammation, the main characteristic of IBD (Wang et al., 2001; Ashcroft et al., 2003; Boyce et al., 2007; Fouque-Aubert et al., 2008). Additionally, genome-wide association metadata analysis identified a gene near the TNFS11 that encodes RANKL and its expression is increased in CD patients (Dixon et al., 2007; Sanseau et al., 2012). Prolia is a monoclonal antibody that inhibits RANK/RANKL pathway in bone remodeling procedures (Miller, 2011; Dempster et al., 2012). Therefore, we decided to use Prolia to target RANKL inhibition for obtaining a decreased level of inflammatory response in colitic

conditions. To confirm the effectiveness of Prolia-treatment, we looked at fecal and colon mucosa-associated microbiota as well as several inflammatory markers, in PBS 1%, Ethanol 30%, and DNBS/PBS treatments of vehicles and Prolia groups, which there was no evidence of adverse effects of these treatments on the inflammatory response and the microbiome dysbiosis. After verifying the effect of Prolia on microbiome/host immune response interactions, we defined its effects on DNBS/Ethanol-induced colitic conditions. Our results demonstrated that the inhibition of RANKL did not show any adverse pro-inflammatory effect on DAI, macroscopic/microscopic scores, the level of CRP and colonic MPO. On sacrifice day, mice from DNBS/Ethanol group treated with Prolia did not show any symptoms of ameliorating colitis. CRP and MPO levels as well as microscopic and macroscopic scores were also not modified following administration of Prolia. However, Prolia administration significantly reduced ($P<0.02$) the mRNA expression and protein concentration of IL6, IL1B, and TNF-a. This reduction in pro-inflammatory cytokines levels at the colonic mucosa can be interpreted as a RANKL blockade that led to the inhibition of the activation of immune cells possessing the RANK receptors, such as macrophages and dendritic cells. As vital members of innate immunity, majority of these antigen-presenting cells induce IL-1β, IL-6, TNF-α, therefore, their downregulation is probably due to reduced activation of such APC (Wang et al., 2001; Ashcroft et al., 2003; Moelants et al., 2013). Lending support to this hypothesis, Aschroft et al. (2003) reported that in IL-2-deficient mice, which develop spontaneous $CD4^+$ T cells activated autoimmune disease associated within multi-organ inflammation and excessive gastrointestinal tract T-cell infiltration, administration of Fc-OPG not only increased bone density but also ameliorated ulceration in the gastrointestinal tissue as well as reduced level of mucosal T-cell infiltration. Also, RANKL contributes to B-cell maturation

and development, since the RANKL (-/-) mice are illustrated with a reduced numbers of B-cells in the spleen and with defective transition of pro-B to pre-B resulting in an impaired antibody immune response in the invasion of pathogenic bacteria (Young-Yun Kong et al., 1999). Yun et al. (2001) reported that the *OPG* (-/-) mice demonstrated an increase in B-cell proliferation with altered antibody response due to specific isotype imperfection in IgG immunoglobulins class switching, although in contrast, OPG transgenic mice showed neither B-cell maturation nor innate immune-related cell deficiency (Stolina et al., 2007). All the above studies confirm the anti-inflammatory effect of RANKL inhibitor, which is in consistent with our data regarding reduction of pro-inflammatory cytokines.

In the next part of our project, we examined the modulatory role of Prolia on DNBS/Ethanol-induced microbiome dysbiosis. Prolia reduced the altered species richness and avoided dysbiosis in DNBS/Ethanol treatment when compared with other induction models. In DNBS/Ethanol-induced colon samples, Prolia reduced the increased number of species due to DNBS/Ethanol administration. Furthermore, having looked at beta-diversity comparing different treatments, Prolia attenuated microbiota dysbiosis within both colon mucosa and feces. Bacterial taxa that were promoted by Prolia treatment consisted of increased level of f. Lachnospiraceae from p. Firmicutes in contrast with decreased level of o. RF32, f. Entereobacteriaceae, and g. *Bilophila* from p. Proteobacteria, g. *Bacteroides,* g. *Rikenellaceae* from p. Bacteroidetes, and g. *Akkermansia* from p. Verrucomicrobia. Prolia also increased o. Clostridiales, which their decrease was one of the symptoms of experimental models of CD (Oberc et al., 2015; Abeles et al., 2016).

Understanding the correlations between altered inflammatory pathways due to vehicle or Prolia treatment and the most abundant (0.1 % of the community) taxa within the colonic

mucosa and feces were of high importance in this study. As shown in figure 12, vehicle group was characterized with a dysbiotic community with high number of correlations between several bacterial taxa and immunological factors compared to Prolia group illustrating the alleviated effect of Prolia under DNBS/Ethanol-induced colitis.

To address the second objective of this project, which was to assess the role of experimental model of colitis on inflammatory response and gut microbiota, the DNBS model of colitis was used. Although there are several models that can recapitulate CD (Kim et al., 1992; Mizoguchi, 2012), two main hapten-induced models are the DNBS and the TNBS, which both are administered intrarectally. Clinically, DNBS/Ethanol-induced colitis results in severe inflammatory response in the colon and rectum of rats (Morampudi et al., 2014). Compared to DNBS, TNBS due its highly oxidative nature is a hazardous chemical with explosion potentiality in contact with sodium and potassium. The binding affinity of DNBS is higher with proteins compared to TNBS, which selectively binds only to the lysine ε-amino group (Goyal et al., 2014; Morampudi et al., 2014).

Previously in animal studies Ethanol 30 or 50 percent were used extensively to dissolve DNBS. However, what was not considered in those earlier studies was the potential role of Ethanol in causing dysbiosis in the gut independent of DNBS. As such, varying concentrations of Ethanol could possibly result in different colonic inflammatory response (Morampudi et al., 2014). In addition, for the first time, here we reported the role of DNBS alone (dissolved in PBS 1%) on inflammatory markers, colonic mucosa-associate and fecal microbiota. Our study revealed that pro-inflammatory cytokines and inflammatory markers as well as colonic mucosa-associated and fecal microbiota, were not significantly affected when DNBS or Ethanol administered alone. DNBS dissolved in Ethanol 30% was the only group

that showed all the symptoms of disease. Samples taken from colon mucosa were used to measure inflammatory response at the site of induction of colitis. Disease activity index, macroscopic and microscopic assessment plus histology images from H&E staining showed a transmural infiltration of immune cells, a disruption of epithelial barrier and a depletion of goblet cells, which together confirms the activation of colonic inflammatory response following DNBS/Ethanol administration. Also, analysis of the inflammatory mediators including serum CRP level, a marker for systemic inflammation, and colonic MPO level showed significant up-regulation in the case of DNBS/Ethanol induced colitis. In line with previous studies, considerable neutrophil accumulation and increased levels in serum CRP level and colonic MPO have been reported in DNBS/Ethanol-induced colitis (Ghia et al., 2008; Borrelli et al., 2015). Also, in parallel with previous studies (Yan et al., 2002; Ghia et al., 2007; Harel et al., 2011; Rabbi et al., 2016), potential pro-inflammatory mediators such as *il6, il1b, tnfa* at both mRNA and protein level are up-regulated confirming the inflammatory response induced by DNBS/Ethanol. IL-1β secreted by a large number of immune cells, such as DC, macrophages, fibroblasts and endothelial cells, leads to activation of T cells and NK cells, proliferation of B cells, nitric oxide production and increase in the level of adhesion molecule expressions (Beck et al., 1997). On the other hand, IL-6 promotes activation of inflammatory cells, such as T and B-lymphocytes, and several reports declare the increased level of IL-6 in CD patients (Beck et al., 1997; Neurath, 2014). The suggested mechanism behind DNBS/Ethanol role can be explained by the fact that Ethanol administration is needed to disrupt the colonic mucosal barrier and consequently let the DNBS to penetrate into lamina propria in order to haptenize the local colonic and gut bacterial proteins to acquire immunogenic characteristic (Morampudi et al., 2014). DNBS by its high-binding affinity to

lysine e-amino group shifts those membrane-bound proteins to the haptenized proteins, thus, initiating the activation of antigen presenting cells inside the colon mucosa to overexpress pro-inflammatory cytokines, such as INF-γ, IL-β, IL-12, TNF-α and nitric oxide (NO) through induction of inducible nitric oxide synthase (iNOS) as well as T-helper 1-mediated innate immune response. Weight loss and diarrhea are some of the symptoms of such transmural colonic inflammatory immune response due to DNBS administration (Kim et al., 2012; Goyal et al., 2014; Morampudi et al., 2014).

Resident gut microbiota are key-role players in IBD pathogenesis since approximately all IBD murine models requisite microbiome presence for development of colitis and likewise germfree mice show no signs for initiation of colitic conditions (Rath et al., 2001). Building on these observations, in our study we evaluated both fecal and mucosa-associated microbial communities alterations by evaluating species richness and diversities, patterns of microbiota clustering, prediction of functional capacities of the gut microbiome. Our results elucidated signature bacterial species in DNBS/Ethanol treated mice compared to control groups that were highly correlated with inflammatory immune responses.

