Gut microbiota profile and immune responses in piglet and mice models of inflammatory bowel disease: The impact of antepartum use of antibiotics, prebiotics and probiotics.

by

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ABSTRACT

A major challenge facing scientists is to find a full understanding of the factors behind inflammatory bowel disease' [IBD; including ulcerative colitis (UC) and Crohn's disease (CD)] pathogenesis and optimal treatment. This is demonstrated by the fact that despite many years of research, IBD is still an idiopathic disease whose cure is far from reach. IBD is currently recognized as a global burden and could be due to genetic, environmental, microbiota or immune-related factors. The gut microbiota co-evolve with the host and therefore play an important role in the development and maturation of the immune system. Hence, changes in the composition, distribution and functional activities of the gut microbiota might predispose to IBD. In five studies, the role of adherent invasive *Escherichia coli (*AIEC) strain UM146 and antepartum antibiotics on gut microbiota dysbiosis and susceptibility to colitis, and therapeutic impact of prebiotics (resistant starch; RS) and probiotics (*E. coli* UM 2 & 7), in mice and pig models of colitis were investigated. Weaner pigs received 1 % degraded carrageenan gum (CG) for 21 days and were inoculated with AIEC on day 8. CG-induced colitis caused bacterial dysbiosis similar to observations in IBD patients; however, AIEC did not show clear impacts. In the second study, weaner pigs received 1 % CG for 21 days and RS and the *E. coli* probiotics therapies were administered from day 8 until end of the study. Inclusion of RS and *E. coli* probiotics showed minimal effects in modulation of bacterial dysbiosis and inflammation. The third study investigated protective and therapeutic effects of RS in a 40-day experiment. Inclusion of RS modulated inflammation, histological damage and bacterial dysbiosis. The fourth study investigated impact of 5 % dextran sodium sulphate (DSS)-induced colitis in mice for 5 days; DSS caused inflammation and bacterial dysbiosis. In the last study,

pregnant mice were injected with antibiotic cefazolin (160 mg/kg bw) 6 days before due date and the offspring were exposed to 4 % DSS-induced colitis at 7 weeks of age. Antepartum antibiotics influenced offspring intestinal bacterial colonization predisposing them to colitis later in life. Overall, this work demonstrates the critical role of DSS/CG-induced colitis and antepartum antibiotics in disrupting existing or initial composition of gut microbiota, and the role this disruption might play in the development of IBD. Also, the work highlights significant role of RS in IBD treatment and hold implications and potential for advancement in the management of IBD.

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Glory and Honour to God for the great things He has done for me!

DEDICATION

This thesis is dedicated to my dear mother, Beatrice Mweela Munyaka of blessed memory and to my dad Mr. Francis Munyaka Kang'esa.

FOREWORD

This thesis was written following a manuscript format and is composed of five manuscripts. Part of the literature review (section 2.6.2.7 to 2.6.2.7.5 of this thesis) is also published as a review paper in Frontiers in Pediatricts. 2014. 2:109. All manuscripts have been formatted based on the Frontiers in Microbiology Journal. The titles of these manuscripts and list of contributing authors are as follows:

Manuscript 1: Peris M Munyaka, Shadi Sepehri, Ehsan Khafipour and Jean-Eric Ghia. 2016. Degraded carrageenan gum and adherent invasive *Escherichia coli* in a piglet model of experimental inflammatory bowel disease: impact on intestinal mucosa-associated microbiota. Frontiers in Microbiology.7:462.

Manuscript 2: Peris M Munyaka, Jean-Eric Ghia and Ehsan Khafipour. Mucosa associated microbiota dysbiosis in a pig model of experimental colitis: impact of prebiotics (resistant starch) and *Escherichia coli* (UM2 and UM7) probiotics. Target journal: Beneficial microbes.

Manuscript 3: Peris M Munyaka, Jean-Eric Ghia and Ehsan Khafipour. Protective and therapeutic use of resistant starch (MSPrebiotic) modulates gut microbiota profile, histological structure and local gene expression in a pig model of experimental colitis. Target journal: PLoS One.

Manuscript 4: Peris M Munyaka, Mohammad Fazle Rabbi, Ehsan Khafipour and Jean-Eric Ghia. 2016. Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbiota dysbiosis in mice. Journal of Basic Microbiology. 56:1-13.

Manuscript 5: Peris M Munyaka, Nour Eissa, Charles Noah Bernstein, Ehsan Khafipour and Jean-Eric Ghia. 2015. Antepartum antibiotic treatment increases offspring susceptibility to experimental colitis: a role of the gut microbiota. PLoS One. 10(11):e0142536.

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LIST OF ABBREVIATIONS

ACE	Abundance-based coverage estimator
AIEC	Adherent invasive *Escherichia coli*
AOM	Azoxymethane
APC	Antigen presenting cell
ATB	Antepartum antibiotics
BW	Body weight
CCAC	Canadian council on animal care
CD	Crohn's disease
CG	degraded carrageenan gum
CRP	C-reactive protein
d	Day
DC	Dendritic cells
DIA	Disease index activity
DNA	Deoxyribonucleic acid
DNBS	Dinitrobenzene sulfonic acid
dsDNA	Double stranded deoxyribonucleic acid
DSS	Dextran sulphate sodium
ELISA	Enzyme linked immunosorbent assay
FC	Fecal consistency
FISH	Fluorescent in-situ hybridization
FMT	Fecal microbiota transplantation
FOS	Fructo-oligosaccharides

Foxp3	Forkhead box p3
FS	Fecal score
GBS	Group B streptococci
GI	Gastrointestinal
GIT	Gastrointestinal tract
GWAS	Genome wide associated studies
h	Hour
HPLC	High quality reagents and chemicals
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN	Interferon
IL	Interleukin
KEGG	Kyoto encyclopedia of genes and genomes
Lefse	Linear discriminant analysis with effect size
MAM	Mucosa-associated microbiota
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
N	Nitrogen
NCBI	National center for biotechnology information
NEC	Necrotizing enterocolitis
nMDS	Non-metric multidimensional scaling
NRC	National research council
NSAID	Nonsteroidal anti-inflammatory drugs
NSTI	Nearest sequence taxon index

OTU	Operational taxonomic units
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
PMN	Polymorphonuclear
QIIME	Quantitative insights into microbial ecology
qRT-PCR	Quantitative real time polymerase chain reaction
ROM	Reactive oxygen metabolite
rRNA	Ribosomal ribonucleic acid
RS	Resistant starch
RT-PCR	Reverse transcriptase polymerase chain reaction
SAS	Statistical analysis system
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficiency
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
T-RFLP	Terminal restriction fragment length polymorphism
T1D	Type 1 diabetes
TGF	Transforming growth factor

Th	T helper
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
UC	Ulcerative colitis
VFA	Volatile fatty acids
VIP	Variable influence on projection

LIST OF COPYRIGHTED MATERIALS FOR WHICH PERMISSION WAS OBTAINED

1. Chapter 6, Manuscript IV: Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice.

NB: Chapter 2 section 2.6.2.7 to 2.6.2.7.5, and chapters 3 and 7, are published under the creative commons license in which case no permission is required to re-use the material.

CHAPTER ONE

GENERAL INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the small and large intestines encompassing two main clinical entities: Crohn's disease (CD) and ulcerative (UC) colitis (Strober et al., 2007; Cosnes et al., 2011; Manichanh et al., 2012). Crohn's disease affects the small bowel and colon with discontinuous ulceration and full thickness bowel wall inflammation often including granulomas. Patients with Crohn's disease can also develop obstructing strictures of the bowel and inflammatory connections (fistulae) between segments of bowel or between the bowel and skin and other organs (Fuss, 2004). In comparison, ulcerative colitis primarily affects the colon, with a continuous inflammation of the mucosa nearly always involving the rectum and extending proximally (Fuss, 2004). The symptoms are similar to Crohn's disease, although fistula development does not occur (Abraham and Cho, 2009). Usually both conditions are chronic and relapsing, however, ulcerative colitis may be curable by surgical removal of the colon whereas surgery for Crohn's disease treats bowel blockage, fistula complications, and intractable bleeding and pain, but is not used for cure (McLeod, 2003).

Both CD and UC are associated with multiple pathogenic factors including environmental changes, an array of susceptibility gene variants, a qualitatively and quantitatively abnormal gut microbiota and a broadly dysregulated immune response (Strober et al., 2007; Nagalingam et al., 2011; Manichanh et al., 2012; Molodecky et al., 2012). However, in spite of this realization and the identification of seemingly pertinent environmental, genetic, microbial and immune factors, a full understanding of IBD pathogenesis is still out of reach and, consequently, treatment is far from optimal. Therefore, more research is needed to further

investigate the pathogenic factors of IBD and to find alternative treatment measures. Indeed, with respect to the involvement of gut microbiota in the pathogenesis of IBD, bacterial dysbiosis characterized by disruption of microbial equilibrium in the gut has been pointed out (Seksik et al., 2005; Seksik et al., 2006; Prideaux et al., 2013; Thorkildsen et al., 2013; de Souza and Fiocchi, 2016). This dysbiosis could result from various intrinsic or extrinsic factors that might influence the existing gut microbiota composition, or the nature of initial microbial colonization and subsequent succession such as: maternal use of antibiotics, antibiotic use at different stages of life, stress, mode of delivery, infections/ infectious agents, diet and other lifestyle practices (Dominguez-Bello et al., 2010; Agus et al., 2014a; Munyaka et al., 2014). In the context of infectious agents, increased abundance of members of the Enterobacteriaceae family, especially *Escherichia coli*, have been reported in humans and dogs with IBD and also in experimental animal models of IBD (Darfeuille-Michaud et al., 1998; Sellon et al., 1998; Darfeuille-Michaud et al., 2004; Mylonaki et al., 2005; Kotlowski et al., 2007; Lupp et al., 2007; Sepehri et al., 2007; Xenoulis et al., 2008; Sepehri et al., 2011; Wright et al., 2015). Adherent invasive *E. coli* (AIEC), a member of Enterobacteriaceae family has been associated with Crohn's disease, and some strains of AIEC including LF82 and UM146 have been isolated from IBD patients and characterized (Miquel et al., 2010; Krause et al., 2011; Sepehri et al., 2011; Desilets et al., 2015). Strain UM146 was previously isolated in our lab from a CD patient and shown to be able to invade and replicate within macrophages, which is characteristic of an AIEC (Krause et al., 2011; Sepehri et al., 2011). However, despite the repeated association of the AIEC pathotype with the intestinal mucosa of patients with CD (Darfeuille-Michaud et al., 1998; Darfeuille-Michaud et al., 2004; Agus et al., 2014b), it is still difficult to pinpoint whether AIEC triggers intestinal inflammation leading to the disease, or if they colonize the gut mucosa as a consequence of pre-existing

inflammation (Martin et al., 2004; Agus et al., 2014b). Therefore, a cause and consequence relationship between *E. coli* strains and IBD is yet to be determined.

Early life events especially the antepartum use of antibiotics, a common practice in clinical settings in North America (Anonymous, 2010; Persaud et al., 2014b; Azad et al., 2015) and possibly in other parts of the world, could be a potential cause of dysbiosis to the unborn, a condition that might predispose the offspring to IBD conditions later in life (Munyaka et al., 2014; Munyaka et al., 2015). In this case, although antepartum antibiotics are short-term, they are given at a critical time when newborn acquisition of gut microbiota, which is also known to influence the initial immune system development, is just beginning. Thus, antepartum antibiotic exposure may have far-reaching implications on maturation of neonatal immune system (Noverr and Huffnagle, 2005; Renz et al., 2012). In this regard, increased incidences of atopic diseases, irritable bowel syndrome (de Silva-Sanigorski et al., 2010), and inflammatory bowel disease (IBD) have all been reported in children exposed to antibiotics (Droste et al., 2000; Shaw et al., 2010; Roberts et al., 2011; Villarreal et al., 2012; Metsälä et al., 2013; Russell et al., 2013; Stensballe et al., 2013). However, despite the high association between perinatal or neonatal antibiotic use on the microbial colonization and future risk for atopic diseases/reactions and other disease conditions, the impact of antepartum antibiotic use on the process of intestinal microbiota development and future susceptibility to IBD remain elusive or poorly understood.

Current medical treatment of IBD involves use of antibiotics and compounds with immunomodulation or anti-inflammatory properties (Bernstein, 2015; Chandel et al., 2015; Shahidi et al., 2016); however, not all patients respond well to these therapies and IBD continues to cause significant morbidity. This has led to research into alternative therapeutic

measures including use of different types of probiotics, prebiotics and or synbiotics, but the results are inconsistent or inconclusive, hence, there is need for further investigations (Steed et al., 2008; Sinagra et al., 2013; Ghouri et al., 2014; Bernstein, 2015; Peterson et al., 2015; Wasilewski et al., 2015).

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014); whereas prebiotics represents a diverse range of indigestible dietary carbohydrates that are not digested and absorbed in the upper digestive tract and, so, pass into the large bowel where they undergo microbial fermentation (Higgins and Brown, 2013; Giuberti et al., 2015). Synbiotics are products that contain both probiotics and prebiotics, and evidence suggests that prebiotics can selectively stimulate growth of beneficial gut bacteria, for example *Lactobacillus* and *Bifidobacterium* (Patel and DuPont, 2015), and also act as substrates for probiotics (Amit, 2007).

Given the complexity of IBD, the use of animal models is an important tool to study the involvement of various factors in the pathogenesis of IBD and, importantly, to test new therapeutic options, the interactions of different therapeutic measures with the gut microbiota, and consequently with the immune system, and how these might influence the course of IBD. Therefore, a number of animal species have been used and are still widely utilized for research in IBD including the primates, zebra fish, mice, rat, guinea pigs and domestic pigs. Primates are thought to be more closer to humans, and hence, more ideal for use as human models; however, due to restrictions and constraints associated with their use, rodents and pigs are the animals used most commonly in IBD research. The pig is generally accepted to be closer to humans than other laboratory or domestic animals in terms of gastrointestinal anatomy, physiology, nutrition, and intestinal microbial ecology (Graham and Åman, 1987; Miller and

Ullrey, 1987; Rowan et al., 1994; Topping and Clifton, 2001; Heinritz et al., 2013; Gonzalez et al., 2015; Wang and Donovan, 2015). On another note, mice and or rats are commonly used models since even though they are not so close to humans, they are easy to handle and genetically manipulate in order to mimic different aspects related to IBD.

Chemically induced models of intestinal inflammation are among the most commonly used animal models of IBD as the onset of inflammation is immediate and the procedure is relatively straightforward. Even though they have limitations like all other models, they present some important immunological and histopathological aspects of IBD in humans (Solomon et al., 2010). The most widely used and characterized experimental model of erosion and inflammation related to UC in mice is the dextran sulphate sodium (DSS)-induced colitis, which is developed by DSS administration either in the drinking water (Solomon et al., 2010; Chassaing et al., 2014a) or via intragastric catheter (Lee et al., 2009b; Kim et al., 2010). Treatment with dextran sulfate sodium triggers development of colitis in mice and rats by binding to medium-chain-length fatty acids present in the colon and inducing inflammation (Ghia et al., 2006; Ghia et al., 2007a; Ghia et al., 2007b; Ghia et al., 2009; Laroui et al., 2012; Rabbi et al., 2014). However, given the widespread use of DSS, not many studies have so far investigated the compositional shifts in gut microbiota and changes in their metabolic capacity in relation to DSS treatment in mice or rat models (Nagalingam et al., 2011; Berry et al., 2012; Samanta et al., 2012; Smith et al., 2012; De Fazio et al., 2014; Liang et al., 2014; Mar et al., 2014; Schwab et al., 2014; Hakansson et al., 2015). Another commonly used chemical model although less characterized is degraded carrageenan gum (CG). Carrageenan gum is a high molecular weight sulphated polysaccharide derived from red seaweeds, and its degraded forms can induce intestinal inflammation, and have been widely used to model colitis in several

species including mice, rats, rabbits, and guinea pigs (Marcus and Watt, 1971; Kitano et al., 1986; Marcus et al., 1989; Tobacman, 2001b; Benard et al., 2010). However, the focus of these studies has been on the dynamics and profile of mucosal response in relation to CG-induced colitis and its similarity to IBD patients. Therefore, there is no detailed and clear understanding of the structural and functional alterations of the intestinal microbiota in response to CG-induced colitis.

It was therefore hypothesized that: 1) Degraded carrageenan gum (CG) and dextran sodium sulphate (DSS) models of colitis induce gut microbiota dysbiosis comparable to microbial changes observed in UC patients; 2) Adherent invasive *E. coli (*AIEC) strain UM146 can cause or exacerbate bacterial dysbiosis and inflammation in a pig model of experimental colitis; 3) Antepartum use of antibiotics could influence the nature of offspring gut microbiaota colonization, predisposing them to colitis later in life; 4) Prebiotics, probiotics and or synbiotics can attenuate gut microbial dysbiosis and inflammation in experimental models of colitis. The main aim of the research described in this thesis was to investigate the role of adherent invasive *E. coli (*AIEC) strain UM146, and antepartum use of antibiotics on gut microbiota profile and immune responses, and therapeutic roles of prebiotics and probiotics, in mice and pig models of experimental colitis. Specific objectives were:

a) To investigate the impact of degraded carrageenan gum (CG) on gut microbiota profile, and role of AIEC strain UM146 in a pig model of experimental colitis.
b) To investigate therapeutic role of Probiotics (*E. coli* strains UM2 and UM7) and prebiotics (resistant starch) in a pig model of experimental colitis.
c) To elucidate the impact of DSS-induced colitis on gut microbiota profile in mice model of experimental colitis.

d) To determine the impact of antepartum use of antibiotics on offspring gut microbial profile and future susceptibility to experimental colitis.

CHAPTER TWO

LITERATURE REVIEW

2.1. Overview of inflammation

The inflammatory response consists of an innate system of cellular and humoral responses following injury in which the body attempts to restore the tissue to its pre-injury state (Ward, 2010). Inflammation is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines (Feghali and Wright, 1997). The acute inflammatory response consists of release of water, salt, and proteins from the vascular compartment; activation of endothelial cells; adhesion between the leukocytes and the vascular endothelium; recruitment of leukocytes; activation of tissue macrophages; activation and aggregation of platelets; complement activation; clotting and fibronolytic systems; and release of proteases and oxidants from phagocytic cells (Ward, 2010). The acute phase response is marked by changes in the concentrations of acute phase proteins, whose concentrations increases (positive acute phase proteins) or decreases (negative acute phase proteins) by at least 25% in inflammatory disorders, as well as behavioral, psychological, nutritional, and biochemical changes (Gabay and Kushner, 1999). Upon activation of the endothelial cells, pro-inflammatory cytokines and chemokines are released, which chemotactically attract and activate polymorphonuclear (PMN) leukocytes (Ward, 2010). Howevrer, although acute inflammation is a beneficial host defense mechanism protecting the body from infection and other insults, in many situations the acute inflammatory response becomes prolonged or excessive leading to serious damage of tissues and organs (Ward, 2010). Therefore, our health relies on its well functioning and the outcome of the inflammatory process is not always successful resolution and repair of damaged tissue. Chronic

inflammatory diseases develop as a result of genetic and environmental interplay, which is still only partially understood and represent a major challenge. In this context, there has been a drastic increase in the prevalence of chronic inflammatory diseases, particularly in the western world, possibly due to genetic and epigenetic factors in combination with changes in diet and lifestyle (Yach et al., 2004; Renz et al., 2011). Therefore, understanding how the highly complex and sophisticated signaling circuits are regulated in inflammation and chronic inflammatory disease is of crucial importance in order to discover and develop new therapeutic strategies.

2.2. Mediators of inflammation - overview

Crohn's disease and ulcerative colitis were considered prototypically as T helper (Th)1 or Th2-associated diseases, respectively, however, Th17 cytokines have been identified as a common player in both disease phenotypes (Monteleone et al., 2011; Strober and Fuss, 2011; Neurath, 2014). Traditionally it has been believed that the adaptive immune system is the key contributor to the pathogenesis of IBD (Xavier and Podolsky, 2007), however, research has also shown that cells mediating innate immunity are as well altered in IBD patients (Cader and Kaser, 2013), and along with proven efficacy of anti-TNF-α drugs in UC, a crucial role for defective innate immunity has been established for therapeutic interventions (Rahimi et al., 2007). Innate immune pathogenesis causes activation of the inflammatory cytokine cascade that leads to chemotaxis and reactive oxygen metabolite (ROM) cascade, which further causes oxidative stress, resulting in aggravation of the inflammation initiated tissue damage (Frink et al., 2007). Also, lamina propria dendritic cells (DCs) and macrophages are key antigen-presenting cells (APCs) that are found in the inflamed mucosa in IBD. Following activation, which occurs in response to components of the commensal microbiota and Toll-like receptor (TLR) signalling, these cells produce large amounts of proinflammatory cytokines, such as interleukin (IL)- 1β,

IL- 6, IL- 18 and tumour necrosis factor (TNF) -α (Ng et al., 2011). Hence, given the key role of several cytokines in regulating mucosal immune responses, special attention has been directed to this family of mediators.

Some of the proinflammatory cytokines most commonly involved in innate immune pathogenesis of IBD are TNF-α, interferon (IFN) -γ, IL-8, IL-6, IL1-β and IL-12. Additionally, IL-17 released from innate lymphoid cells (ILCs) has a controversial role as an effector or protector (Cader and Kaser, 2013). In this regard, some studies have shown its pathogenic role (Fujino et al., 2003) whereas others have suggested a protective role in different models of IBD (Yang et al., 2008; O'Connor et al., 2009; Eken et al., 2014). Cytokine IL-1 is produced by a wide array of cells, including macrophages, monocytes, endothelial cells and fibroblasts. It promotes T cell activation, NK cell activation, B cell proliferation and upregulates adhesion molecule expression as well as eicosanoid and nitric oxide production (Beck and Wallace, 1997). IL-2 is a key factor in the regulation of T lymphocyte proliferation, and it also influences activation of macrophages, proliferation of B lymphocytes and natural killer (NK) cells (Beck and Wallace, 1997). IL- 6 is produced by lamina propria macrophages and CD4+ T cells and its production is increased in experimental colitis and in patients with IBD (Atreya et al., 2000; Kai et al., 2005). The main actions of IL-6 include activation of T and B lymphocytes and induction of hepatic acute phase reactants (Baumann and Gauldie, 1994). However, IL- 6 may also have important homeostatic functions by stimulating the proliferation and expansion of intestinal epithelial cells (IECs). IL-8 is a potent chemotaxin for neutrophils (Sartor, 1994), whereas like IL-1, TNF-α is released from macrophages' early inflammatory response and has numerous roles including activation of macrophages, neutrophils, eosinophils and lymphocytes (Beck and Wallace, 1997). IFN-γ, another important cytokine produced by APC has an established pro-

inflammatory role in the pathogenesis of IBD and its also associated with antiproliferative and immunomodulatory activities (Sartor, 1994). Members of the IL- 12 family of heterodimeric cytokines, such as IL- 12, IL- 23, IL- 27 and IL- 35, are produced by APCs during intestinal inflammation. For instance, both DCs and macrophages showed augmented production of IL- 12 (which is composed of p35 and p40 subunits; also known as IL- 12 subunit-α and IL- 12 subunit-β, respectively) (Monteleone et al., 1997; Ng et al., 2011).

Some anti-inflammatory cytokines are involved in the regulation of inflammatory responses including IBD pathogenesis, such as IL-4, IL-10, IL-13, and TGF-β. IL-4 is primarily produced by mast cells and T lymphocytes, and it has numerous actions including the ability to down-regulate IFN-γ, IL-1 and TNF-α production, and stimulate IL-10 synthesis (Beck and Wallace, 1997). In humans, IL-10 is mainly produced by Th2 cells but it can also be produced by both Th1 cells and monocytes (Kuhn et al., 1993; Kucharzik et al., 1995; Schreiber et al., 1995) IL-10 has numerous actions, however, its main role appears to involve inhibition of macrophage and T cell function (Beck and Wallace, 1997), although there have been conflicting reports on IL-10 levels. IL-13 is a lymphocyte-derived cytokine that appears to have some immunosuppressive actions that are similar to IL-4 and IL-10 (Beck and Wallace, 1997), whereas TGF-β is a cytokine with several anti-inflammatory properties, including modulation of the functions and secretory activities of macrophages and other immunocytes and it is also a promoter of wound healing (Beck and Wallace, 1997).

2.3. Overview of inflammatory bowel disease (IBD)

2.3.1. Definition

The idiopathic IBD is a chronic inflammatory condition of the gastrointestinal tract encompassing two major entities of chronic intestinal disorders: Crohn's disease and ulcerative

colitis. Both CD and UC primarily affect the intestines, and are characterized by periods of remission and relapse (Fiocchi, 1998; 2005; Danese and Fiocchi, 2011; Baumgart and Sandborn, 2012). However, CD mostly involves the ileum and colon, but it can affect, often discontinuously, any region of the gastrointestinal tract whilst UC involves the rectum and may affect a section, or the entire colon in a continuous pattern. In addition, transmural inflammation often characterizes CD, whereas in UC the inflammation is typically limited to the mucosa, and CD can be associated with intestinal granulomas, strictures, and fistulas, but these are not typical findings in UC (Abraham and Cho, 2009).

2.3.2. Prevalence and incidence

Since the middle of the twentieth century, the incidence of UC and CD has increased in the Western world (North America, Europe, Australia and New Zealand) (Molodecky et al., 2012). However, there is also a rising increase in both the incidence and prevalence of IBD in other parts of the world (including Asia, South America and the Middle East) especially with increased industrialization, making IBD a global disease with increasing prevalence (Molodecky et al., 2012; M'Koma, 2013; Kaplan, 2015). In this context, the incidence and prevalence of IBD varies within different geographic regions.

2.3.3. Etiopathology

Pathogenesis of IBD is well known to be complex and hence, the precise cause of IBD has not yet been elucidated. However, several possible factors have been highlighted, including: host genetics, various immunological, environmental, bacterial or microbial factors, and the complicated regulatory mechanisms associated with mucosal immunity (Allez and Mayer, 2004; Nell et al., 2010; Jostins et al., 2012; Knights et al., 2013; Huttenhower et al., 2014). In this context, animal model studies suggest that inflammation in IBD patients likely occurs as a result

of either excessive effector T cell function or deficient regulatory T cell function, associated with the overproduction of pro-inflammatory cytokines, such as TNF-α, IL-12 or a deficiency in the production or function of known regulatory/immunosuppressive cytokines, such as IL-10 (Matsumoto et al., 1998; Hovhannisyan et al., 2011; Mayne and Williams, 2013; Yamada et al., 2016). In addition, a primary defect in innate immune mechanisms associated with impaired mucosal barrier function and/or bacterial clearance at the epithelial interface facilitates loss of bacterial compartmentalization and immune tolerance leading to a life-long risk for inadequate and recurrent adaptive immune activation towards luminal gut antigens and the development of chronic tissue damage (Bouma and Strober, 2003; Allez and Mayer, 2004; Sartor, 2008; Asquith and Powrie, 2010). This may consequently result in (or is accompanied by) changes in the normal composition and functions of gut microbiota, so called dysbiosis (Manichanh et al., 2006; DeGruttola et al., 2016).