Several meta-analyses have identified dysbiotic patterns of gut bacteria in both UC and CD patients (Halfvarson et al., 2017; Knights et al., 2013). Decreased species richness and bacterial species diversities within fecal and mucosa-associated microbiome (MAM) is a characteristic of IBD patients. Similar pattern was discovered in rodents under DSS experimental colitis (Andoh et al., 2005; Moschen et al., 2005; Xenoulis et al., 2008; Zenewicz et al., 2008; Samanta et al., 2012; Wills et al., 2014). These data are consistent with our alpha- and beta-diversity results, when communities' richness was compared between DNBS/Ethanol and its controls. In transient fecal microbiota, there was a significant decrease

in species richness under DNBS/Ethanol and it was clustered far from its controls. This distinctive clustering pattern was also seen in colon mucosa, although, DNBS/Ethanol administration increased the species richness in colon mucosa compared to controls. The dysbalanced patterns seen in microbiome profile of fecal and colon samples in colitic conditions could be suggested as dysbiosis state. This may be due depletion of commensal gut bacteria following DNBS/Ethanol administration, which provided an opportunity for opportunistic/pathogenic bacteria to grow more due to higher availability of nutrients, space, and oxygen. Furthermore, our data suggests the impact of DNBS/Ethanol on the microbiome composition may vary across different anatomical sites of murine gut. Having determined the alterations in the microbial community compositions at the lower taxonomic levels, several bacteria genera showed more relative abundance fluctuations within each treatment. Several taxa that were positively associated with DNBS/Ethanol-induced colitis in colon mucosa were: o. RF32, g. *Sutterella,* f. Entereobacteriaceae, and g. *Bilophila* from p. Proteobacteria, g. *Bacteroides,* f. Rikenellaceae from p. Bacteroidetes, g. *Coprococcus* from p. Firmicutes, and g. *Akkermansia* from p. Verruocomicrobia. The results showed the presence of Firmicutes's members associated with control group (Fig 9a). In the fecal microbiota, f. Ruminococcaceae, g. *Coprobacillus,* g. *Blautia* and g. *Enterococcus* from p. Firmicutes, f. Rikenellaceae and g. *Parabacteroides* from p. Bacteroidetes, YS2 from p. Cyanobacteria, in addition to the taxa mentioned in colon samples, were positively associated with DNBS/Ethanol group. The first important message to convey would be the positive association of p. Firmicutes members, such as f. Lachinospiraceae, g. *Ruminococcus,* g. *Lactobacillus,* g. *Clostridium,* and g. *Turibacter* with control samples, and their significant deficiency in DNBS/Ethanol group which result in a separate clusteration of DNBS/Ethanol.

Our data is confirmed by several studies demonstrated the decrease in majority of Firmicutes members, such as g. *Clostridium* in CD patients with an increased abundance in the p. Proteobacteria (Manichanh et al., 2006; Oberc et al., 2015). The role of g. *Clostridium* is widely investigated in butyrate production, a short chain fatty acid and source of energy for intestinal epithelium with the potentiality to acidify the intestinal lumen and therefore protect it against certain pathogenic bacteria, such as *Salmonella* and *Escherichia coli* (Topping et al., 2001; Vogt et al., 2015). Also, consistent with our findings, Schwab et al. (2014) reported several genera from p. Bacteroidetes, such as g. *Bacteroides* as a key role player in the onset of mouse model of colitis. Similarly several clinical studies reported the bold presence of mentioned bacteria in both CD and UC patients (Ott et al., 2004; Andoh et al., 2005; Swidsinski et al., 2005; Gophna et al., 2006; Sepehri et al., 2007; Bibiloni et al., 2008). The depletion in proportion of relative abundance of g. *Bacteroides* and f. Lachnospiraceae in tissue samples from resected GI tract of CD and UC patients were also reported in Frank et al. (2007) study. Additionally, the increased level of f. Enterobacteriaceae was highlighted in various studies in experimental model of CD or after enteric pathogens infection (Lupp et al., 2007; Stecher et al., 2012; Hill et al., 2014). Similar findings regarding association of f. Enterobacteriaceae with IBD, especially CD patients were also reported and support our findings (Ott et al., 2004; Manichanh et al., 2012; Nagalingam et al., 2012; Stecher et al., 2012; Berry et al., 2015). A study by Gophna et al. (2006) demonstrated the overrepresentation of p. Proteobacteria and p. Bacteroidetes and underrepresentation of o. Clostriales in biopsy samples from CD patients, the same pattern was also observed in our study. Additionally, g. *Roseburia*, g. *Faecalibacterium*, and f. Ruminococcaceae, known as some of producers of short-chain fatty acids (SCFA), were reduced whereas g.

Escherichia/Shigella were particularly elevated in the ileum of CD patients (Kinross et al., 2011; Morgan et al., 2012; Oberc et al., 2015). These data confirm the observed enrichment in the abundance of these bacteria within the DNBS/Ethanol colon mucosa-associated microbiota. Based on PICRUSt analysis, our data predicted a reduced amino acid synthesis. This phenomenon is in parallel with prediction of several elevated pathways such as: amino acid uptake, carbohydrate level, lipid uptake, and those metabolism pathways in charge of mucins overproduction during CD. Also, the glutathione uptake pathway, with its role to store oxidative stress from inflammation, is enriched in ileal CD patients and in our DNBS induced colitis (Keshavarzian et al., 2003).

The mechanism behind how these microbiota shifts contribute to pathophysiological state during CD and what initiates such dysbalanced microbiota in parallel with excessive inflammatory response is still an active area of research. One idea is the loss of SCFA producing bacteria lead to less enterocyte survival at epithelial layer and increase the permeability of this layer through loosen tight junction because of impaired butyrate synthesis and then as a result activate inflammatory response pathways (Peng et al., 2009). Also, survival of facultative anaerobic Proteobacteria, such as members of Entereobacteriaceae, and *Bilophila* with their higher robustness to reactive-oxygen species gives them priority to compete during inflammatory response with predominant anaerobic Firmicutes and Bacteroidetes (Miyoshi et al., 2017) . It seems that microbiome dysbiosis works as both as a cause and a consequence of IBD pathogenesis. However, the idea of whole microbiome genome sequencing flourished to look at the composition and abundance of all present gut bacteria at the same time rather than putting one pathogenic bacterium in charge of all IBD or CD pathogenesis. This is when network analysis is required to look at not only all correlated

bacteria, but also the archaea, the fungi communities and how a continuous disturbance in one member can affect the equilibrium state of the host. Furthermore, more clinical studies are required since future therapies are mostly focused on deteriorating harmful microbes and restoring a protective gut microbiome in IBD susceptible individuals.

Chapter Six

Conclusion, Significance, and Future Directions

6.1. Conclusion

Our experiments confirmed that DNBS solubilized in Ethanol 30% mimics a CD model of colitis based on inducing an inflammatory response and a gut microbiota dysbiosis. It also confirmed the neutral effect of DNBS or Ethanol alone on the immune response and the gut microbiota profile. Most importantly, by blocking the pro-inflammatory RANK/RANKL pathway through administration of Denosumab (Prolia™), which acts like OPG, the inflammatory response was decreased significantly as illustrated by cytokine levels. This is in parallel with Prolia's alleviating the effects on gut microbiota dysbiosis induced by experimental colitis, in which, Prolia-treatment resulted in prevention of dysbiosis in gut microbiome under DNBS/Ethanol induction model. All these data confirmed the effective role of anti-RANKL on modulating DNBS/Ethanol-induced colitis in murine model through the regulation of immune activation and gut microbiota.

6.2. Significance

This study depicted how DNBS experimental model of colitis can affect the colonic mucosa and fecal microbiota in murine model. It also highlighted the role of Ethanol for DNBS dissolvent in activating this experimental model. On the other hand, Prolia for the first time was used to target inflammation in an experimental model of CD. Our data confirmed the anti-inflammatory role of inhibitor of RANK/RANKL pathway. Prolia not only contributes to reduction of osteoclastogenesis for arthritis patients (Dempster et al., 2012), but

also according to this study, it reduced the inflammatory response and modulated the dysbalance in gut microbiota in DNBS/Ethanol-induced model of colitis. More experiments need to be performed to define the exact cellular mechanisms of action.

6.3. Future Directions

Keystone and foundation bacterial species that are crucial in development or prevention of gut microbiota dysbiosis can be cultured and inoculated into germ-free or spontaneous colitic mice to more precisely study their function and role in development of colitis and activation of immune response. Metabolomics and proteomics analysis can be performed to better understand how bacterial dysbiosis contributes to development of CD. Combination of microbiome, metabolome and proteomics data using network analysis can help us to identify the influential members of a defined community that can be used a therapeutic approach for treatment of IBD. Although Prolia has been used in treatment of arthritis patients, its safety for therapeutic use under CD condition requires further assessment.

Chapter Seven

References

Abeles, S. R., Jones, M. B., Santiago-Rodriguez, T. M., Ly, M., Klitgord, N., Yooseph, S., .Pride, D. T. (2016). Microbial diversity in individuals and their household contacts following typical antibiotic courses. *Microbiome, 4*(1), 39. doi:10.1186/s40168-016-0187-9

Abraham, C., & Cho, J. H. (2009). Inflammatory bowel disease. *New England Journal of Medicine, 361*(21), 2066-2078. doi:10.1056/NEJMra0804647

Abraham, C., & Medzhitov, R. (2011). Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology, 140*(6), 1729-1737. doi:10.1053/j.gastro.2011.02.012

Abreu, M. T. (2013). The genetics and pathogenesis of inflammatory bowel disease. *Gastroenterology & Hepatology, 9*(8), 521-523.