2.4. Dysbiosis of gut microbiota in intestinal inflammation

Under normal physiological conditions, the gut microbiota is highly resilient to perturbations, including moderate fluctuations in response to a change in dietary patterns, and short-term application of drugs or antibiotics. Given the co-evolution of microbiota and host, the homeostatic balance of the intestinal microbiota is extremely beneficial to the host, however if there is a change in the microbial composition that causes a drastic imbalance between the beneficial and potentially pathogenic bacteria, the gut becomes vulnerable to pathogenic insult with gut microbial alterations. This imbalance in the microbial equilibrium is termed “dysbiosis”, which has been further defined as a disturbance to gut microbiota homeostasis due to an imbalance in the microbiota itself, changes in their functional composition and metabolic activities, or changes in their local distribution (Manichanh et al., 2006; Frank et al., 2007;

Willing et al., 2010; Lepage et al., 2011; Bien et al., 2013; Knights et al., 2013). In general, dysbiosis can be categorized into three different types: 1) Loss of beneficial organisms, 2) Excessive growth of potentially harmful organisms, and 3) Loss of overall microbial diversity (Gevers et al., 2014). It has been found that these three types are not mutually exclusive and can occur at the same time, which is most often the case. Dysbiosis has been shown to correlate well with disease activity in various studies and subsets of IBD patients (Gevers et al., 2014). However, the "egg or hen" question related to the cause or consequence in the context of inflammation-driven changes in the microbiota remains unanswered, as dysbiosis has also been implicated in other immune-mediated pathologies, such as obesity, allergic disorders, Type 1 diabetes mellitus, autism, and colorectal cancer, in both humans and animal models (Kostic et al., 2015; Butto and Haller, 2016; DeGruttola et al., 2016).

2.5. Dysbiosis of gut microbiota in IBD

Dysbiosis in IBD is characterized by a decrease in overall species richness and evenness, often characterized by the alteration in the abundance of phylum Firmicutes, especially reduction in members of Lachnospiraceae family, such as *Roseburia* and Clostridium cluster XIVa and IV, with *Faecalibacterium prausnitzii* as a prominent representative species (Frank et al., 2007; Sokol et al., 2008; Sokol et al., 2009; Willing et al., 2010; Gevers et al., 2014; Lopez-Siles et al., 2015). Bifidobacteria and Lactobacilli have also been shown to decrease in IBD conditions (Braun et al., 2009; Rigottier-Gois, 2013). On the other hand, family Veillonellaceae seem to be overrepresented (Gevers et al., 2014), whereas members of Bacteroidetes phylum, such as *Bacteroides fragilis* and *Bacteroides vulgatus* gain in abundance (Takaishi et al., 2008), with concomitant overrepresentation of phyla Fusobacteria (Strauss et al., 2011; Gevers et al., 2014) and Proteobacteria, especially family Enterobacteriaceae (Strauss et al., 2011; Bien et al., 2013;

Gevers et al., 2014; Minamoto et al., 2015). However, despite the availability of these data from different studies, consensus about specific disease- relevant taxa in IBD is still far from reach.

2.6. Effectors of dysbiosis

Dysbiosis is manifested in a number of ways involving changes in the microbial composition, such as, loss of function (characterized by reduced bacterial diversity and reduction in specific indicator species); gain of function (characterized by expansion of pathobionts); and change in microbial functional properties (Butto et al., 2015). Alterations in microbiota composition might result from endogenous components, such as genetic predisposition, and exposure to exogenous factors, such as antibiotics (Keeney et al., 2014; Munyaka et al., 2014; Vangay et al., 2015), drugs (Syer and Wallace, 2014), psychological and physical stress (Bailey et al., 2010; Bailey et al., 2011; Cryan and Dinan, 2012), exposure to pathogens/infectious agents, dietary factors (Day and Lopez, 2015; Kaakoush et al., 2015), as well as early life exposures (Munyaka et al., 2014).

2.6.1. Endogenous effectors of dysbiosis

2.6.1.1. Genetic susceptibility in IBD

Genome-wide association studies (GWAS) have identified a variety of target genes that point towards a disruption of microbe–host interactions including genetic loci associated with microbial sensing and clearance as well as resilience mechanisms to cope with accumulating cell stress (Jostins et al., 2012; Liu et al., 2015). Defects in these functions lead to a dysfunctional mucosal interface and chronic activation of adaptive immune effectors and ultimately to dysbiosis, potentially due to different mechanisms, such as: microbial factors, loss of barrier function (CDH1, MUC19), failure to maintain intestinal epithelial cell homeostasis (XBP1; ORMDL3) specifically targeting Paneth cells, loss of innate mechanisms for microbial clearance

(NOD2, ATG16L1, IRGM), shift towards aggressive immune responses and loss of tolerance (TNFS15, IL-10RB, IL-23R), and persistence of pathogenic antigens (Jostins et al., 2012;Liu et al., 2015). Also, a recent analysis of a variety of different mouse models using 3D-stereomicroscopy identified unique patterns of focal lesions with variable degrees of disease-associated abnormalities driven by the host genetic background (i.e. SAMP/Yit vs. TNF$^{\Delta ARE}$ mice) (Rodriguez-Palacios et al., 2015). Therefore, the identification of genetic risk factors has paved way to a better understanding of the defects in host defense mechanisms implicated in the IBD phenotype (Butto et al., 2015), and hence, the specific configuration of host susceptibility, including genetics, can be the underlying cause for the formation and propagation of focal lesions in the gut mucosa, resulting in spatial sites of inflammation, disrupting mucosal homeostasis and potentially leading to disease onset.

2.6.2. Exogenous effectors of dysbiosis

2.6.2.1. Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used medications worldwide for routine relief of pain or fever, to manage various forms of arthritis and inflammatory intestinal disorders, and to prevent or treat alimentary cancers (Maroon et al., 2010; van Laar et al., 2012; Conrozier et al., 2014; Burr et al., 2016). Despite their effectiveness for managing these varied and highly prevalent conditions, NSAIDs may cause damage to the gastrointestinal (GI) tract. Administration of NSAIDs causes a dysbiosis characterized by a reduction of the predominantly gram-positive phylum Firmicutes and a corresponding increase of gram-negative bacteria (Hagiwara et al., 2004; Makivuokko et al., 2010; Whitfield-Cargile et al., 2016). Specifically, NSAID administration decreases various members of the class Clostridia and increases members of the class Bacteroidia (Blackler et al., 2015; Whitfield-Cargile et al.,

2016). In addition, germ-free rats lacking intestinal microbiota do not develop NSAID enteropathy, whereas they develop NSAID-induced intestinal lesions when colonized with gram-negative bacteria (Uejima et al., 1996). However, concurrent administration of NSAIDs and antimicrobials targeting gram-negative bacteria reduces the severity of NSAID-induced gastrointestinal lesions in rats (Koga et al., 1999). In another study, co-administration of indole with NSAID indomethacin in mice resulted in a reduction in severity of mucosal injury and it maintained or even increased some members of the phylum Firmicutes (Whitfield-Cargile et al., 2016). Collectively, these reports supports changes in gut microbiota resulting from the use of NSAIDs.

2.6.2.2. Psychological and physical stress

Stress which has been defined as a threat to homeostasis and caused by events in the surrounding environment or from within, could have both short- and long-term effects on the functions of the GI tract, and consequently disrupt the composition of gastrointestinal microbiota. For example, stress may influence the immune system and gut epithelial tissue with notable effects including increased gut permeability and gut sensitization to luminal antigens, as well as changes in bacterial adherence to the mucous membrane (Buret, 2006; Konturek et al., 2011). A significant difference in the community structure of microbiota in mice subjected to a prolonged restraint stress in comparison to non-stressed mice has also been reported (Bailey et al., 2010). In addition, social disruption stress was found to reduce diversity and richness of some microbes in mice (Bailey et al., 2011).

2.6.2.3. Dietary factors

Several studies have shown that dietary factors alter the microbial community resulting in biological changes to the host (Brown et al., 2012; Martinez-Medina et al., 2014). In this context,

"Western" diet, enriched in total fat, animal protein, n-6 poly- unsaturated fatty acids and refined sugars, is considered a risk factor for the development of CD (Chapman-Kiddell et al., 2010). In addition, the Western diet is associated with dysbiosis, which affects both host gastrointestinal tract metabolism and immune homeostasis (Sekirov et al., 2010; Martinez-Medina et al., 2014), and promotes AIEC gut colonisation in genetically susceptible mice (Martinez-Medina et al., 2014). Also, switching mice fed a low-fat plant polysaccharide-rich diet to a Western diet, the microbiota composition shifted to an overgrowth of Firmicutes including *Clostridium innocuum*, *Eubacterium dolichum*, *Catenibacterium mitsuokai* and *Enterococcus* spp., as well as a significant reduction in several *Bacteroides* spp. (Turnbaugh et al., 2009). Furthermore, diets rich in complex carbohydrates show increased levels of beneficial *Bifidobacterium* spp. such as *Bifidobacterium longum* subspecies *longum*, *Bifidobacterium breve* and *Bifidobacterium thetaiotaomicron* (Pokusaeva et al., 2011;Bondue and Delcenserie, 2015) than diets higher in fat or protein (Cummings et al., 1979; Smith and Macfarlane, 1997; Santacruz et al., 2009), whereas refined sugars on the other hand, mediate the overgrowth of opportunistic bacteria like *Clostridium difficile* (Berg et al., 2013) and *C. perfringens*. Interestingly, European children were found to have a microbiota depleted of Bacteroidetes and enriched in Enterobacteriaceae compared to rural African children, and this was attributed to dietary differences (De Filippo et al., 2010).

2.6.2.4. Exposure to pathogens or infectious agents

A 'healthy' microbiota plays an important role in the defense against infectious pathogens, and mechanisms associated with colonization resistance facilitate pathogen clearance from the intestinal tract. However, phenomena that alter microbiota composition, such as host inflammation or antibiotic treatment, can be exploited by opportunistic pathogens, such as

Salmonella enterica serovar Typhimurium, *C. difficile* (Keeney and Finlay, 2011; Ng et al., 2013), *Enterococcus* spp. (Kinnebrew et al., 2010) and *Escherichia* spp. (Patwa et al., 2011), which benefit from the resulting perturbation of the microbiota. Once dysbiosis is established, pathogens can rapidly outcompete commensals due to factors in their genomes (e.g. those encoding bacterial toxins, antimicrobial resistance, adhesion factors) that confer greater resistance to host defense mechanisms (e.g. antimicrobial peptides, reactive oxygen species and phagocyte killing), and better utilization of the gut nutrient environment (Raffatellu et al., 2009; Winter et al., 2010; Keeney and Finlay, 2011). It has also been shown that, some infectious or pathobiont bacteria are frequently associated with IBD, and may cause changes in the microbial environment of the susceptible host, and thereby trigger development of chronic inflammation. However, it is still debated whether bacterial infection is primary or secondary to the underlying immune dysregulation in IBD patients.

2.6.2.5. Infectious agents associated with IBD

Because IBD is an inflammatory disease of the gastrointestinal tract, it has been speculated that luminal factors are involved and researchers have been trying to discover specific microbes that could be responsible. As a result, three major pathogens have been found to be associated the most with IBD, including: *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which was once thought to be a potential infectious agent associated with the pathogenesis of CD (Prantera et al., 1994; Hermon-Taylor et al., 1998; Collins et al., 2000; Pierce, 2009); Adherent-Invasive *E. coli* (AIEC), which has been frequently found in IBD patients with active phases and has been suggested to increase the inflammatory response (Darfeuille-Michaud et al., 1998; Darfeuille-Michaud et al., 2004; Kotlowski et al., 2007; Peterson et al., 2008; Sepehri et al., 2011; Wright et al., 2015); and *C. difficile*, which has been

detected in patients with UC relapse as well as remission (Dorman et al., 1982; Clayton et al., 2009). While some studies have shown that there may be an increase in comorbidity with these bacteria and IBD, other studies have shown that these results are inconsistent (Shanahan and Bernstein, 2009; Mondot et al., 2011; Bien et al., 2013). Therefore, there is still no direct evidence that any of these bacteria are the sole cause of IBD, and hence, it is possible that IBD may be caused by an imbalance of commensal microbiota associated with more complex interactions between the host and the entire intestinal microbiota rather than specific microbes (Shanahan and Bernstein, 2009).

Mycobacterium avium subspecies *paratuberculosis* is suspected as an etiologic factor for CD, because of CD's similarity to Johne´s disease (Collins et al., 2000), a chronic ileitis in cattle caused by MAP. Additionally, in one study, remission was shown to occur in the majority of patients with CD after treatment by anti-mycobacterial antibiotics (Prantera et al., 1994). However, although MAP has been isolated from patients with CD, its detection in granuloma and other tissues is very difficult.