Ali, S., & Tamboli, C. P. (2008). Advances in epidemiology and diagnosis of inflammatory bowel diseases. *Curr Gastroenterol Rep, 10*(6), 576-584.

Ali, T., Lam, D., Bronze, M. S., & Humphrey, M. B. (2009). Osteoporosis in inflammatory bowel disease. *American Journal of Medicine, 122*(7), 599-604. doi:10.1016/j.amjmed.2009.01.022

Alizadeh, M., Munyaka, P., Yitbarek, A., Echeverry, H., & Rodriguez-Lecompte, J. C. (2017). Maternal antibody decay and antibody-mediated immune responses in chicken pullets fed prebiotics and synbiotics. *Poultry Science, 96*(1), 58-64. doi:10.3382/ps/pew244

Anastasilakis, A. D., Toulis, K. A., Goulis, D. G., Polyzos, S. A., Delaroudis, S., Giomisi, A., & Terpos, E. (2009). Efficacy and safety of denosumab in postmenopausal women with osteopenia or osteoporosis: a systematic review and a meta-analysis. *Hormone and Metabolic Research, 41*(10), 721-729. doi:10.1055/s-0029-1224109

Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature, 390*(6656), 175-179. doi:10.1038/36593

Anderson, M. J. (2005). PERMANOVA-Permutational multivariate analysis of variance.

Andoh, A., Zhang, Z., Inatomi, O., Fujino, S., Deguchi, Y., Araki, Y., Fujiyama, Y. (2005). Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology, 129*(3), 969-984. doi:10.1053/j.gastro.2005.06.071

Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Romagnani, S. (2007). Phenotypic and functional features of human Th17 cells. *Journal of Experimental Medicine, 204*(8), 1849-1861. doi:10.1084/jem.20070663

Ashcroft, A. J., Cruickshank, S. M., Croucher, P. I., Perry, M. J., Rollinson, S., Lippitt, J. M., Carding, S. R. (2003). Colonic dendritic cells, intestinal inflammation, and T cell-mediated bone destruction are modulated by recombinant osteoprotegerin. *Immunity, 19*(6), 849-861.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L., & Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *Journal of Experimental Medicine, 190*(7), 995-1004.

Atreya, R., & Neurath, M. F. (2017). Current and Future Targets for Mucosal Healing in Inflammatory Bowel Disease. *Visc Med, 33*(1), 82-88. doi:10.1159/000458006

Azzopardi, N., & Ellul, P. (2013). Risk factors for osteoporosis in Crohn's disease: infliximab, corticosteroids, body mass index, and age of onset. *Inflammatory Bowel Diseases, 19*(6), 1173-1178. doi:10.1097/MIB.0b013e31828075a7

Backhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., Finlay, B. B. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe, 12*(5), 611-622. doi:10.1016/j.chom.2012.10.012

Bar, F., Sina, C., Hundorfean, G., Pagel, R., Lehnert, H., Fellermann, K., & Buning, J. (2013). Inflammatory bowel diseases influence major histocompatibility complex class I (MHC I) and II compartments in intestinal epithelial cells. *Clinical and Experimental Immunology, 172*(2), 280-289. doi:10.1111/cei.12047

Barcelo-Batllori, S., Andre, M., Servis, C., Levy, N., Takikawa, O., Michetti, P., Felley-Bosco, E. (2002). Proteomic analysis of cytokine induced proteins in human intestinal epithelial cells: implications for inflammatory bowel diseases. *Proteomics, 2*(5), 551-560. doi:10.1002/1615-9861(200205)2:5<551::AID-PROT551>3.0.CO;2-O

Baumgart, D. C., & Sandborn, W. J. (2012). Crohn's disease. *Lancet, 380*(9853), 1590-1605. doi:10.1016/S0140-6736(12)60026-9

Beck, P. L., & Wallace, J. L. (1997). Cytokines in inflammatory bowel disease. *Mediators of Inflammation, 6*(2), 95-103. doi:10.1080/09629359791785

Becker, C., Dornhoff, H., Neufert, C., Fantini, M. C., Wirtz, S., Huebner, S., Neurath, M. F. (2006). Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis. *Journal of Immunology, 177*(5), 2760-2764.

Becker, C., Neurath, M. F., & Wirtz, S. (2015). The Intestinal Microbiota in Inflammatory Bowel Disease. *ILAR J, 56*(2), 192-204. doi:10.1093/ilar/ilv030

Bernstein. (2006). Inflammatory Bowel Diseases as Secondary Causes of Osteoporosis. *Curr Osteoporos Rep*. doi:10.1007/s11914-996-0031-4

Bernstein. (2005). Serum osteoprotegerin is increased in Crohn's disease: A population‐based case control study. *Inflammatory Bowel Diseases, 11*. doi:10.1097/01.MIB.0000164015.60795.ca

Bernstein, C. N., Blanchard, J. F., Leslie, W., Wajda, A., & Yu, B. N. (2000). The incidence of fracture among patients with inflammatory bowel disease. A population-based cohort study. *Annals of Internal Medicine, 133*(10), 795-799.

Berry, D., Kuzyk, O., Rauch, I., Heider, S., Schwab, C., Hainzl, E., Loy, A. (2015). Intestinal Microbiota Signatures Associated with Inflammation History in Mice Experiencing Recurring Colitis. *Frontiers in Microbiology, 6*, 1408. doi:10.3389/fmicb.2015.01408

Bibiloni, R., Tandon, P., Vargas-Voracka, F., Barreto-Zuniga, R., Lupian-Sanchez, A., Rico-Hinojosa, M. A., Tannock, G. W. (2008). Differential clustering of bowel biopsy-associated bacterial profiles of specimens collected in Mexico and Canada: what do these profiles represent? *Journal of Medical Microbiology, 57*(Pt 1), 111-117. doi:10.1099/jmm.0.47321-0

Borrelli, F., Romano, B., Petrosino, S., Pagano, E., Capasso, R., Coppola, D., Izzo, A. A. (2015). Palmitoylethanolamide, a naturally occurring lipid, is an orally effective

intestinal anti-inflammatory agent. *British Journal of Pharmacology, 172*(1), 142-158. doi:10.1111/bph.12907

Boyce, B. F., & Xing, L. (2007). The RANKL/RANK/OPG pathway. *Curr Osteoporos Rep, 5*(3), 98-104.

Brown, G. M., & McAuley, J. S. (1952). Cortisone therapy in ulcerative colitis. *Journal of the American Medical Association, 150*(16), 1587-1591.

Bryda, E. C. (2013). The Mighty Mouse: the impact of rodents on advances in biomedical research. *Missouri Medicine, 110*(3), 207-211.

Buehring, B., Viswanathan, R., Binkley, N., & Busse, W. (2013). Glucocorticoid-induced osteoporosis: an update on effects and management. *Journal of Allergy and Clinical Immunology, 132*(5), 1019-1030. doi:10.1016/j.jaci.2013.08.040

Butto, L. F., & Haller, D. (2016). Dysbiosis in intestinal inflammation: Cause or consequence. *International Journal of Medical Microbiology, 306*(5), 302-309. doi:10.1016/j.ijmm.2016.02.010

Cader, M. Z., & Kaser, A. (2013). Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut, 62*(11), 1653-1664. doi:10.1136/gutjnl-2012-303955

Cankaya, M., Cizmeci Senel, F., Kadioglu Duman, M., Muci, E., Dayisoylu, E. H., & Balaban, F. (2013). The effects of chronic zoledronate usage on the jaw and long bones evaluated using RANKL and osteoprotegerin levels in an animal model. *International Journal of Oral and Maxillofacial Surgery, 42*(9), 1134-1139. doi:10.1016/j.ijom.2013.02.008

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J, 6*(8), 1621-1624. doi:10.1038/ismej.2012.8

Card, T., West, J., Hubbard, R., & Logan, R. F. (2004). Hip fractures in patients with inflammatory bowel disease and their relationship to corticosteroid use: a population based cohort study. *Gut, 53*(2), 251-255.

CCAC, C. C. o. A. C. (1993). Canadian Council on Animal Care.

Chaudhry, A., Rudra, D., Treuting, P., Samstein, R. M., Liang, Y., Kas, A., & Rudensky, A. Y. (2009). CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science, 326*(5955), 986-991. doi:10.1126/science.1172702

Compston, J. (2003). Osteoporosis associated with gastrointestinal diseases. *European Journal of Gastroenterology and Hepatology, 15*(8), 841-843. doi:10.1097/01.meg.0000059186.46867.55

Cook, L. C., Hillhouse, A. E., Myles, M. H., Lubahn, D. B., Bryda, E. C., Davis, J. W., & Franklin, C. L. (2014). The role of estrogen signaling in a mouse model of inflammatory bowel disease: a Helicobacter hepaticus model. *PloS One, 9*(4), e94209. doi:10.1371/journal.pone.0094209

Cooper, H. S., Murthy, S. N., Shah, R. S., & Sedergran, D. J. (1993). Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Laboratory Investigation, 69*(2), 238-249.

Cuzzocrea, S., McDonald, M. C., Mazzon, E., Mota-Filipe, H., Centorrino, T., Terranova, M. L., Thiemermann, C. (2001). Calpain inhibitor I reduces colon injury caused by dinitrobenzene sulphonic acid in the rat. *Gut, 48*(4), 478-488.