E. coli strains have been suspected as a possible reason for the onset of disease in IBD since the 1970's (Keighley et al., 1978) when a modification of luminal bacteria concentrations in CD patients with evidence of a dramatic increase in *E. coli* species was reported. Since then, several studies have found increased numbers of *E. coli* species (including adhesive *E. coli* species) with virulence properties in IBD patients in comparison to healthy control groups (Burke and Axon, 1988; Giaffer et al., 1992), and a number of studies suggest a link between the prevalence of *E. coli* spp. and IBD relapses (Boudeau et al., 1999; La Ferla et al., 2004). In addition, increased abundance of members of the Enterobacteriaceae family, especially *E. coli*, have been observed in humans and dogs with IBD and also in experimental animal models of

IBD (Darfeuille-Michaud et al., 1998; Sellon et al., 1998; Darfeuille-Michaud et al., 2004; Schuppler et al., 2004; Mylonaki et al., 2005; Kotlowski et al., 2007; Lupp et al., 2007; Xenoulis et al., 2008; Sepehri et al., 2011; Wright et al., 2015). Moreover, specific strains of members of the adherent invasive *E. coli* (AIEC) including LF82 and UM146, have been isolated from IBD patients and characterized (Miquel et al., 2010; Krause et al., 2011; Sepehri et al., 2011; Desilets et al., 2015). Darfeuille-Michaud and colleagues (Darfeuille-Michaud et al., 1998) showed high prevalence of *E. coli* isolated from ileal biopsies of CD patients, whereas Martin et al. (Martin et al., 2004) showed increased mucosa associated gram-negative bacteria in colonic biopsies obtained from patients with CD, majority of which were identified as *E. coli*, and infection with *E. coli* O157:H7 was found to mimic right-sided colonic CD (Ilnyckyj et al., 1997).

One of the histological characteristics of CD is the presence of epithelioid granulomatous inflammation of the intestine (Greenstein, 2003; Scanu et al., 2007). In this context, an in vitro model of human granulomas showed that AIEC (strain LF82) has the ability to survive and replicate within infected macrophages, inducing aggregation of infected macrophages as well as their fusion to form multinucleated giant cells, along with the subsequent recruitment of lymphocytes (Meconi et al., 2007), which are histologically linked to CD. Also, specific pathogenic strains of *E. coli* with the ability to infect, invade host cells, multiply and damage the host tissues (Boudeau et al., 1999; La Ferla et al., 2004) have been reported in colonic biopsies from patients with CD.

2.6.2.6. Antibiotics

Antibiotic use is an obvious common and significant cause of major alterations in normal GI tract microbiota (Hawrelak and Myers, 2004). The potential for an antimicrobial agent to influence gut microbiota is related to a number of factors including: spectrum of activity,

pharmacokinetics, dosage, and length of administration (Gill et al., 1998; Zhu et al., 2010; Baldoni et al., 2014). Overall, it is possible that microbiota alterations induced by a particular antibiotic might be more severe in individuals with compromised health or who have been subjected to multiple courses of antibiotics.

Several studies confirm that antibiotic administration can result in gut microbiota dysbiosis. In this context, broad-spectrum antibiotics can affect the abundances of the bacteria in the gut community, causing rapid and significant reduction in taxonomic richness, diversity and evenness (Dethlefsen et al., 2008a; Dethlefsen and Relman, 2011). Once antibiotic treatment has been stopped, the microbiota may present a certain degree of resilience, being capable of returning to a composition similar to the original one, but the initial state is often not totally recovered. In fact, antibiotic-induced microbiota alterations can remain after long periods of time, spanning to months and even years (De La Cochetiere et al., 2005; Jernberg et al., 2007; Dethlefsen et al., 2008a; Dethlefsen and Relman, 2011; Jernberg et al., 2013).

Beyond altering the composition of taxa, antibiotics also affect the gene expression, protein activity and overall metabolism of the gut microbiota (Franzosa et al., 2015), and these changes can occur at a much faster pace than those involving replacement of taxa in the community (Perez-Cobas et al., 2013a; Perez-Cobas et al., 2013b). Moreover, the induced changes can drive the functionality of the microbiota toward states similar to those observed under disease conditions. In this direction, the microbiota of individuals treated with β-lactams have been shown to have a repertoire of enzymatic activities for carbohydrate degradation that results in an unbalanced sugar metabolism, similar to that observed in obese individuals (Hernandez et al., 2013). Experimental approaches have also confirmed that antibiotics rapidly alter the physiological state and activity of the gut microbiota. In this context, *ex vivo* incubations

of fecal samples with different antibiotics showed an increase in the proportion of gut microbiota cells with damaged membranes, changes in active populations of the microbiota, and expression of genes involved in antibiotic resistance and stress response (Maurice et al., 2013). The authors also reported increased expression for genes related to genetic information processing (e.g., transcription and translation) in the case of antibiotics that inhibit translation, such as tetracycline and the macrolides. Generally, the substantial effects documented for antibiotics on the functioning of the gut microbiota stress their likely impact on the physiological processes that depend on the activities performed by the microbes.

2.6.2.7. Early life exposures

2.6.2.7.1. Impacts of the prenatal period on gut microbiota

The intrauterine environment and the unborn infant are generally thought to be sterile until delivery. However, some studies have reported presence of bacteria in the intrauterine environment, which suggest that these bacteria may influence gut microbiota of the infant before birth (Mackie et al., 1999; Jimenez et al., 2005; Penders et al., 2006; DiGiulio et al., 2008; Jimenez et al., 2008). For example, *Lactobacillus* and *Bifidobacterium* DNA were detected in the placenta of vaginally and caesarean section-delivered infants (Satokari et al., 2009), and *Enterococcus faecium* strains that were orally inoculated to pregnant mice were later detected in the amniotic fluid and meconium of the pups following delivery (DiGiulio et al., 2008). In addition, a unique placental microbiome niche similar to the human oral microbiome composed of non-pathogenic commensal microbiota from different phyla including Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria were characterized from a population-based cohort of placental specimens (Aagaard et al., 2014; Aagaard, 2014). This

suggests that the placenta is not actually sterile and that oral microbiota may play a major role in the colonization of the placenta although the mechanism through which oral microbes find their way into the placenta remains to be elucidated. It is therefore possible that bacteria in the intrauterine environment could result in prenatal colonization of the meconium (Madan et al., 2012; Li et al., 2014). Jimenez and colleagues showed that the presence of bacterial species in the meconium, such as *E. coli, E. faecium,* and *Staphylococcus epidermidis,* could result from the translocation of the mother's gut bacteria via the bloodstream (Jimenez et al., 2005). Despite these findings, it is not clear whether colonization of the infant's gut microbiota starts before birth, because the presence of bacteria in the amniotic fluid could also be an indication of undetected infection, which may increase the risk of miscarriages or preterm delivery (DiGiulio et al., 2008). In this case, an association of the placental microbiome with a remote history of antenatal infections such as urinary tract infection in the first trimester and preterm birth has been reported (Aagaard et al., 2014; Aagaard, 2014). However, as reviewed by Li et al. (Li et al., 2014), *Bifidobacterium* has been reported in meconium, amniotic fluid, foetal membranes, umbilical cord blood, and placenta of healthy mothers and infants with no detectable or known clinical infections or inflammation.

External factors during pregnancy may also influence future development and behaviour of the infant. For example, infant monkeys born from mothers stressed during pregnancy had significantly lower counts of *Bifidobacterium* and *Lactobacillus* (Bailey et al., 2004). Probiotic administration (*Lactobacillus rhamnosus*) to mothers during late pregnancy also resulted in increased fecal *B. longum* counts in their infants (Lahtinen et al., 2009), although, it is not clear whether these microbes were acquired from the mother during pregnancy, during birth, or after birth. Several other variables during pregnancy, including the use of antibiotics in the perinatal

period, have been associated with delayed colonization by some microbes especially *Bifidobacterium* and *Lactobacillus* species (Westerbeek et al., 2006; Faa et al., 2013). This may have long-term impacts since these species are considered to have beneficial properties; for example, allergies, irritable bowel syndrome (IBS), and IBD, have all been frequently reported in antibiotic-exposed children (Droste et al., 2000; Shaw et al., 2010; Roberts et al., 2011; Russell et al., 2012; Villarreal et al., 2012; Metsälä et al., 2013; Russell et al., 2013; Stensballe et al., 2013). Roberts et al. (Roberts et al., 2011) showed that children born to mothers who smoked have a higher risk of IBD, which may be due to disturbed microbial colonization since the cessation of smoking was correlated with increased Firmicutes and Actinobacteria, and a lower proportion of Bacteroidetes and Proteobacteria (Biedermann et al., 2013).

The length of the gestational period may also play an important role in initial infant gut microbial colonization. Colonization in preterm infants has been shown to take place slowly, have a low diversity, and several inter individual differences as opposed to that of full-term infants (Sakata et al., 1985; Li et al., 2014). It is also mostly dominated by potential pathogens including *Clostridium* species, *E. coli*, *Enterococcus*, *Streptococcus*, *Klebsiella*, and *Staphylococcus* (Sakata et al., 1985; Gewolb et al., 1999; Westerbeek et al., 2006; Li et al., 2014). Healthy full-term infants are usually colonized by beneficial microbes, such as *Bifidobacterium* and *Lactobacillus*, which are not present or are detected in low levels in preterm infants (Butel et al., 2007). The delayed rate of colonization could result from the events surrounding the delivery, because most preterm infants are delivered through emergency or elective caesarean delivery. Infants born by caesarean section, notably electively, have been shown to have low bacterial richness and diversity (Azad et al., 2013), which could be a result of less exposure to the mothers' delivery fluids, delayed oral feeding, and high hygienic care of the

preterm infants and use of antibiotics that may consequently lead to colonization by few resistant/notorious microbes that are potentially pathogenic (Li et al., 2014).

2.6.2.7.2. Delivery mode

During the birth process and immediately after birth, microbes from the mother and surrounding environment colonize the GI tract of the infant leading to the development of a dense complex microbiota (Mackie et al., 1999). The mode of delivery (vaginally or by caesarean section) has been demonstrated to have a strong influence on early gut microbiota colonization (Matamoros et al., 2013). A review by Mackie and colleagues (Mackie et al., 1999) showed that, in cases of vaginal delivery, a longer birth process has been associated with the presence of viable microbes in the stomach and mouth of the infant, and the same *E. coli* serotypes were found in both the mouths of babies and in their mothers' feces immediately after birth. This implies that the proximity of the birth canal and the anus play an important role in the transmission of microbes from the mother to the infant. In addition, bacteria present in the mother's vagina immediately before birth were reported in the nasopharynxes of over 50% of babies born vaginally (Mackie et al., 1999).

Children born by caesarean section are also exposed to their mothers' microbiota, but initial exposure is most likely to non-maternally derived environmental isolates from equipment, air, and other infants, with the nursing staff serving as vectors for transfer (Biasucci et al., 2008; Biasucci et al., 2010). A number of studies have described altered fecal or intestinal microbiota profiles in caesarean section-delivered infants beginning at 1 day after birth and persisting to 6 weeks (Kozyrskyj et al., 2011), 6 months, and even 7 years of age (Jakobsson et al., 2014). Infants delivered by caesarean section have a lower total microbial diversity within the first 2 years of life associated with a less abundance and diversity of phylum Bacteroidetes

(Jakobsson et al., 2014). They are also less often colonized by *Bifidobacterium*, *Bacteroides*, and *E. coli*, but are more frequently colonized by both *Clostridium* cluster I and *C. difficile* (Penders et al., 2013; Jakobsson et al., 2014). It has also been shown that skin microbes including *Staphylococcus, Corynebacterium*, and *Propionibacterium* dominate the gut microbiota of caesarean-delivered infants, while vaginally delivered infants have a higher prevalence of vaginal-related microbes, such as *Lactobacillus, Prevotella*, and *Sneathia* (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2011; Khafipour and Ghia, 2013). Generally, children born by caesarean section have an altered intestinal microbial colonization and studies have highlighted that this may be associated with a subsequent increased risk of developing various diseases including asthma and/or type 1 diabetes (T1D). This could be due to poor development of the immune system since infants born through caesarean section have also been reported to have remarkably lower levels of the Th1-related chemokines CXL10 and CXL11 in their blood, which may translate to less protection (Jakobsson et al., 2014). A meta-analysis of the association between caesarean section and childhood asthma involving studies with different designs, conducted in different countries and using different measures of asthma reported an increased risk of asthma after caesarean section (Thavagnanam et al., 2008), whereas a 20% increase in the risk of childhood onset of T1D was reported in another meta-analysis of children born through caesarean section (Dominguez-Bello et al., 2010). An additional meta-analysis investigating the use of antibiotics in infants reported that the use of antibiotics in childhood was associated with asthma and wheezing (Penders et al., 2011).