D'Argenio, V., & Salvatore, F. (2015). The role of the gut microbiome in the healthy adult status. *Clinica Chimica Acta, 451*(Pt A), 97-102. doi:10.1016/j.cca.2015.01.003

Danese, S., & Fiocchi, C. (2011). Ulcerative colitis. *New England Journal of Medicine, 365*(18), 1713-1725. doi:10.1056/NEJMra1102942

de Souza, H. S., & Fiocchi, C. (2016). Immunopathogenesis of IBD: current state of the art. *Nature Reviews: Gastroenterology & Hepatology, 13*(1), 13-27. doi:10.1038/nrgastro.2015.186

De Voogd, F. A., Gearry, R. B., Mulder, C. J., & Day, A. S. (2016). Osteoprotegerin: A novel biomarker for inflammatory bowel disease and gastrointestinal carcinoma. *Journal of Gastroenterology and Hepatology, 31*(8), 1386-1392. doi:10.1111/jgh.13324

Debelius, J., Song, S. J., Vazquez-Baeza, Y., Xu, Z. Z., Gonzalez, A., & Knight, R. (2016). Tiny microbes, enormous impacts: what matters in gut microbiome studies? *Genome Biology, 17*(1), 217. doi:10.1186/s13059-016-1086-x

DeGruttola, A. K., Low, D., Mizoguchi, A., & Mizoguchi, E. (2016). Current Understanding of Dysbiosis in Disease in Human and Animal Models. *Inflammatory Bowel Diseases, 22*(5), 1137-1150. doi:10.1097/MIB.0000000000000750

Dempster, D. W., Lambing, C. L., Kostenuik, P. J., & Grauer, A. (2012). Role of RANK ligand and denosumab, a targeted RANK ligand inhibitor, in bone health and osteoporosis: a review of preclinical and clinical data. *Clinical Therapeutics, 34*(3), 521-536. doi:10.1016/j.clinthera.2012.02.002

Derakhshani, H., De Buck, J., Mortier, R., Barkema, H. W., Krause, D. O., & Khafipour, E. (2016). The Features of Fecal and Ileal Mucosa-Associated Microbiota in Dairy Calves during Early Infection with Mycobacterium avium Subspecies paratuberculosis. *Frontiers in Microbiology, 7*, 426. doi:10.3389/fmicb.2016.00426

Derakhshani, H., Tun, H. M., & Khafipour, E. (2016). An extended single-index multiplexed 16S rRNA sequencing for microbial community analysis on MiSeq illumina platforms. *Journal of Basic Microbiology, 56*(3), 321-326. doi:10.1002/jobm.201500420

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology, 72*(7), 5069-5072. doi:10.1128/AEM.03006-05

Dethlefsen, L., Huse, S., Sogin, M. L., & Relman, D. A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biology, 6*(11), e280. doi:10.1371/journal.pbio.0060280

Dickson, I. (2017). Gut microbiota: Diagnosing IBD with the gut microbiome. *Nature Reviews: Gastroenterology & Hepatology, 14*(4), 195. doi:10.1038/nrgastro.2017.25

Dixon, A. L., Liang, L., Moffatt, M. F., Chen, W., Heath, S., Wong, K. C., Cookson, W. O. (2007). A genome-wide association study of global gene expression. *Nature Genetics, 39*(10), 1202-1207. doi:10.1038/ng2109

Doecke, J. D., Simms, L. A., Zhao, Z. Z., Huang, N., Hanigan, K., Krishnaprasad, K., Radford-Smith, G. L. (2013). Genetic susceptibility in IBD: overlap between ulcerative colitis and Crohn's disease. *Inflammatory Bowel Diseases, 19*(2), 240-245. doi:10.1097/MIB.0b013e3182810041

Dulai, P. S., Singh, S., Jiang, X., Peerani, F., Narula, N., Chaudrey, K., Colombel, J. F. (2016). The Real-World Effectiveness and Safety of Vedolizumab for Moderate-Severe Crohn's Disease: Results From the US VICTORY Consortium. *American Journal of Gastroenterology, 111*(8), 1147-1155. doi:10.1038/ajg.2016.236

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics, 27*(16), 2194-2200. doi:10.1093/bioinformatics/btr381

Eken, A., Singh, A. K., Treuting, P. M., & Oukka, M. (2014). IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. *Mucosal Immunology, 7*(1), 143-154. doi:10.1038/mi.2013.33

Elson, C. O., Sartor, R. B., Tennyson, G. S., & Riddell, R. H. (1995). Experimental models of inflammatory bowel disease. *Gastroenterology, 109*(4), 1344-1367.

Fiocchi, C. (1998). Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology, 115*(1), 182-205.

Fiocchi, C. (2005). Inflammatory bowel disease pathogenesis: therapeutic implications. *Chinese Journal of Digestive Diseases, 6*(1), 6-9. doi:10.1111/j.1443-9573.2005.00191.x

Fitzpatrick, L. R., Meirelles, K., Small, J. S., Puleo, F. J., Koltun, W. A., & Cooney, R. N. (2010). A new model of chronic hapten-induced colitis in young rats. *Journal of Pediatric Gastroenterology and Nutrition, 50*(3), 240-250. doi:10.1097/MPG.0b013e3181cb8f4a

Fizazi, K., Carducci, M., Smith, M., Damião, R., Brown, J., Karsh, L., Goessl, C. (2011). Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. *The Lancet, 377*(9768), 813-822. doi:10.1016/s0140-6736(10)62344-6

Fletcher, J. (2016). Vitamin D deficiency in patients with inflammatory bowel disease. *British Journal of Nursing, 25*(15), 846-851. doi:10.12968/bjon.2016.25.15.846

Flores, G. E., Caporaso, J. G., Henley, J. B., Rideout, J. R., Domogala, D., Chase, J., Fierer, N. (2014). Temporal variability is a personalized feature of the human microbiome. *Genome Biology, 15*(12), 531. doi:10.1186/s13059-014-0531-y

Fouque-Aubert, A., & Chapurlat, R. (2008). Influence of RANKL inhibition on immune system in the treatment of bone diseases. *Joint, Bone, Spine: Revue du Rhumatisme, 75*(1), 5-10. doi:10.1016/j.jbspin.2007.05.004

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of*

Sciences of the United States of America, 104(34), 13780-13785. doi:10.1073/pnas.0706625104

Franke, A., McGovern, D. P., Barrett, J. C., Wang, K., Radford-Smith, G. L., Ahmad, T., Parkes, M. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature Genetics, 42*(12), 1118-1125. doi:10.1038/ng.717

Frink, M., Hsieh, Y. C., Hsieh, C. H., Pape, H. C., Choudhry, M. A., Schwacha, M. G., & Chaudry, I. H. (2007). Keratinocyte-derived chemokine plays a critical role in the induction of systemic inflammation and tissue damage after trauma-hemorrhage. *Shock, 28*(5), 576-581.

Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., Strober, W. (1996). Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of Immunology, 157*(3), 1261-1270.

Fuss, I. J., & Strober, W. (2008). The role of IL-13 and NK T cells in experimental and human ulcerative colitis. *Mucosal Immunology, 1 Suppl 1*, S31-33. doi:10.1038/mi.2008.40

Galili, T. (2015). dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics, 31*(22), 3718-3720. doi:10.1093/bioinformatics/btv428

Gerlach, K., Hwang, Y., Nikolaev, A., Atreya, R., Dornhoff, H., Steiner, S., Neurath, M. F. (2014). TH9 cells that express the transcription factor PU.1 drive T cell-mediated

colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nature Immunology, 15*(7), 676-686. doi:10.1038/ni.2920

German, A. J., Hall, E. J., Kelly, D. F., Watson, A. D., & Day, M. J. (2000). An immunohistochemical study of histiocytic ulcerative colitis in boxer dogs. *Journal of Comparative Pathology, 122*(2-3), 163-175. doi:10.1053/jcpa.1999.0353

Gevers, D., Kugathasan, S., Denson, L. A., Vazquez-Baeza, Y., Van Treuren, W., Ren, B., Xavier, R. J. (2014). The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe, 15*(3), 382-392. doi:10.1016/j.chom.2014.02.005

Ghia, J. E., Blennerhassett, P., & Collins, S. M. (2007). Vagus nerve integrity and experimental colitis. *American Journal of Physiology: Gastrointestinal and Liver Physiology, 293*(3), G560-567. doi:10.1152/ajpgi.00098.2007

Ghia, J. E., Blennerhassett, P., & Collins, S. M. (2008). Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. *Journal of Clinical Investigation, 118*(6), 2209-2218. doi:10.1172/JCI32849

Ghia, J. E., Blennerhassett, P., Deng, Y., Verdu, E. F., Khan, W. I., & Collins, S. M. (2009). Reactivation of inflammatory bowel disease in a mouse model of depression. *Gastroenterology, 136*(7), 2280-2288 e2281-2284. doi:10.1053/j.gastro.2009.02.069

Ghia, J. E., Li, N., Wang, H., Collins, M., Deng, Y., El-Sharkawy, R. T., Khan, W. I. (2009). Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology, 137*(5), 1649-1660. doi:10.1053/j.gastro.2009.08.041

Gibson, T. B., Ng, E., Ozminkowski, R. J., Wang, S., Burton, W. N., Goetzel, R. Z., & Maclean, R. (2008). The direct and indirect cost burden of Crohn's disease and

ulcerative colitis. *Journal of Occupational and Environmental Medicine, 50*(11), 1261-1272. doi:10.1097/JOM.0b013e318181b8ca

Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F., & Veldhuyzen van Zanten, S. J. (2006). Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *Journal of Clinical Microbiology, 44*(11), 4136-4141. doi:10.1128/JCM.01004-06

Goyal, N., Rana, A., Ahlawat, A., Bijjem, K. R., & Kumar, P. (2014). Animal models of inflammatory bowel disease: a review. *Inflammopharmacology, 22*(4), 219-233. doi:10.1007/s10787-014-0207-y

Gu, S., Chen, D., Zhang, J. N., Lv, X., Wang, K., Duan, L. P., Wu, X. L. (2013). Bacterial community mapping of the mouse gastrointestinal tract. *PloS One, 8*(10), e74957. doi:10.1371/journal.pone.0074957

Gu, Z., Eils, R., & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics, 32*(18), 2847-2849. doi:10.1093/bioinformatics/btw313

Halfvarson, J., Brislawn, C. J., Lamendella, R., Vazquez-Baeza, Y., Walters, W. A., Bramer, L. M., Jansson, J. K. (2017). Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol, 2*, 17004. doi:10.1038/nmicrobiol.2017.4

Hammer, Ø., Harper, David A.T., and Paul D. Ryan. (2001). Past: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica, 4*.