2.6.2.7.3. Breastfeeding, formula feeding, solid food at weaning, and geographical location

The succession of microbial colonization in the intestinal tract most occurs during the early development stages especially the first year of life. During this period, the feeding mode

shifts from breastfeeding to formula feeding and/or to the introduction of solid food; however, individual instances of gut colonization may vary in terms of microbiota richness and diversity (Mackie et al., 1999; Kozyrskyj et al., 2011; Valles et al., 2014). Dynamic balances exist between the GI microbiota, host physiology, and diet that directly influence the initial acquisition, developmental succession, and eventual stability of the gut ecosystem (Koenig et al., 2011). Breastfeeding modulates the gut microbiota (Stark and Lee, 1982), and this might confer some protective effects to the infant against various forms of diseases or disorders (Kramer, 2011), because evidence exists for an entero-mammary pathway that transfers diverse microbes from the mother's gut to the baby through breast milk (Donnet-Hughes et al., 2010; Hunt et al., 2011; Jost et al., 2013; Jost et al., 2014). However, human milk is known to contain complex polysaccharides that act as selective prebiotics and therefore promote the colonization of the infant gut with beneficial microbiota (Zivkovic et al., 2011; Garrido et al., 2012), as opposed to children fed with formula. Formula feeding has been associated with an increased microbial richness of species in infants at four months of age with overrepresentation of *C. difficile*, a known gastrointestinal pathogen (Penders et al., 2007b; Azad et al., 2013). In addition, formula feeding induces intestinal hypertrophy and accelerates maturation of hydrolysis capacities; it increases intestinal permeability and bacterial translocation. Therefore, the microbiota may not be the principal actor. However, a recent publication observed more than two times increased numbers of bacteria cells in breast-fed infants, compared to formula-fed ones (Bezirtzoglou et al., 2011). It is therefore clear that breastfeeding may encourage proliferation or colonization by bacteria that may have protective effects on the growing infant, while formula feeding may predispose children to potential pathogens.

The gut microbiota of children is also influenced by the nature of food (other than

formula) they receive, which could also be stratified by income status, mode of upbringing, or geographical location. The microbiota of children in Burkina Faso was found to be dominated by Bacteroidetes, compared with that of Italians, which was dominated by Firmicutes, and this was attributed to dietary differences as well as geographical location (De Filippo et al., 2010). Similarly, the biodiversity of microbiota from USA was lower than that from Malawians or Venezuelan Amerindians (Yatsunenko et al., 2012). Moreover, the effect of the natural environment, such as housing conditions, has also been investigated in animals. In this case, genetically related piglets were housed in either indoor or outdoor environments and sequencing of the 16S rRNA revealed that *Lactobacillus* strains were dominant in the gut of pigs raised outdoors, compared with hygienic indoor pigs, which had reduced *Lactobacillus* and more potentially pathogenic phylotypes (Mulder et al., 2009).

2.6.2.7.4. Mother and immediate family members

Influence of the mother on the child's microbiota is evident during the first year after birth (Matamoros et al., 2013). This effect is reported to be stronger within the first month of life during which the infant's intestinal microbiota is both functionally and phylogenetically close to that of the mother. However, at 1 year of age, phylogenetic differences appear while the similarities persist at the functional level (Vaishampayan et al., 2010). With respect to fecal microbiota, close similarities were found between the mother and the infant during the first six months after birth of the infant, which was mainly due to the presence of *Bifidobacterium bifidum, B. breve,* and *Staphylococcus aureus* (Gronlund et al., 2011).

After birth, environmental, oral, and skin microbes from the mother are mechanically transferred to the newborn in different ways, which may influence the diversity of intestinal microbiota in the neonate. For example, biodiversity in the homes, in the surrounding

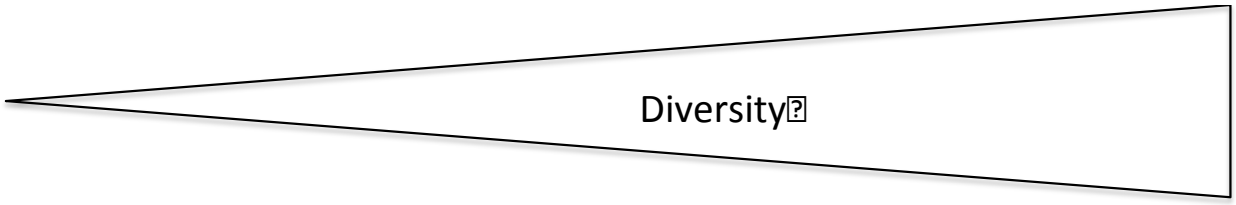
environment and in family members who have a close or constant contact with the baby have a direct impact on the diversity of microbes that are transferred to the infant (Sjogren et al., 2009; Ege et al., 2011; Hanski et al., 2012). In addition, hygienic practices (e.g., cleaning of baby's pacifier through sucking or by other methods) may influence the microbial diversity, and differences in microbial diversity have been associated with the development of allergy and/or asthma later in life (Sherriff et al., 2002; Hesselmar et al., 2013). Numerous population-based studies have also confirmed an inverse relationship between allergy prevalence and various measures of "hygiene," such as growing up on a farm, early day care, and low socioeconomic standards (Benn et al., 2004; von Mutius, 2007). There are also observed differences in the composition of the gut microbiota between infants living in countries with a high and a low prevalence of allergy and between healthy and allergic infants, even very early in life before they have developed any clinical symptoms of disease (Bjorksten, 2009). Furthermore, an increasing number of older siblings are associated with colonization of *Lactobacillus* and *Bacteroides* at 5 weeks of age, all of which are associated with beneficial effects (Penders et al., 2013). The mother and family at large, therefore, play a major role in the initial microbial colonization in infants who may have a health impact on the child. **Table 2.1** shows a summary of the factors discussed in this section.

2.6.2.7.5. Long-term effects of early life microbial colonization

The gut microbiota are generally associated with the development and maturation of the immune system (Kondrashova and Hyoty, 2014), which acquires most of its information from exposure to certain subsets of micro- and macro-organisms (Hwang et al., 2012). For example, early colonization with *E. coli* and *Bifidobacterium* is associated with higher numbers of CD20 + B cells that express the memory marker CD27 at 4 and 18 months of age

Table 2.1. Summary of factors affecting colonization of gut microbiota in infants

a) Factors affecting colonization of gut microbiota before birth	**b)** Factors affecting colonization of gut microbiota during/at birth	**c)** Factors affecting colonization of gut microbiota after birth
- Intra-uterine environment - Maternal exposures or practices such as stress, antibiotic use, smoking - Length of gestation period (term vs preterm)	- Mode of delivery (caesarean section vs vaginal delivery). - The environment at the time of delivery. - Contact with the mother or health care staff.	- Breastfeeding vs formula feeding. - Weaning or food supplementation. - Antibiotic exposure. - Home or family setting (rural vs urban) - Home structure (contact with the mother and other family members including siblings and close contact relatives.



(Lundell et al., 2012). Disruption of exposure to these organisms is at least partly responsible for the immunoregulatory deficits that underlie the increased prevalence of conditions, such as chronic immune inflammatory diseases (i.e., IBD), asthma, and atopic dermatitis (Bach, 2002; Kozyrskyj et al., 2011; Penders et al., 2013).

Molecular microbiology techniques suggest that a high diversity of the gut microbiota in childhood could be more important as opposed to low diversity, which is associated with increased risk of subsequent allergic diseases, since repeated exposure to different bacterial antigens would enhance development of immune regulation through inhibition of responses to inappropriate targets such as gut contents and allergens (Bisgaard et al., 2011; Abrahamsson et al., 2012). In this regard, the microbial diversity and composition of 47 infants as analyzed using barcoded 16S rRNA 454 pyrosequencing in stool samples at 1 week, 1 month, and 12 months of age, revealed that low total diversity of the gut microbiota during the first month of life was associated with asthma in the children at 7 years of age (Abrahamsson et al., 2014). Also, a low gut microbial diversity during the first month of life was associated with subsequent sensitization and atopic eczema at 2 years of age (Abrahamsson et al., 2012). In addition, reduced bacterial diversity of the infant's intestinal microbiota was associated with increased risk of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia, in the first 6 years of life (Bisgaard et al., 2011). Early-life exposures, including those known to impact gastrointestinal microbiome composition, such as antibiotic administration have also been associated with increased risk for childhood asthma due to altered microbiota profiles or long-term reduction in microbial diversity (Johnson et al., 2005; Joffe and Simpson, 2009; Kozyrskyj et al., 2011). Alterations of the intestinal microbiota in preterm infants characterized by low microbial diversity and abundance of potentially pathogenic bacteria have been highlighted in the

development of necrotizing enterocolitis (NEC), although there are discrepancies among different studies (Carlisle and Morowitz, 2013; Torrazza and Neu, 2013). Hällström and colleagues (Hallstrom et al., 2004) reported a link between caesarean delivery and disturbed intestinal colonization, with an increased frequency of *Enterococcus* species and *Candida albicans*, and the probable occurrence of NEC in preterm infants. These findings may not be exclusively due to a disturbed intestinal microbiota because other confounding factors may also be responsible for NEC; however, further investigation will provide useful information on this topic and further clarify the existing discrepancies.

The birth order or family size and the presence or absence of pets has also been implicated in the initial microbial colonization in infants. Over two decades ago, Strachan observed that children who had older siblings were less likely to manifest hay fever as adults, compared to firstborn children, which could be due to a protective effect of infections brought home by the older siblings (Strachan, 1989). Similarly, early life exposure to livestock or pets particularly dogs, significantly decreased the risk for asthma and/or allergic reactions since dog ownership was associated with a distinct house dust microbial exposure (Ownby et al., 2002; von Mutius, 2007). In addition, mice exposed to dog-associated house dust were found to be protected against airway allergen challenge, because they exhibited less Th2 cytokine production, fewer activated T cells, and a distinct gut microbiome composition that was highly enriched for *Lactobacillus johnsonii*, which itself can confer airway protection when orally supplemented as a single species (Fujimura et al., 2014).

Selective microbial targets have been associated with infants developing eczema (Forno et al., 2008). Yap and colleagues (Yap et al., 2014) evaluated the composition of fecal microbiota of infants who developed eczema in the first 5 years of life compared with healthy controls and

reported that longitudinal analysis of fecal microbiota composition at 3 days, 1 and 3 months, and 1 year of life showed a higher abundance of Enterobacteriaceae and *C. perfringens* in children who developed eczema in the first 2 years of life, whereas a lower abundance of *Bifidobacterium* was observed in those who developed eczema at 5 years of age. The authors suggested that relative abundance of specific microbial targets might contribute to the subsequent development of eczema in childhood. Studies have also associated gut microbiota with the development of T1D (Kondrashova and Hyoty, 2014). For example, metagenomic analysis revealed that the proportion of Bacteroidetes was increased in children with T1D while the proportion of Firmicutes was increased in normal healthy children (Hwang et al., 2012). Conversely, the relative proportion of Bacteroidetes is decreased in obese people compared with lean people, while the proportion of Firmicutes is increased in obese people (Hwang et al., 2012). In addition, colonization with clostridia, at the age of 5 and 13 weeks is associated with an increased risk of developing atopic dermatitis in the subsequent 6 months of life (Penders et al., 2013). Breastfeeding and formula feeding play major roles in defining the initial microbial colonization, and a previous meta-analysis showed that breast-fed children have a lower risk of being overweight compared to formula-fed children and that the duration of breastfeeding is inversely and linearly associated with the risk of overweight (Harder et al., 2005).

Use of antibiotics is generally known to change the gut microbiota. A meta-analysis on antibiotic use in infants reported that wheezing and asthma were related to antibiotic use (Penders et al., 2011). In addition, high-throughput sequencing revealed an incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin (Fouhy et al., 2012). The use of antibiotics in this context has been implicated in the development of IBD and current research has shown that children with IBD are more likely

to have received antibiotics in their first year of life as compared to healthy controls (Shaw et al., 2010), suggesting that microbial dysbiosis associated with early antibiotic exposure in neonates may be a predisposing factor to IBD, including other disease conditions, such as wheezing and asthma. **Table 2.2** shows a summary of the factors affecting gut microbiota and their effects in neonates, infants or children.

2.7. Clinical treatment of IBD

2.7.1. Immunomodulatory and anti-inflammatory treatment

A number of agents with anti-inflammatory or immunomodulatory properties have been utilized in treatment of IBD. In this context, both rectal and oral therapies of 5-Aminosalicylates (5-ASA) have been shown to be effective in inducing and maintaining remission in UC, but its use in CD is not clear; however, the combination of both oral and rectal therapies is deemed better than either of them in UC (Bernstein, 2015). Also, corticosteroids have been shown to be effective in both UC and CD for inducing remission, and are among the less expensive agents that can be prescribed; however, they are associated with adverse effects and some of their effects are short-term (Ford et al., 2011; Bernstein, 2015). Other agents that are also used include thiopurines, methotrexate and calcineurin inhibitors although they also have limitations like the other agents (Bernstein, 2015).