Hanauer, S. B. (2006). Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflammatory Bowel Diseases, 12 Suppl 1*, S3-9.

Harel, E., Rubinstein, A., Nissan, A., Khazanov, E., Nadler Milbauer, M., Barenholz, Y., & Tirosh, B. (2011). Enhanced transferrin receptor expression by proinflammatory cytokines in enterocytes as a means for local delivery of drugs to inflamed gut mucosa. *PloS One, 6*(9), e24202. doi:10.1371/journal.pone.0024202

Hawkins, J. V., Emmel, E. L., Feuer, J. J., Nedelman, M. A., Harvey, C. J., Klein, H. J., Billings, P. C. (1997). Protease activity in a hapten-induced model of ulcerative colitis in rats. *Digestive Diseases and Sciences, 42*(9), 1969-1980.

Heinritz, S. N., Mosenthin, R., & Weiss, E. (2013). Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. *Nutrition Research Reviews, 26*(2), 191-209. doi:10.1017/S0954422413000152

Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Schulzke, J. D. (2005). Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology, 129*(2), 550-564. doi:10.1016/j.gastro.2005.05.002

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews: Gastroenterology & Hepatology, 11*(8), 506-514. doi:10.1038/nrgastro.2014.66

Hisamatsu, T., Kanai, T., Mikami, Y., Yoneno, K., Matsuoka, K., & Hibi, T. (2013). Immune aspects of the pathogenesis of inflammatory bowel disease. *Pharmacology and Therapeutics, 137*(3), 283-297. doi:10.1016/j.pharmthera.2012.10.008

Houpt, K. A., Houpt, T. R., & Pond, W. G. (1979). The pig as a model for the study of obesity and of control of food intake: a review. *Yale Journal of Biology and Medicine, 52*(3), 307-329.

Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Boyle, W. J. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proceedings of the National Academy of Sciences of the United States of America, 96*(7), 3540-3545.

Iqbal, N., Oliver, J. R., Wagner, F. H., Lazenby, A. S., Elson, C. O., & Weaver, C. T. (2002). T helper 1 and T helper 2 cells are pathogenic in an antigen-specific model of colitis. *Journal of Experimental Medicine, 195*(1), 71-84.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Littman, D. R. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell, 139*(3), 485-498. doi:10.1016/j.cell.2009.09.033

Jahnsen, J., Falch, J. A., Mowinckel, P., & Aadland, E. (2004). Bone mineral density in patients with inflammatory bowel disease: a population-based prospective two-year follow-up study. *Scandinavian Journal of Gastroenterology, 39*(2), 145-153.

Jean-Jacques Body, T. F., Robert E Coleman, Allan Lipton, Filip Geurs, Michelle Fan, Donna Holloway, Mark C Peterson and Pirow J Bekker. (2006). A study of the biological receptor activator of nuclear factor-kappaB ligand inhibitor, denosumab, in patients with multiple myeloma or bone metastases from breast cancer. *Clinical Cancer Research*. doi:10.1158/1078-0432

Jimi, E., Akiyama, S., Tsurukai, T., Okahashi, N., Kobayashi, K., Udagawa, N., Suda, T. (1999). Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *Journal of Immunology, 163*(1), 434-442.

Jones, D. H., Kong, Y. Y., & Penninger, J. M. (2002). Role of RANKL and RANK in bone loss and arthritis. *Annals of the Rheumatic Diseases, 61 Suppl 2*, ii32-39.

Joshi, S. V., Vyas, B. A., Shah, P. D., Shah, D. R., Shah, S. A., & Gandhi, T. R. (2011). Protective effect of aqueous extract of Oroxylum indicum Linn. (root bark) against DNBS-induced colitis in rats. *Indian Journal of Pharmacology, 43*(6), 656-661. doi:10.4103/0253-7613.89821

Josse, R., Khan, A., Ngui, D., & Shapiro, M. (2013). Denosumab, a new pharmacotherapy option for postmenopausal osteoporosis. *Current Medical Research and Opinion, 29*(3), 205-216. doi:10.1185/03007995.2013.763779

Kaplan, G. G. (2015). The global burden of IBD: from 2015 to 2025. *Nature Reviews: Gastroenterology & Hepatology, 12*(12), 720-727. doi:10.1038/nrgastro.2015.150

Kaser, A., Lee, A. H., Franke, A., Glickman, J. N., Zeissig, S., Tilg, H., Blumberg, R. S. (2008). XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell, 134*(5), 743-756. doi:10.1016/j.cell.2008.07.021

Kawada, M., Arihiro, A., & Mizoguchi, E. (2007). Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. *World Journal of Gastroenterology, 13*(42), 5581-5593.

Kelsen, J. R., & Sullivan, K. E. (2017). Inflammatory Bowel Disease in Primary Immunodeficiencies. *Current Allergy and Asthma Reports, 17*(8), 57. doi:10.1007/s11882-017-0724-z

Keshavarzian, A., Banan, A., Farhadi, A., Komanduri, S., Mutlu, E., Zhang, Y., & Fields, J. Z. (2003). Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. *Gut, 52*(5), 720-728.

Khafipour, E., Li, S., Plaizier, J. C., & Krause, D. O. (2009). Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Applied and Environmental Microbiology, 75*(22), 7115-7124. doi:10.1128/AEM.00739-09

Khan, W. I., Blennerhasset, P. A., Varghese, A. K., Chowdhury, S. K., Omsted, P., Deng, Y., & Collins, S. M. (2002). Intestinal nematode infection ameliorates experimental colitis in mice. *Infection and Immunity, 70*(11), 5931-5937.

Khor, B., Gardet, A., & Xavier, R. J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature, 474*(7351), 307-317. doi:10.1038/nature10209

Kim, H. S., & Berstad, A. (1992). Experimental colitis in animal models. *Scandinavian Journal of Gastroenterology, 27*(7), 529-537.

Kim, J. J., Shajib, M. S., Manocha, M. M., & Khan, W. I. (2012). Investigating intestinal inflammation in DSS-induced model of IBD. *J Vis Exp*(60). doi:10.3791/3678

Kinross, J. M., Darzi, A. W., & Nicholson, J. K. (2011). Gut microbiome-host interactions in health and disease. *Genome Medicine, 3*(3), 14. doi:10.1186/gm228

Kitajima, S., Morimoto, M., Sagara, E., Shimizu, C., & Ikeda, Y. (2001). Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. *Experimental Animals, 50*(5), 387-395.

Kleinschek, M. A., Boniface, K., Sadekova, S., Grein, J., Murphy, E. E., Turner, S. P., Kastelein, R. A. (2009). Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *Journal of Experimental Medicine, 206*(3), 525-534. doi:10.1084/jem.20081712

Ko, J. K., & Cho, C. H. (2005). The diverse actions of nicotine and different extracted fractions from tobacco smoke against hapten-induced colitis in rats. *Toxicological Sciences, 87*(1), 285-295. doi:10.1093/toxsci/kfi238

Ko, J. K., Lam, F. Y., & Cheung, A. P. (2005). Amelioration of experimental colitis by Astragalus membranaceus through anti-oxidation and inhibition of adhesion molecule synthesis. *World Journal of Gastroenterology, 11*(37), 5787-5794.

Kobayashi, T., Steinbach, E. C., Russo, S. M., Matsuoka, K., Nochi, T., Maharshak, N., Plevy, S. E. (2014). NFIL3-deficient mice develop microbiota-dependent, IL-12/23-driven spontaneous colitis. *Journal of Immunology, 192*(4), 1918-1927. doi:10.4049/jimmunol.1301819

Kostic, A. D., Gevers, D., Siljander, H., Vatanen, T., Hyotylainen, T., Hamalainen, A. M., Xavier, R. J. (2015). The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe, 17*(2), 260-273. doi:10.1016/j.chom.2015.01.001

Krela-Kazmierczak, I., Szymczak, A., Lykowska-Szuber, L., Eder, P., & Linke, K. (2016). Osteoporosis in Gastrointestinal Diseases. *Adv Clin Exp Med, 25*(1), 185-190. doi:10.17219/acem/33746

Kremer, B., Mariman, R., van Erk, M., Lagerweij, T., & Nagelkerken, L. (2012). Temporal colonic gene expression profiling in the recurrent colitis model identifies early and

chronic inflammatory processes. *PloS One,* *7*(11), e50388. doi:10.1371/journal.pone.0050388

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., & Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell, 75*(2), 263-274.

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Boyle, W. J. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell, 93*(2), 165-176.

Lakhan, S. E., & Kirchgessner, A. (2010). Neuroinflammation in inflammatory bowel disease. *Journal of Neuroinflammation, 7*, 37. doi:10.1186/1742-2094-7-37

Lam, S. H., Chua, H. L., Gong, Z., Lam, T. J., & Sin, Y. M. (2004). Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. *Developmental and Comparative Immunology, 28*(1), 9-28.

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology, 31*(9), 814-821. doi:10.1038/nbt.2676

Lederberg, J. (2001). Ome Sweet'Omics--A Genealogical Treasury of Words. *The Scientist*.

Legaki, E., & Gazouli, M. (2016). Influence of environmental factors in the development of inflammatory bowel diseases. *World Journal of Gastrointestinal Pharmacology and Therapeutics, 7*(1), 112-125. doi:10.4292/wjgpt.v7.i1.112

Leung, J. M., Davenport, M., Wolff, M. J., Wiens, K. E., Abidi, W. M., Poles, M. A., Loke, P. (2014). IL-22-producing CD4+ cells are depleted in actively inflamed colitis tissue. *Mucosal Immunology, 7*(1), 124-133. doi:10.1038/mi.2013.31

Liu, T. C., & Stappenbeck, T. S. (2016). Genetics and Pathogenesis of Inflammatory Bowel Disease. *Annual Review of Pathology, 11*, 127-148. doi:10.1146/annurev-pathol-012615-044152

Loddo, I., & Romano, C. (2015). Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis. *Frontiers in Immunology, 6*, 551. doi:10.3389/fimmu.2015.00551

Lopetuso, L. R., Petito, V., Graziani, C., Schiavoni, E., Paroni Sterbini, F., Poscia, A., Gasbarrini, A. (2017). Gut Microbiota in Health, Diverticular Disease, Irritable Bowel Syndrome, and Inflammatory Bowel Diseases: Time for Microbial Marker of Gastrointestinal Disorders? *Digestive Diseases*. doi:10.1159/000477205

Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C., & Finlay, B. B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe, 2*(3), 204.

M'Koma, A. E. (2013). Inflammatory bowel disease: an expanding global health problem. *Clinical Medicine Insights: Gastroenterology, 6*, 33-47. doi:10.4137/CGast.S12731

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., Vermeire, S. (2014). A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut, 63*(8), 1275-1283. doi:10.1136/gutjnl-2013-304833

Maloy, K. J., & Powrie, F. (2011). Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature, 474*(7351), 298-306. doi:10.1038/nature10208

Manichanh, C., Borruel, N., Casellas, F., & Guarner, F. (2012). The gut microbiota in IBD. *Nature Reviews: Gastroenterology & Hepatology, 9*(10), 599-608. doi:10.1038/nrgastro.2012.152

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Dore, J. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut, 55*(2), 205-211. doi:10.1136/gut.2005.073817

Mansfield, K. G., Lin, K. C., Xia, D., Newman, J. V., Schauer, D. B., MacKey, J., Carville, A. (2001). Enteropathogenic Escherichia coli and ulcerative colitis in cotton-top tamarins (Saguinus oedipus). *Journal of Infectious Diseases, 184*(6), 803-807. doi:10.1086/322990

Manzel, A., Muller, D. N., Hafler, D. A., Erdman, S. E., Linker, R. A., & Kleinewietfeld, M. (2014). Role of "Western diet" in inflammatory autoimmune diseases. *Current Allergy and Asthma Reports, 14*(1), 404. doi:10.1007/s11882-013-0404-6

Mariman, R., Kremer, B., van Erk, M., Lagerweij, T., Koning, F., & Nagelkerken, L. (2012). Gene expression profiling identifies mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics. *Inflammatory Bowel Diseases, 18*(8), 1424-1433. doi:10.1002/ibd.22849

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., & Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics, 13*, 31. doi:10.1186/1471-2105-13-31

Mazmanian, S. K., Round, J. L., & Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature, 453*(7195), 620-625. doi:10.1038/nature07008

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS One, 8*(4), e61217. doi:10.1371/journal.pone.0061217

Mestas, J., & Hughes, C. C. (2004). Of mice and not men: differences between mouse and human immunology. *Journal of Immunology, 172*(5), 2731-2738.

Miller, P. D. (2011). A review of the efficacy and safety of denosumab in postmenopausal women with osteoporosis. *Therapeutic Advances in Musculoskeletal Disease, 3*(6), 271-282. doi:10.1177/1759720X11424220

Miyoshi, J., & Chang, E. B. (2017). The gut microbiota and inflammatory bowel diseases. *Translational Research: The Journal of Laboratory and Clinical Medicine, 179*, 38-48. doi:10.1016/j.trsl.2016.06.002

Mizoguchi, A. (2012). Animal models of inflammatory bowel disease. *Progress in Molecular Biology and Translational Science, 105*, 263-320. doi:10.1016/B978-0-12-394596-9.00009-3

Mizoguchi, A., & Mizoguchi, E. (2008). Inflammatory bowel disease, past, present and future: lessons from animal models. *Journal of Gastroenterology, 43*(1), 1-17. doi:10.1007/s00535-007-2111-3

Modigliani, R., Mary, J. Y., Simon, J. F., Cortot, A., Soule, J. C., Gendre, J. P., & Rene, E. (1990). Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives. *Gastroenterology, 98*(4), 811-818.

Moelants, E. A., Mortier, A., Van Damme, J., & Proost, P. (2013). Regulation of TNF-alpha with a focus on rheumatoid arthritis. *Immunology and Cell Biology, 91*(6), 393-401. doi:10.1038/icb.2013.15

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., Kaplan, G. G. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology, 142*(1), 46-54 e42; quiz e30. doi:10.1053/j.gastro.2011.10.001

Mondal, K., & Kugathasan, S. (2017). IBD: Genetic differences in Crohn's disease susceptibility and outcome. *Nature Reviews: Gastroenterology & Hepatology, 14*(5), 266-268. doi:10.1038/nrgastro.2017.24

Monteleone, G., Biancone, L., Marasco, R., Morrone, G., Marasco, O., Luzza, F., & Pallone, F. (1997). Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology, 112*(4), 1169-1178.

Monteleone, G., Kumberova, A., Croft, N. M., McKenzie, C., Steer, H. W., & MacDonald, T. T. (2001). Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *Journal of Clinical Investigation, 108*(4), 601-609. doi:10.1172/JCI12821

Morampudi, V., Bhinder, G., Wu, X., Dai, C., Sham, H. P., Vallance, B. A., & Jacobson, K. (2014). DNBS/TNBS colitis models: providing insights into inflammatory bowel disease and effects of dietary fat. *J Vis Exp*(84), e51297. doi:10.3791/51297

Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., Huttenhower, C. (2012). Dysfunction of the intestinal microbiome in inflammatory

bowel disease and treatment. *Genome Biology, 13*(9), R79. doi:10.1186/gb-2012-13-9-r79

Moschen, A. R., Kaser, A., Enrich, B., Ludwiczek, O., Gabriel, M., Obrist, P., Tilg, H. (2005). The RANKL/OPG system is activated in inflammatory bowel disease and relates to the state of bone loss. *Gut, 54*(4), 479-487. doi:10.1136/gut.2004.044370

Munyaka, P. M., Sepehri, S., Ghia, J. E., & Khafipour, E. (2016). Carrageenan Gum and Adherent Invasive Escherichia coli in a Piglet Model of Inflammatory Bowel Disease: Impact on Intestinal Mucosa-associated Microbiota. *Frontiers in Microbiology, 7*, 462. doi:10.3389/fmicb.2016.00462

Mylonaki, M., Rayment, N. B., Rampton, D. S., Hudspith, B. N., & Brostoff, J. (2005). Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflammatory Bowel Diseases, 11*(5), 481-487.

Nagalingam, N. A., & Lynch, S. V. (2012). Role of the microbiota in inflammatory bowel diseases. *Inflammatory Bowel Diseases, 18*(5), 968-984. doi:10.1002/ibd.21866

Nahidi, L., Leach, S. T., Sidler, M. A., Levin, A., Lemberg, D. A., & Day, A. S. (2011). Osteoprotegerin in pediatric Crohn's disease and the effects of exclusive enteral nutrition. *Inflammatory Bowel Diseases, 17*(2), 516-523. doi:10.1002/ibd.21361

Nakamura, K., Sakuragi, N., Takakuwa, A., & Ayabe, T. (2016). Paneth cell alpha-defensins and enteric microbiota in health and disease. *Biosci Microbiota Food Health, 35*(2), 57-67. doi:10.12938/bmfh.2015-019

Narula, N., & Marshall, J. K. (2012). Management of inflammatory bowel disease with vitamin D: beyond bone health. *J Crohns Colitis, 6*(4), 397-404. doi:10.1016/j.crohns.2011.10.015

Nemati, S., & Teimourian, S. (2017). An Overview of Inflammatory Bowel Disease: General Consideration and Genetic Screening Approach in Diagnosis of Early Onset Subsets. *Middle East J Dig Dis, 9*(2), 69-80. doi:10.15171/mejdd.2017.54

Neuprez, A., Rompen, E., Crielaard, J. M., & Reginster, J. Y. (2014). Teriparatide therapy for denosumab-induced osteonecrosis of the jaw in a male osteoporotic patient. *Calcified Tissue International, 95*(1), 94-96. doi:10.1007/s00223-014-9858-3

Neurath, M. (2008). IL-12 family members in experimental colitis. *Mucosal Immunology, 1 Suppl 1*, S28-30. doi:10.1038/mi.2008.45

Neurath, M., Fuss, I., & Strober, W. (2000). TNBS-colitis. *International Reviews of Immunology, 19*(1), 51-62.