2.7.2. Cytokine-based therapies in IBD treatment

As reviewed previously (Neurath, 2014), cytokines seem to have a major role in driving intestinal inflammation, local complications and systemic and extra-intestinal manifestations in patients with IBD, and therefore, proinflammatory cytokines such as TNF are the key target for IBD therapy. In addition, novel agents that target cytokines or cytokine signalling cascades are

Table 2.2. A summary of factors affecting colonization of gut microbiota in neonates and infants or children, specific microbial effects, and the resultant health conditions

Factor	**Observed effect on microbiota**	**Specific health condition/disorder/disease**
Intra-uterine environment	- Presence of bacteria in the uterus. - Presence of bacteria in the amniotic fluid. - Presence of bacteria in the meconium.	- Remote history of antenatal infections such as urinary tract infection during the first trimester. - Preterm birth.
Stress during pregnancy	- Low counts of beneficial bacteria (e.g *Bifidobacterium, Lactobacillus*).	- Allergic reactions.
Probiotic use during pregnancy	- Increased colonization by beneficial bacteria. - Increased bacterial diversity.	- Reduced incidences of allergic reactions.
Antibiotic use during pregnancy	- Delayed colonization or reduced abundance of beneficial bacteria.	- Increased allergic reactions (asthma, allergic sensitization, allergic rhinitis). - Irritable bowel syndrome (IBS). - Inflammatory bowel disease (IBD).
Smoking during pregnancy	- Microbial dysbiosis (decrease in Firmicutes and Actinobacteria and an increase in Bacteroidetes and Proteobacteria.	- Increased risk of IBD.
Length of gestation period - preterm	- Slow rate of bacterial colonization. - Reduced bacterial diversity. - High inter-individual differences in colonization. - Increased level of potential pathogenic bacteria.	- Necrotic enterocolitis (NEC).
Length of gestation period- term	- Increased abundance of beneficial bacteria. - High bacterial diversity.	- Less incidences of NEC.
Caesarean delivery	- Reduced bacterial richness and diversity. - Reduced colonization by beneficial bacteria. - Increased colonization by potential	- Increased risk of asthma, allergic reactions, Type 1 diabetes, atopic eczema, obesity and NEC. - Low levels of Th1 responses.

	pathogens.	
Vaginal delivery	- Increased microbial diversity. - Increased colonization by beneficial bacteria. - Reduced colonization by potential pathogens.	- Decreased risk of asthma, allergic reactions, Type 1 diabetes, atopic eczema, obesity and NEC.
Antibiotic use in neonates and infants	- Reduced bacterial richness and diversity. - Reduced colonization by beneficial bacteria. - Increased colonization by potential pathogens.	- Increased risk of IBD, asthma, wheeze, eczema, atopic dermatitis.
Formula feeding	- Colonization by potential pathogens.	- Atopic dermatitis, overweight.
Breastfeeding	- Colonization by beneficial microbiota. - Increased bacterial diversity.	- Protection against various diseases and disorders.
Diet/solid food as stratified by geographical location, income status, hygiene level	- Children from developing countries with low income and less hygiene practices have high microbial diversity rich in beneficial bacteria.	- Fewer incidences of allergic reactions.
Contact with mother, dust, pets, increasing number of older siblings	- Increased bacterial diversity. - Colonization by beneficial bacteria.	- Decreased incidences of allergic reactions, hay fever, and asthma.

currently being tested in clinical trials, suggesting that cytokine blockade will remain a crucial field of interest for IBD therapy (Neurath, 2014). However, anti-cytokine therapies (such as antibodies specific for TNF, IL- 12 or IL- 23) and cytokine signalling blockers (such as tofacitinib) only seem to have beneficial clinical effects in certain subgroups of patients (Mannon et al., 2004; Danese and Fiocchi, 2011; Sandborn et al., 2012a; Sandborn et al., 2012b). This may be due to the fact that cytokine networks are more complex and cytokines are subject to multiple layers of regulation by microbial, genetic and immunological factors. A key feature of the mucosal cytokine network is its dynamic fluidity and ability to traverse spatial boundaries, and it is likely that the blockade of a single cytokine in patients with IBD may lead to the development of alternative compensatory pro-inflammatory cytokine pathways (Neurath, 2014). Furthermore, the pathological mechanisms that drive mucosal inflammation are likely to differ between patients, which would make the targeting of a single pro-inflammatory cytokine less effective. This led to testing of a combination therapy of TNF-α and IFN-γ antibodies or other combination of cytokines as an alternative approach to target the disease (Morrison, 2007). However another limitation of cytokine therapy is that it possesses serious side effects that require extensive monitoring, especially in countries that have burden of tuberculosis and hepatitis, which can be reactivated as a result of immune suppression (Pai et al., 2005). Therefore, in most cases cytokine therapies are kept as a third line of action when the escalating response is required, despite being the most effective in the available treatment options (Rogler, 2015).

2.7.3. Antibiotics treatment

A significant benefit of the antibiotics in IBD patients has been reported in different studies indicating a role of gut microbiota in IBD. Antibiotics may influence the course of IBD by decreasing concentrations of bacteria in the gut lumen and altering the composition of

intestinal microbiota to favor beneficial bacteria (Sartor, 2004; Scott et al., 2015). They can also target specific bacteria that are hypothesized to be implicated in the pathogenesis of IBD such as administration of ciprofloxacin, aminoglycosides or rifaximin against virulent *E. coli* and other gram negative enteric bacteria, metronidazole for anaerobes (specifically *Bacteroides fragilis*), or anti tuberculous drugs designed to treat mycobacterial infection (MAP), which has been hypothesized to have a role in the development of CD (Kerman and Deshpande, 2014). However, antibiotic therapy is also associated with negative outcomes. Concerns that antibiotics may elicit IBD by altering the gut microbiota have been raised and some studies found a link between antibiotics and CD (Ungaro et al., 2014). In this case, a strong association between CD and prior antibiotic use in children has been reported, suggesting a link between early life changes in intestinal microbiota and the development of the gut immune system (Hviid et al., 2011). Also, a dramatic increase in concentrations of mucosal bacteria 1 wk after cessation of antibiotic therapy that remained higher over a period of 5 months was observed in IBD patients as compared to patients without antibiotic treatment (Swidsinski et al., 2008). Another caveat of antibiotic treatment is the development of antibiotic resistance, as two thirds of gram negative isolates from abdominal abscess isolates in patients with CD were found to be resistant to ciprofloxacin (Park et al., 2014), whereas another study demonstrated rifaximin resistance in IBD associated ileal *E. coli* strains, that was significantly associated with prior rifaximin treatment and was found to be correlated with a specific mutation in bacterial efflux pump (Kothary et al., 2013).

2.8. Alternative therapeutic interventions targeting the intestinal microbiota dysbiosis in IBD

As shown above, current medical treatment of IBD involves the use of antibiotics and compounds with immunomodulation or anti-inflammatory properties (Bernstein, 2015; Chandel

et al., 2015; Shahidi et al., 2016); however, although these agents have reasonable efficacy, they may produce significant side effects associated with chronic immune suppression, and also, not all patients respond well to these therapies and IBD continues to cause significant morbidity. In addition, the current therapies are mostly directed against the overly aggressive adaptive immune response of the host, but fail to correct potential environmental triggers such as the intestinal microbiota that induces and perpetuates these disorders. This has led to research into therapeutic measures that given the role of the gastrointestinal microbiota in driving inflammation in IBD, may change or restore the balance of commensal luminal bacteria and eliminate bacterial antigens as an alternative/adjunctive IBD treatment. Some of the alternative therapies explored are discussed below.

2.8.1. Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefit on the host (Hill et al., 2014). According to Food and Agricultural Organization (FAO) and WHO guidelines and the recent consensus by the expert committee (Hill et al., 2014), probiotics should be able to survive stomach gastric juices (acid and bile), proliferate and colonize the digestive tract, maintain viability for the duration of the shelf-life during storage, and be safe and effective for human consumption.

Although the list of probiotics keep increasing, microorganisms most commonly used as probiotics belong to the heterogeneous group of lactic acid bacteria (*Lactobacillus*, *Enterococcus*) and to the genus *Bifidobacterium* (Holzapfel et al., 2001). Other less commonly used probiotic microorganisms are strains of: *Streptococcus*, *E. coli*, *Bacillus* and *Saccharomyces* (Saad et al., 2013). Also, probiotics can utilize prebiotics or starch to gain a competitive advantage over pathogenic bacteria that lack the ability to utilize starch in the hindgut (Amit,

2007).

While the precise reasons behind the probiotics' therapeutic benefit are incompletely understood and described, the proposed mechanisms include: improving host mucosal barrier function, production of antimicrobial metabolites displacing/blocking deleterious microbes from the mucosal surface, competition for nutrients, changes in environmental conditions (reduction of luminal pH), production of growth substrates (e.g. vitamins, short chain fatty acids), and modulation of the mucosal immune response (Alvarez-Olmos and Oberhelman, 2001; Mack et al., 2003; Servin, 2004; Candela et al., 2005; Rioux et al., 2005; Tien et al., 2006; Amit, 2007; Collado et al., 2007a; Collado et al., 2007b; Vanderpool et al., 2008; Power et al., 2014).

Specific bacterial strains have been suggested to play a protective role against IBD, by competing with pathogenic microbes or directly preventing their colonization in the gut, as well as through their anti-inflammatory properties (Kostic et al., 2014). *E. coli Nissle* 1917, a nonpathogenic *E. coli* is the best studied among probiotics as a single strain. Application of probiotic *E. coli Nissle* 1917 strain in patients with CD did not cause any significant differences in the duration of remission compared with the control group, whereas in four randomized clinical trials, *E. coli Nissle* 1917 worked as effectively as mesalazine therapy in the maintenance of remission in UC patients (Kruis et al., 1997; Rembacken et al., 1999; Kruis et al., 2004; Henker et al., 2008).

Administration of *Saccharomyces boulardii* strain with mesalazine in patients with CD significantly reduced the incidence of remission compared with mesalazine-treated group (Guslandi et al., 2000). In addition, *S. boulardii* did not prove better than placebo in improving the rates of remission induction and disease activity scores in patients with active CD (Bourreille et al., 2013).

Another well-studied probiotic single strains belong to the *Lactobacillus* group although the results are not very promising. *Lactobacillus rhamnosus* GG (*L. rhamnosus GG)* was compared to mesalazine for the maintenance of remission in UC, and similar relapse rates after 6 and 12 months of treatment were observed with the two treatments, but a significantly longer relapse-free time was obtained with *L. rhamnosus* GG (Zocco et al., 2006). Also rectal administration of *Lactobacillus* was attempted in left-sided UC in an 8 week-treatment with *Lactobacillus reuteri* ATCC 5573, and significantly higher rates of clinical remission than placebo were reported in children (Oliva et al., 2012). On the other hand, *Lactobacillus rhamnosus* GG failed to show any benefit over placebo when used as an adjunct to conventional therapy both for the induction and the maintenance of remission in two small randomized controlled trials conducted in patients with active CD (Prantera et al., 2002; Schultz et al., 2004). Similarly, *L. rhamnosus GG* did not show superiority over placebo in prolonging the time to relapse in a pediatric population with CD in remission (Bousvaros et al., 2005; Prantera et al., 2006). Furthermore, *Lactobacillus johnsonii* LA1 was not effective in preventing postoperative recurrences of CD in two randomized placebo-controlled trials (Marteau et al., 2006; Van Gossum et al., 2007).

Probiotic combinations have also been widely investigated in patients with UC. The most extensively studied combination is represented by VSL#3, (a probiotic cocktail of *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium brevis, Bifidobacterium infantis, Bifidobacterium longum*, and *Streptococcus thermophiles*). Use of VSL#3 strengthened integrity of the intestinal epithelial barrier by increasing expression of proteins responsible for the formation of tight junctions (Mennigen et al., 2009) in a murine model of colitis, and resulted in the induction and

maintenance of remission in children (Miele et al., 2009) and in adults (Shen et al., 2014) with UC but this was not observed in the case of other probiotics (Shen et al., 2014). Moreover, VSL#3 has also been found to increase bacterial diversity in GIT of IBD patients (Kuhbacher et al., 2006). In addition, several other studies, including randomized placebo-controlled trials, have clearly demonstrated the efficacy of VSL#3 probiotic mixture, administered for 6–12 weeks, in inducing remission in patients with mildly-to-moderately active UC treated with conventional therapy (Tursi et al., 2004; Bibiloni et al., 2005; Tursi et al., 2010) or not receiving any other therapy (Sood et al., 2009; Lee et al., 2012). However, VSL#3 probiotic mixture supplementation did not result in a reduction in pediatric post-surgical relapse compared to mesalamine-treated CD patients (Campieri et al., 2000).

Lactobacilli mixtures have not shown clinical efficacy in the treatment of UC or CD. Supplementation of sulfasalazine with *Lactobacillus delbruekii* and *Lactobacillus fermentum* was effective in decreasing colonic levels of IL-6, NF-κB, TNF-α, as well as leukocyte recruitment and fecal calprotectin levels in patients with mild-to-moderate active UC, but without any significant clinical impact (Hegazy and El-Bedewy, 2010). Also, *Bifidobacterium lactis* strain BB-12 and *L. acidophilus* strain LA-5 failed to maintain higher rates of remission than placebo in patients with left-sided UC (Wildt et al., 2011). Furthermore, a combination of *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A and *Bacillus mesentericus* TO-A displayed unclear results in the induction of remission in patients with mild-to-moderate UC that were unresponsive or intolerant to standard therapies (Tsuda et al., 2007).

These studies have indicated mixed results with respect to use of different probiotics in IBD, therefore, there is still little evidence to support the use of probiotics in the treatment or maintenance of remission in CD or UC (Meijer and Dieleman, 2011; Naidoo et al., 2011). The

paucity of randomized controlled trials and the small patient numbers in the studies are the main limitations (Rolfe et al., 2006; Butterworth et al., 2008).