Neurath, M. F. (2014). Cytokines in inflammatory bowel disease. *Nature Reviews: Immunology, 14*(5), 329-342. doi:10.1038/nri3661

Neurath, M. F. (2017). Current and emerging therapeutic targets for IBD. *Nature Reviews: Gastroenterology & Hepatology, 14*(5), 269-278. doi:10.1038/nrgastro.2016.208

Nguyen, D., & Xu, T. (2008). The expanding role of mouse genetics for understanding human biology and disease. *Disease Models & Mechanisms, 1*(1), 56-66. doi:10.1242/dmm.000232

O'Connor, W., Jr., Kamanaka, M., Booth, C. J., Town, T., Nakae, S., Iwakura, Y., Flavell, R. A. (2009). A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nature Immunology, 10*(6), 603-609. doi:10.1038/ni.1736

Oberc, A., & Coombes, B. K. (2015). Convergence of External Crohn's Disease Risk Factors on Intestinal Bacteria. *Frontiers in Immunology, 6*, 558. doi:10.3389/fimmu.2015.00558

Oehlers, S. H., Flores, M. V., Hall, C. J., Swift, S., Crosier, K. E., & Crosier, P. S. (2011). The inflammatory bowel disease (IBD) susceptibility genes NOD1 and NOD2 have conserved anti-bacterial roles in zebrafish. *Disease Models & Mechanisms, 4*(6), 832-841. doi:10.1242/dmm.006122

Oksanen, R. K., Pierre Legendre, Bob O'Hara, M. Henry H. Stevens. (2007). Community Ecology Package.

Olaison, G., Sjodahl, R., & Tagesson, C. (1990). Glucocorticoid treatment in ileal Crohn's disease: relief of symptoms but not of endoscopically viewed inflammation. *Gut, 31*(3), 325-328.

Olate, S., Uribe, F., Martinez, F., Almeida, A., & Unibazo, A. (2014). Osteonecrosis of the jaw in patient with denosumab therapy. *International Journal of Clinical and Experimental Medicine, 7*(10), 3707-3709.

Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Folsch, U. R., Schreiber, S. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut, 53*(5), 685-693.

Park, J. H., Peyrin-Biroulet, L., Eisenhut, M., & Shin, J. I. (2017). IBD immunopathogenesis: A comprehensive review of inflammatory molecules. *Autoimmun Rev, 16*(4), 416-426. doi:10.1016/j.autrev.2017.02.013

Parks, D. H., Tyson, G. W., Hugenholtz, P., & Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics, 30*(21), 3123-3124. doi:10.1093/bioinformatics/btu494

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-

activated protein kinase in Caco-2 cell monolayers. *Journal of Nutrition, 139*(9), 1619-1625. doi:10.3945/jn.109.104638

Perse, M., & Cerar, A. (2012). Dextran sodium sulphate colitis mouse model: traps and tricks. *J Biomed Biotechnol, 2012*, 718617. doi:10.1155/2012/718617

Peterson, D. A., Frank, D. N., Pace, N. R., & Gordon, J. I. (2008). Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. *Cell Host Microbe, 3*(6), 417-427. doi:10.1016/j.chom.2008.05.001

Pichler, J., Hanslik, A., Huber, W. D., Aufricht, C., & Bidmon-Fliegenschnee, B. (2014). Paediatric patients with inflammatory bowel disease who received infliximab experienced improved growth and bone health. *Acta Paediatrica, 103*(2), e69-75. doi:10.1111/apa.12448

Pizarro, T. T., Michie, M. H., Bentz, M., Woraratanadharm, J., Smith, M. F., Jr., Foley, E., Cominelli, F. (1999). IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *Journal of Immunology, 162*(11), 6829-6835.

Pouillart, P. R., Depeint, F., Abdelnour, A., Deremaux, L., Vincent, O., Maziere, J. C., Dupas, J. L. (2010). Nutriose, a prebiotic low-digestible carbohydrate, stimulates gut mucosal immunity and prevents TNBS-induced colitis in piglets. *Inflammatory Bowel Diseases, 16*(5), 783-794. doi:10.1002/ibd.21130

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2--approximately maximum-likelihood trees for large alignments. *PloS One, 5*(3), e9490. doi:10.1371/journal.pone.0009490

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature, 464*(7285), 59-65. doi:10.1038/nature08821

Rabbi, M. F., Munyaka, P. M., Eissa, N., Metz-Boutigue, M. H., Khafipour, E., & Ghia, J. E. (2016). Human Catestatin Alters Gut Microbiota Composition in Mice. *Frontiers in Microbiology, 7*, 2151. doi:10.3389/fmicb.2016.02151

Rahimi, R., Nikfar, S., & Abdollahi, M. (2007). Meta-analysis technique confirms the effectiveness of anti-TNF-alpha in the management of active ulcerative colitis when administered in combination with corticosteroids. *Medical Science Monitor, 13*(7), PI13-18.

Rapozo, D. C., Bernardazzi, C., & de Souza, H. S. (2017). Diet and microbiota in inflammatory bowel disease: The gut in disharmony. *World Journal of Gastroenterology, 23*(12), 2124-2140. doi:10.3748/wjg.v23.i12.2124

Rath, H. C., Schultz, M., Freitag, R., Dieleman, L. A., Li, F., Linde, H. J., Sartor, R. B. (2001). Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infection and Immunity, 69*(4), 2277-2285. doi:10.1128/IAI.69.4.2277-2285.2001

Reinshagen, M. (2008). Osteoporosis in inflammatory bowel disease. *J Crohns Colitis, 2*(3), 202-207. doi:10.1016/j.crohns.2008.01.005

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Caporaso, J. G. (2014). Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ, 2*, e545. doi:10.7717/peerj.545

Robinson, A. M., Sakkal, S., Park, A., Jovanovska, V., Payne, N., Carbone, S. E., Nurgali, K. (2014). Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in guinea pig TNBS-induced colitis. *American Journal of Physiology: Gastrointestinal and Liver Physiology, 307*(11), G1115-1129. doi:10.1152/ajpgi.00174.2014

Roda, G., Sartini, A., Zambon, E., Calafiore, A., Marocchi, M., Caponi, A., Roda, E. (2010). Intestinal epithelial cells in inflammatory bowel diseases. *World Journal of Gastroenterology, 16*(34), 4264-4271.

Rodriguez de Santiago, E., Albillos Martinez, A., & Lopez-Sanroman, A. (2017). Infections in inflammatory bowel disease. *Medicina Clínica, 148*(9), 415-423. doi:10.1016/j.medcli.2016.12.041

Rogler, G. (2017). Resolution of inflammation in inflammatory bowel disease. *Lancet Gastroenterol Hepatol, 2*(7), 521-530. doi:10.1016/S2468-1253(17)30031-6

Roshandel, D., Holliday, K. L., Pye, S. R., Boonen, S., Borghs, H., Vanderschueren, D., Group, E. S. (2010). Genetic variation in the RANKL/RANK/OPG signaling pathway is associated with bone turnover and bone mineral density in men. *Journal of Bone and Mineral Research, 25*(8), 1830-1838. doi:10.1002/jbmr.78

Russell, S. L., Gold, M. J., Hartmann, M., Willing, B. P., Thorson, L., Wlodarska, M., Finlay, B. B. (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep, 13*(5), 440-447. doi:10.1038/embor.2012.32

Saad, F., Brown, J. E., Van Poznak, C., Ibrahim, T., Stemmer, S. M., Stopeck, A. T., Dansey, R. (2012). Incidence, risk factors, and outcomes of osteonecrosis of the jaw: integrated

analysis from three blinded active-controlled phase III trials in cancer patients with bone metastases. *Annals of Oncology, 23*(5), 1341-1347. doi:10.1093/annonc/mdr435

Saleiro, D., Murillo, G., Benya, R. V., Bissonnette, M., Hart, J., & Mehta, R. G. (2012). Estrogen receptor-beta protects against colitis-associated neoplasia in mice. *International Journal of Cancer, 131*(11), 2553-2561. doi:10.1002/ijc.27578

Samanta, A. K., Torok, V. A., Percy, N. J., Abimosleh, S. M., & Howarth, G. S. (2012). Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. *Journal of Microbiology, 50*(2), 218-225. doi:10.1007/s12275-012-1362-8

Sandborn, W. J., Ghosh, S., Panes, J., Vranic, I., Su, C., Rousell, S., Study, A. I. (2012). Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *New England Journal of Medicine, 367*(7), 616-624. doi:10.1056/NEJMoa1112168

Sands, B. E., & Grabert, S. (2009). Epidemiology of inflammatory bowel disease and overview of pathogenesis. *Medicine and Health, Rhode Island, 92*(3), 73-77.

Sanseau, P., Agarwal, P., Barnes, M. R., Pastinen, T., Richards, J. B., Cardon, L. R., & Mooser, V. (2012). Use of genome-wide association studies for drug repositioning. *Nature Biotechnology, 30*(4), 317-320. doi:10.1038/nbt.2151

Sartor, R. B. (2004). Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology, 126*(6), 1620-1633.

Schule, S., Rossel, J. B., Frey, D., Biedermann, L., Scharl, M., Zeitz, J., Swiss, I. B. D. c. s. (2016). Prediction of low bone mineral density in patients with inflammatory bowel

diseases. *United European Gastroenterol J, 4*(5), 669-676. doi:10.1177/2050640616658224

Schwab, C., Berry, D., Rauch, I., Rennisch, I., Ramesmayer, J., Hainzl, E., Urich, T. (2014). Longitudinal study of murine microbiota activity and interactions with the host during acute inflammation and recovery. *ISME J, 8*(5), 1101-1114. doi:10.1038/ismej.2013.223

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology, 12*(6), R60. doi:10.1186/gb-2011-12-6-r60

Seifarth, C., Borner, L., Siegmund, B., Buhr, H. J., Ritz, J. P., & Grone, J. (2017). Impact of staged surgery on quality of life in refractory ulcerative colitis. *Surgical Endoscopy, 31*(2), 643-649. doi:10.1007/s00464-016-5010-y

Seksik, P., Rigottier-Gois, L., Gramet, G., Sutren, M., Pochart, P., Marteau, P., Dore, J. (2003). Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut, 52*(2), 237-242.

Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology, 14*(8), e1002533. doi:10.1371/journal.pbio.1002533

Sepehri, S., Kotlowski, R., Bernstein, C. N., & Krause, D. O. (2007). Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflammatory Bowel Diseases, 13*(6), 675-683. doi:10.1002/ibd.20101

Siakavellas, S. I., & Bamias, G. (2012). Role of the IL-23/IL-17 axis in Crohn's disease. *Discovery Medicine, 14*(77), 253-262.

Smith, M. R., Saad, F., Coleman, R., Shore, N., Fizazi, K., Tombal, B., Goessl, C. (2012). Denosumab and bone-metastasis-free survival in men with castration-resistant prostate cancer: results of a phase 3, randomised, placebo-controlled trial. *The Lancet, 379*(9810), 39-46. doi:10.1016/s0140-6736(11)61226-9

Sokol, H., Leducq, V., Aschard, H., Pham, H. P., Jegou, S., Landman, C., Beaugerie, L. (2016). Fungal microbiota dysbiosis in IBD. *Gut*. doi:10.1136/gutjnl-2015-310746

Sollid, L. M., & Johansen, F. E. (2008). Animal models of inflammatory bowel disease at the dawn of the new genetics era. *PLoS Medicine, 5*(9), e198. doi:10.1371/journal.pmed.0050198

Stanker, L. H., & Hnasko, R. M. (2015). A Double-Sandwich ELISA for Identification of Monoclonal Antibodies Suitable for Sandwich Immunoassays. *Methods in Molecular Biology, 1318*, 69-78. doi:10.1007/978-1-4939-2742-5_7

Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M. J., Pickard, D. J., Hardt, W. D. (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences of the United States of America, 109*(4), 1269-1274. doi:10.1073/pnas.1113246109

Stolina, M., Dwyer, D., Ominsky, M. S., Corbin, T., Van, G., Bolon, B., Kostenuik, P. (2007). Continuous RANKL Inhibition in Osteoprotegerin Transgenic Mice and Rats Suppresses Bone Resorption without Impairing Lymphorganogenesis or Functional Immune Responses. *The Journal of Immunology, 179*(11), 7497-7505. doi:10.4049/jimmunol.179.11.7497

Strober, W., Fuss, I., & Mannon, P. (2007). The fundamental basis of inflammatory bowel disease. *Journal of Clinical Investigation, 117*(3), 514-521. doi:10.1172/JCI30587

Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L. P., & Lochs, H. (2005). Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *Journal of Clinical Microbiology, 43*(7), 3380-3389. doi:10.1128/JCM.43.7.3380-3389.2005

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews, 81*(3), 1031-1064.

Tremelling, M., & Parkes, M. (2007). Genome-wide association scans identify multiple confirmed susceptibility loci for Crohn's disease: lessons for study design. *Inflammatory Bowel Diseases, 13*(12), 1554-1560. doi:10.1002/ibd.20239

Vasquez, A. (2014). Reply to "role of Western diet in inflammatory autoimmune diseases" by Manzel et al. In current allergy and asthma reports (volume 14, issue 1, January 2014). *Current Allergy and Asthma Reports, 14*(8), 454. doi:10.1007/s11882-014-0454-4

Vogt, S. L., Pena-Diaz, J., & Finlay, B. B. (2015). Chemical communication in the gut: Effects of microbiota-generated metabolites on gastrointestinal bacterial pathogens. *Anaerobe, 34*, 106-115. doi:10.1016/j.anaerobe.2015.05.002

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261-5267. doi:10.1128/AEM.00062-07

Wang, R., Zhang, L., Zhang, X., Moreno, J., Luo, X., Tondravi, M., & Shi, Y. (2001). Differential regulation of the expression of CD95 ligand, receptor activator of nuclear factor-kappa B ligand (RANKL), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-alpha during T cell activation. *Journal of Immunology, 166*(3), 1983-1990.

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Lander, E. S. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature, 420*(6915), 520-562. doi:10.1038/nature01262

Wei, T. (2016). Visualization of a Correlation Matrix.

West, G. A., Matsuura, T., Levine, A. D., Klein, J. S., & Fiocchi, C. (1996). Interleukin 4 in inflammatory bowel disease and mucosal immune reactivity. *Gastroenterology, 110*(6), 1683-1695.

Wills, E. S., Jonkers, D. M., Savelkoul, P. H., Masclee, A. A., Pierik, M. J., & Penders, J. (2014). Fecal microbial composition of ulcerative colitis and Crohn's disease patients in remission and subsequent exacerbation. *PloS One, 9*(3), e90981. doi:10.1371/journal.pone.0090981

Wirtz, S., Neufert, C., Weigmann, B., & Neurath, M. F. (2007). Chemically induced mouse models of intestinal inflammation. *Nature Protocols, 2*(3), 541-546. doi:10.1038/nprot.2007.41

Wirtz, S., Popp, V., Kindermann, M., Gerlach, K., Weigmann, B., Fichtner-Feigl, S., & Neurath, M. F. (2017). Chemically induced mouse models of acute and chronic intestinal inflammation. *Nature Protocols, 12*(7), 1295-1309. doi:10.1038/nprot.2017.044

Wu, Y., Yang, M., Fan, J., Peng, Y., Deng, L., Ding, Y., Fu, Q. (2014). Deficiency of osteoblastic Arl6ip5 impaired osteoblast differentiation and enhanced osteoclastogenesis via disturbance of ER calcium homeostasis and induction of ER stress-mediated apoptosis. *Cell Death & Disease, 5*, e1464. doi:10.1038/cddis.2014.427

Xavier, R. J., & Podolsky, D. K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. *Nature, 448*(7152), 427-434. doi:10.1038/nature06005

Xenoulis, P. G., Palculict, B., Allenspach, K., Steiner, J. M., Van House, A. M., & Suchodolski, J. S. (2008). Molecular-phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. *FEMS Microbiology Ecology, 66*(3), 579-589. doi:10.1111/j.1574-6941.2008.00556.x

Yan, X., Sun, M., & Gibb, W. (2002). Localization of nuclear factor-kappa B (NF kappa B) and inhibitory factor-kappa B (I kappa B) in human fetal membranes and decidua at term and preterm delivery. *Placenta, 23*(4), 288-293. doi:10.1053/plac.2002.0789

Yang, X. O., Chang, S. H., Park, H., Nurieva, R., Shah, B., Acero, L., Dong, C. (2008). Regulation of inflammatory responses by IL-17F. *Journal of Experimental Medicine, 205*(5), 1063-1075. doi:10.1084/jem.20071978

Young-Yun Kong, H. Y., Ildiko Sarosi, Hong-Lin Tan, Emma Timms, Casey Capparelli,, & Sean Morony, A. J. O.-d.-S., Gwyneth Van, Annick Itie, Wilson Khoo, Andrew Wakeham, Colin R. Dunstan, David L. Lacey, Tak W. Mak, William J. Boyle & Josef M. Penninger. (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *NATURE 397, 397*, 315-323 doi:10.1038/16852

Yun, T. J., Tallquist, M. D., Aicher, A., Rafferty, K. L., Marshall, A. J., Moon, J. J., Clark, E. A. (2001). Osteoprotegerin, a Crucial Regulator of Bone Metabolism, Also Regulates B Cell Development and Function. *The Journal of Immunology, 166*(3), 1482-1491. doi:10.4049/jimmunol.166.3.1482

Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M., Murphy, A. J., Stevens, S., & Flavell, R. A. (2008). Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity, 29*(6), 947-957. doi:10.1016/j.immuni.2008.11.003

Zhang, Y. Z., & Li, Y. Y. (2014). Inflammatory bowel disease: pathogenesis. *World Journal of Gastroenterology, 20*(1), 91-99. doi:10.3748/wjg.v20.i1.91