2.8.2. Prebiotics

Prebiotics are non-digestible carbohydrates, defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host wellbeing and health." (Roberfroid, 2007; Higgins and Brown, 2013; Giuberti et al., 2015). Substances are considered prebiotics when they meet the following criteria: be neither hydrolyzed nor absorbed in the upper part of the GI tract; be selectively fermented by one or a limited number of potentially beneficial bacteria in the intestine; and be able to alter the colonic microbiota toward a healthier composition (Gibson and Roberfroid, 1995; Roberfroid, 2007). Their protective mechanisms includes the potential to selectively stimulate growth of beneficial gut bacteria, for example *Lactobacillus* and *Bifidobacterium* (Patel and DuPont, 2015), improving intestinal barrier, regulating the mucosal and systemic immune response, reduction of luminal pH, and increasing the production of intestinal SCFAs (Looijer-van Langen et al., 2009). Some examples of commonly used prebiotics include fructans (inulin, fructo-oligosaccharides), galacto-oligosaccharides, and resistant starch (RS). Resistant starch refers to the fraction of starch that escapes digestion in the upper GI tract, therefore passing into the colon where it can be a substrate for fermentation (Higgins and Brown, 2013; Giuberti et al., 2015).

The available data on the use of prebiotics in IBD are still less than those on probiotics. Administration of fructo-oligo-saccharides (FOS) at a dose of 15 g per day for 15 days significantly increased the number of *Bifidobacteria* in the feces, whereas the population of *Bacteroides*, *Fusobacteria*, and *Clostridium* decreased (Gibson and Roberfroid, 1995). In

contrast, in a randomized, double-blinded study in 103 patients with CD, the administration of FOS had no statistically significant effect compared with the control group (Benjamin et al., 2011). In a small nonrandomized clinical trial, a 3-week supplementation with FOS increased Bifidobacteria species content in mucosal and fecal specimens and improved disease activity scores in ten patients with active CD (Lindsay et al., 2006), although a larger placebo controlled clinical trial did not confirm these results (Benjamin et al., 2011). However, administration of oligofructose-enriched inulin for 4 weeks was effective in reducing disease activity and modifying microbiota composition (by increasing the number of *B. longum* strains) in a placebo-controlled clinical trial enrolling patients with mild to moderate active or quiescent CD (Joossens et al., 2012).

Like in the case of probiotics, the efficacy of prebiotics in the management of IBD is still not clear and this warrants more research.

2.8.3. Synbiotics

These are products that contain both probiotics and prebiotics, which are attributed to the additional benefits if prebiotics are combined with probiotics. The rationale to use synbiotics, is based on observations showing the improvement of survival of the probiotic bacteria during the passage through the upper intestinal tract (Pena, 2007). In this context, synbiotic products beneficially affects the host in improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of probiotic bacteria (Cencic and Chingwaru, 2010).

In a double-blind randomized controlled trial, the prebiotic Synergy 1 (containing FOS/inulin mix), in combination with *B. longum*, led to an improvement of sigmoidoscopy scores and a decrease in β-defensin, TNF-α, and IL-1α in biopsy samples from patients with UC

(Furrie et al., 2005). In another trial, patients who received a combination of *B. longum* and the prebiotic Synergy 1 showed significant histological improvement compared with controls (Steed et al., 2010). The authors also reported a significant decrease in TNF-α expression after 3 months but the level of TNF-α did not change further after 6 months of the symbiotic treatment.

In a randomized controlled trial, UC patients in remission or with mildly active disease concomitantly on standard therapy, showed the superiority of a combination of *B. longum* and psyllium over the probiotic or the prebiotic alone in terms of IBD-related quality of life scores or reduction of serum C-reactive protein, respectively (Fujimori et al., 2009). Also, administration of inulin plus a series of beneficial bacterial strains (*B. infantis*, *L. acidophilus*, *L. casei*, *Lactobacillus gasseri*, *L. plantarum*, *Lactobacillus salivarius*, *Lactobacillus sporogenes* and *S. thermophilus*) showed quite good rates of clinical remission in a short treatment series of patients with relapsing mild to- moderate left-sided UC despite maintenance treatment with mesalamine (Guslandi, 2011). Additionally, a one-year supplementation of anti-inflammatory therapy (steroids and salicylates) with a synbiotic composed of *B. breve* strain Yakult and galacto-oligosaccharide resulted in a significant endoscopic score improvement in patients with mild-to-moderate active UC (Ishikawa et al., 2011).

These studies show the potential beneficial effect of synbiotics, future efforts are however needed to obtain more conclusive evidence.

2.8.4. Fecal microbiota transplantation

Fecal microbiota transplantation (FMT), also known as fecal bacteriotherapy, fecal transplantation, fecal microbiota reconstitution, or human probiotic infusion, refers to the process of instilling a liquid suspension of stool from a healthy donor into the patient's upper gastrointestinal tract through a nasogastric/nasoduodenal catheter or gastroscopy, or into the

colon through a colonoscopy or a rectal catheter (Bakken, 2009; Gough et al., 2011; Suwantarat and Bobak, 2013; Emanuelsson et al., 2014).

There has been interest in FMT as an alternative approach for the manipulation of the intestinal microbiota. Indeed, evidence for its use as a treatment for gastrointestinal illness (including pseudomembranous colitis, *C. difficile*-associated diarrhea, antibiotic-associated diarrhea, IBS and IBD) is rapidly accumulating (Gough et al., 2011; Landy et al., 2011; Guo et al., 2012; Suwantarat and Bobak, 2013; Emanuelsson et al., 2014). The authors partly agree that fecal microbiota transplantation could hold great promise as a future treatment, where a dysbiosis of the gut microbiota is responsible for disease, and when standard treatments have failed.

The exact mechanism of action for FMT in the treatment of active IBD and of *C. difficile* infection is unknown, however, by replacing the disrupted microbiota with diverse bacterial populations it is thought to restore the composition and function of the intestinal microbiota in patients (Khoruts et al., 2010; Gough et al., 2011). Nevertheless, FMT as a treatment for IBD is limited by the absence of well-designed randomized controlled trials, with evidence only from a small number of case series and case reports, and therefore, it remains a controversial issue, and there is need to evaluate its safety and develop optimal protocols for use in IBD.

2.9. Models to study IBD

For research purposes, studies using samples from affected human beings would provide the most reliable data; however, this is not always the case especially when intestinal tissues are the required samples due to difficulties in acquiring human tissues. Some of the limitations in the use of human beings when investigating intestinal diseases include: the ethical use of collected human tissue, the often small sample size, the pronounced genetic variability between tissue from various individuals, and the challenges of isolating the intestinal tissue from the whole

organism. As such, comparative animal models are required that can provide information representative of intestinal disease in humans. The models provide large sample sizes, allow for invasive sampling, and some of them have genetically homogeneous backgrounds (i.e. genetically engineered rodent models).

2.9.1. Commonly used animals in IBD research

A number of animal species have been used and are still utilized for research in IBD including: the non-human primates (e.g. monkeys, cotton top tamarins), zebra fish, rodents (e.g. mice, rat, guinea pigs), and domestic pig. Of these animals, non-human primates provide the best and most comparable data to people due to their high degree of genetic and physiological similarity to the human intestine. However their use is associated with considerable drawbacks such as high costs, ethical considerations, and the potential hazards of carrying highly virulent zoonotic agents (Ideland, 2009; Coors et al., 2010), therefore, rodents and pigs are mostly the common animals used in IBD research. Zebra fish are extensively used to study both innate and adaptive immune responses (Lam et al., 2004) in IBD pathogenesis (Oehlers et al., 2011).

Mice are considered a good animal model as they share many specific intestinal genes with people (Waterston et al., 2002; Bryda, 2013). In addition, human and murine intestinal communities exhibit comparable diversity of species within the Firmicutes and Bacteroidetes phyla (Dethlefsen et al., 2007); however, differences exist in the abundance of specific genera between humans and both mice and rats (Heinritz et al., 2013). Mice also have many features analogous to the adaptive immune response such as the presence of similar populations of B cells, T cells, and isotype antibodies (Mestas and Hughes, 2004). Other advantages of mice as an animal model include their small size (e.g. efficiency of husbandry), low costs in breeding, their relatively short estrous cycles and gestation period, and their large litter sizes (Nguyen and Xu,

2008). Rat models have the advantage of being larger than mice thus allowing the acquisition of larger samples for analysis. Nevertheless, several physiological and metabolic differences compared with humans have to be acknowledged such as: rodent's fermentation taking place in their large cecum, while also practicing caecotrophy (Houpt et al., 1979; Graham and Åman, 1987), rodents as small animals require more feed per unit body weight, which translates to faster digesta passage rate, in addition to a lower capacity for fibre digestion compared with humans (Heinritz et al., 2013).

Pigs are commonly used as an alternative monogastric mammalian model, due to their physiological similarity to humans and their intestinal function and morphology is similar to that of human beings (Walters et al., 2012). Similar to humans, the gut microbiota of pigs mainly consists of the Firmicutes and Bacteroidetes phyla, although differences exist in population of specific genera (Leser et al., 2002; Heinritz et al., 2013). In this context, the pig has already been used for a long time as an animal model for research in to human nutrition and biomedicine, and hence, there are diverse areas of application for the pig model (Graham and Åman, 1987; Miller and Ullrey, 1987; Leser et al., 2002; Baker, 2008). Also, in comparison with other animal models including rodents, pigs allow for more invasive sampling in the GIT, induction of disease states, and a variety of nutritional intervention approaches.

2.9.2. Characteristics of a suitable animal to be used for research

For an animal to qualify to be used for research, the animal should be available to different investigators, it should be easy to handle, should be of sufficient size to allow for invasive sampling, it should be relatively inexpensive, it should be able to reproduce fast with ability to produce more off-springs per litter, and it should yield information that could be easily translated to humans.

2.9.3. An ideal animal model for IBD

An ideal animal model of IBD should be able to develop the disease under the same circumstances as experienced by IBD patients, it should bear or exhibit the exact characteristics of the disease as observed in humans or at least show characteristics associated with UC or CD, and should be able to respond to treatment or therapeutic measures the same way the IBD patients would respond. This may not be possible as no single animal model is perfect for studying all components of intestinal inflammation; therefore, models that possess some of these characteristics or at least exhibit characteristics that are close to what is observed in IBD situations are utilized.

2.9.4. Types of animal models of IBD

There are currently many animal models of IBD, and while none of them perfectly phenocopy human IBD, many are useful for studying various aspects of the disease, including disease onset and progression and the wound-healing response.

The most important aspects discovered through IBD animal models are: germ-free animals generally do not develop intestinal inflammation, spontaneous gut inflammation requires a certain genetic background, T cells are involved in most IBD animal models and interactions between T cells and dendritic cells seems to be crucial for the initiation and perpetuation of inflammation (Hoffmann et al., 2002). Overall, IBD animal models can be divided into 5 different categories (Hoffmann et al., 2002): 1; Antigen-induced colitis or colitis induced by microbiota (Iqbal et al., 2002), 2; Chemical inducible forms of colitis (Cooper et al., 1993), 3; Genetic or transgenic colitis models (Kuhn et al., 1993), 4; Adoptive transfer of immune cell models (German et al., 2000; Mansfield et al., 2001), and 5; Spontaneous colitis models (Kobayashi et al., 2014).

2.9.4.1. Antigen-induced colitis or colitis induced by microbiota

As an alternative or in addition to chemical inducers, microbiota or microbiota related antigens have also been used to study intestinal inflammatory diseases in humans. These can be bacterial, viral, protozoal, or helminthic, and can be used to induce both acute and chronic inflammation. Few commonly used bacterial examples are discussed here.

The gram-negative *Salmonella typhimurium* and *Salmonella dublin* are food-borne enteric bacterial pathogens that can cause intestinal diseases by oral infection, which results in systemic infection further causing intestinal inflammation. In this case, it has been shown that the inflammation has similar histopathological characteristics to human UC, including epithelial crypt loss, erosion and neutrophil infiltration (Mizoguchi, 2006; Papanikolaou et al., 2007). The colitis is induced usually after systemic infection within 5–7 days of infection in C57BL/6 mice; therefore, it is perceived that *S. typhimurium* infection is a valuable model to study the acute phase, but not later stages of colitis (Low et al., 2013a).

The commensal adherent-invasive *E. coli* adheres to both small and large intestinal epithelial cells with equal affinity (Jensen et al., 2011). Induction of colonic inflammation in animal models using AIEC infection requires mild epithelial damage, such as low-dose DSS treatment, during the entire course of the infection. The phenotype of the colonic inflammation mimics UC, including body weight loss, presence of blood in stool and colonic neutrophil infiltrations (Mizoguchi, 2006; Low et al., 2013b).

2.9.4.2. Chemical inducible forms of colitis

Animal models of colitis frequently use either DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS) to initiate inflammation. Dinitrobenzene sulfonic acid (DNBS)**,** acetic acid, oxazolone, carrageenan, and azoxymethane have also been used, but to a lesser extent than DSS and TNBS

(Hibi et al., 2002; Mizoguchi, 2012; Goyal et al., 2014; Randhawa et al., 2014). The effectiveness of inducing tissue injury following treatment with chemical agents varies and depends on the molecular weight, concentration, manufacturer, and batch of the chemical (Perse and Cerar, 2012). In addition, the species, gender (Mizoguchi, 2012), and the genetic background of the animal model being challenged influences the degree of tissue injury as well (Wirtz et al., 2007; Lakhan and Kirchgessner, 2010). The method of administration also influences the induction and severity of disease, as some chemicals work well to induce inflammation after ingestion (Mizoguchi, 2012), while others function best when applied directly to the site of infection, such as the rectal administration (Elson et al., 1995b). Furthermore, microorganisms present in the intestine can interact with the chemical inducer and interfere with its ability to effectively induce tissue injury (Kitajima et al., 2001). Nevertheless, chemical inducers cause tissue damage that can effectively represent clinical cases of intestinal inflammation (Randhawa et al., 2014).

2.9.4.2.1. Dextran sulphate sodium (DSS)

Dextran sulphate sodium-induced colitis is a reproducible model that morphologically and symptomatically resembles UC in humans. Supplementing drinking water of rodents with low molecular weight DSS (Solomon et al., 2010; Chassaing et al., 2014a) or via intragastric catheter (Lee et al., 2009b; Kim et al., 2010) in pigs results in symptomatic features resembling UC (Solomon et al., 2010; Laroui et al., 2012; Randhawa et al., 2014). DSS induces inflammation by disrupting the epithelial barrier, causing vascular and mucosal injury through the exposure of the lamina propria to luminal contents and bacterial antigens (Kitajima et al., 2000; Wirtz et al., 2007; Yan et al., 2009; Solomon et al., 2010). This exposure triggers the activation of inflammatory pathways resulting in an increased production of the inflammatory

cytokines, TNF-α, IL-1β, IL-6, IL-10, IL-12 and IFN-γ (Yan et al., 2009). By adjusting the concentration and duration of DSS treatment, the mechanisms involved in both acute and chronic inflammation can be studied. DSS orally administered at a concentration of 1–5 % for approximately 1 week induces acute inflammation in the intestine (Kawada et al., 2007; Mizoguchi, 2012). However, acute colitis may be extrapolated to chronic colitis by administering DSS for a relatively longer period of time in cycled rotations of DSS treatment followed by a one to two week rest between cycles (Wirtz et al., 2007). DSS colitis in acute phase shows weight loss, diarrhea and occult blood in stools, piloerection, anemia, and eventually death, whereas in chronic phase of colitis usually do not reflect severity of inflammation or histological features found in large bowel (Perse and Cerar, 2012). However, the effectiveness of DSS-induced colitis depends on several factors, including molecular weight (5 kDa for mild and 40 kDa for severe colitis), dosage (usually 1–5 %), duration (acute or chronic), strain of animals (C3H/HeJ and Balb/c mice strains are more susceptible), sex of animals (male mice are more susceptible) and microbial environment of animals (e.g., germ-free [GF] vs. specific pathogen-free [SPF]) (Low et al., 2013a). Although the level of tissue injury can vary between treatment groups with DSS, it is still considered a good model for inducing intestinal injury and is commonly used to stimulate UC like lesions in various animal models.

2.9.4.2.2. Trinitrobenzene sulfonic acid (TNBS)

Trinitrobenzene sulfonic acid is a hapten, which when bound with a high molecular tissue protein, turns into an antigen. TNBS is primarily used to establish 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 and Berstad, 1992).

To become chemically active, TNBS needs to be solubilized in ethanol, and this TNBS ethanol mixture induces intestinal inflammation by altering host proteins through the formation of covalent bonds with trinitrophenyl haptens of TNBS (Elson et al., 1995b), a process that stimulates an immune-mediated inflammatory response. The TNBS-ethanol mixture produces 'hapten modified self-antigens' that are recognized by the host immune system and contribute to acute intestinal inflammation (Elson et al., 1995b). However, ethanol also acts as an irritant that contributes to the damage of the epithelial barrier (Kawada et al., 2007). Also, rectal administration of TNBS in 40–50 % ethanol leads to colon shortening, intestinal hemorrhage, epithelial necrosis causing crypt architecture destruction, and transmural inflammation accompanied by an elevated Th1 immune response within the colon (Kawada et al., 2007; Kremer et al., 2012). Rats treated with TNBS often lose weight, present with bloody diarrhea, and exhibit marked mucosal and transmural intestinal inflammation, similarly to people with IBD (Brenna et al., 2013).

Although intestinal inflammation has been established in rodents, swine, and non-human primates using TNBS as the chemical inducer (Mizoguchi, 2012; Robinson et al., 2014), some studies indicate that mice are the best models for investigating TNBS-ethanol induced colitis (Neurath et al., 2000). Also, when selecting the most appropriate mouse strain to examine TNBS induced tissue injury, the genetic background and phenotypic profile of the mouse are important factors to consider. For example, C57BL/6 and DBA/2 strains are relatively resistant to treatment with TNBS, whereas SJL/J, C3HeJ and BALB/c mice produce significant tissue injury following exposure to TNBS (Elson et al., 1995b; Kawada et al., 2007).

In comparison to DSS, TNBS colitis develops as a delayed-type hypersensitivity reaction to haptenized proteins, whereas DSS colitis is the result of a change in epithelial barrier function

(Brenna et al., 2013; Randhawa et al., 2014).

2.9.4.2.3. Dinitrobenzene sulfonic acid (DNBS)

DNBS is a hapten used to induce colonic inflammation, and the feature of colitis in this model is similar to that of the TNBS model with bloody diarrhea and significant loss of body weight evident but is comparatively less hazardous (Dothel et al., 2013). DNBS and TNBS both bind to proteins, but TNBS has an additional active nitro group and binds more readily at lower concentrations. However, DNBS is more selective and binds only to the e –amino group of lysine (Hawkins et al., 1997; Tran et al., 2012). Earlier, DNBS dissolved in 50 % ethanol, was administered as an enema to induce UC, but the method was modified where colitis is induced in lightly anesthetized mice by an intra-rectal injection, delivered 3 cm into the colon via a polyethylene catheter (Cuzzocrea et al., 2001; Tran et al., 2012).

In DNBS model, the physical, histological features, and inflammatory responses are comparable to observations made in DSS and TNBS models (Ko and Cho, 2005; Ko et al., 2005; Joshi et al., 2011).

2.9.4.2.4. Oxazolone

Oxazolone is an alternative chemical agent that can be used to produce ‘hapten-like proteins’ in the host intestine to induce acute intestinal inflammation (Boirivant et al., 1998). Its use results in intestinal lesions associated with a predominant Th2 immune response. The tissue lesions manifested in mice following exposure to oxazolone are similar to UC like lesions in people, with most lesions causing mucosal ulceration, submucosal edema, and tissue hemorrhaging (Boirivant et al., 1998). In mice, oxazolone administration has been attributed to body weight loss, diarrhea, ulcers, and loss of epithelial cells in the large intestine (Boirivant et al., 1998; Wirtz et al., 2007). One of the advantages of using oxazolone to induce tissue injury is

the rapid progression of tissue architecture alteration in comparison to other chemical agents (Boirivant et al., 1998). Indeed, the relatively fast induction of tissue damage makes oxazolone an ideal candidate to study UC-like disease in mice, as histological evidence shows an increase in IL-4, IL-5, and IL-13, cytokines that are indicative of a Th2 immune response (Wirtz et al., 2007). Similarly to TNBS and DSS, the choice of mouse strain will influence the effects of oxazolone treatment on tissues. As an example, oxazolone treated BALB/c mice show increased tissue injury when compared to C57BL/6 mice under the same treatment (Boirivant et al., 1998). Although oxazolone is an effective inducer of acute inflammation, its effectiveness to induce chronic inflammation remains undetermined, as few investigations have examined its potential to cause long-term intestinal inflammation (Boirivant et al., 1998; Gerlach et al., 2014).

2.9.4.2.5. Azoxymethane (AOM).

Administering a combination of chemical inducers of inflammation can enhance the severity of inflammation. In this context, long-term administration of DSS with AOM induces chronic intestinal inflammation that often progresses to colorectal cancer (Sussman et al., 2012). Notably, treatment with DSS and AOM induce intestinal lesions and alter tissue cytokine profiles in mice resulting in increased expression of IL-4 and IFN-γ, consistent to observations in patients with UC (Dieleman et al., 1998). Thus, the combined use of both chemicals is ideal for investigating both inflammatory diseases of the intestine, as well as the pathophysiology of colorectal tumors.

2.9.4.2.6. Acetic acid

Acetic acid produces acute inflammation restricted to the colon and mimics characteristic features of UC. Ulceration and crypt abnormalities can be induced by luminal instillation of dilute acetic acid in a dose-responsive fashion (Low et al., 2013a). The injury in this model is

related to the epithelial necrosis and edema that variably extends into the gastric mucosal layers, depending on the concentrations and length of exposure (Nakao et al., 2014), and is also associated with activation of NF-kB and other inflammatory mediators (Niu et al., 2013). However, the epithelial injury observed within the first 24 h of acetic acid induction is not immunologic in nature (Low et al., 2013a). The advantages of acetic acid-induced colitis are its low cost and the ease of administration.

2.9.4.2.7. Carrageenan gum

Carrageenan is a high molecular weight sulfated polysaccharide, derived from several species of red seaweeds (Rhodophyceae), including Gigartina, Chondrus and Eucheuma. Based on the degree of sulphation and solubility, it is categorized into three distinct sub types i.e. kappa, iota and lambda. Carrageenan has a higher molecular weight (Tong et al., 1980a; Tobacman, 2001b) and it is generally considered safe; however, its degradation by acid hydrolysis to low molecular weights is associated with ulcerations and cancer-promoting effects (Watt et al., 1979; Tobacman, 2001b). Degraded carrageenan has been used in different animal models (mouse, guinea pig, rat, rabbit, rhesus monkey) to chemically induce intestinal ulceration or inflammation (Watt et al., 1979; Tobacman, 2001b), and induces predominantly mucosal and sub mucosal lesions with histological similarity to UC (Elson et al., 1995b).

2.9.4.3. Genetically engineered or transgenic colitis models

Genetically engineered models are grouped in to two categories: i) conventional gene knockout models, in which an existing gene is inactivated or ‘‘knocked-out’’ and replaced with an artificial piece of DNA; ii) conditional knockout models, in which a specific gene is deleted from a single organ of the body rather than the whole body (Reilly et al., 2012; Goyal et al., 2014). Unlike knockout mice, transgenic mouse models of colitis express one or more copies of

the gene of interest resulting in over expression of that particular gene, and they are also either conventional or conditional transgenic animals (Mizoguchi, 2012; Goyal et al., 2014). Many genetically engineered and transgenic mouse and rat models have been developed and the list continues to grow (Blumberg et al., 1999; Hibi et al., 2002; Mizoguchi, 2012; Goyal et al., 2014)

2.9.4.4. Adoptive transfer of immune cell models

This model involves the adoptive transfer of naïve $CD4^+$ T cells ($CD4^+$ $CD45RB^{high}$ T cells) lacking regulatory cells, from donor mice into syngeneic immune deficient (lymphopenic) severe combined immune deficient (SCID) or $Rag1^{-/-}$ recipient mice, which causes mucosal intestinal inflammation (Powrie et al., 1993; Powrie et al., 1994; Powrie, 1995; Leach et al., 1996; Borm and Bouma, 2004; Steinbach et al., 2015). The $CD4^+CD45RB^{high}$ T cell population that largely consists of naïve effector cells is capable of inducing chronic intestinal inflammation, closely resembling key aspects of human IBD (Powrie et al., 1993; Powrie et al., 1994; Powrie, 1995; Leach et al., 1996; Borm and Bouma, 2004; Steinbach et al., 2015).

The model is useful for unraveling many immunologic and genetic factors contributing to disease and provides outstanding new insights into the predominant role of T cells for mucosal immune regulation (Leach et al., 1996; Wirtz and Neurath, 2000). In addition, the method can be manipulated to study aspects of disease onset and progression, as well as the function of innate, adaptive, and regulatory immune cell populations (Kobayashi et al., 2014; Steinbach et al., 2015).

2.9.4.5. Spontaneous colitis models

Spontaneous models can be divided into two related subcategories such as, animals that spontaneously develop mucosal inflammation, and models in which a gene defect or a transgene causes an inappropriate mucosal immune response (Borm and Bouma, 2004). Spontaneous

models represent one of the most attractive model systems for studying intestinal inflammation because similar to human disease, inflammation occurs without any apparent exogenous manipulations. For example, the C3H/HeJBir murine model of colitis is characterized by spontaneous and chronic focal inflammation localized to the right colon and cecal region (Pizarro et al., 2003; Borm and Bouma, 2004). In addition to ulcers and crypt abscesses, increased levels of IFN-γ and IL-2 have been detected in the lamina propria lymphocyte, which shows that the model represents both UC and CD (Pizarro et al., 2003; Borm and Bouma, 2004). The C3H/HeJBir murine model has also been used in combination with inducible colitis models and is therefore valuable for studying and identifying genetic susceptibility factors (Cong et al., 1998; Jurjus et al., 2004).

Another example is the SAMP1/Yit/Fc mice model in which severe inflammation occurs in the terminal ileum, the primary location of CD lesions (Matsumoto et al., 1998). Lesions in this model are characterized by transmural inflammation, granulomatous and alterations in epithelial morphology, and increased epithelial permeability precedes the onset of inflammation (Rivera-Nieves et al., 2003; Sugawara et al., 2005; Modi et al., 2012).

2.10. Summary and perspectives

In light of the existing literature captured in this review and beyond, it is evident that a lot of research has been done in IBD using both human subjects and animal models. However, it is also clear that despite the amount of research work done, IBD still remains to be an important health concern. Several factors have been implicated in the pathogenesis of IBD including: genetic predisposition, dysregulated immune responses, mucosal barrier defects, environmental triggers and alterations in gut microbiota. Research regarding the contributions of genetics, immune responses, and a number of environmental triggers has helped in the improvement of

clinical treatment and management of IBD including the development of more targeted drugs and screening methods. However, IBD incidence and prevalence continues to grow and, therefore, more research needs to be done. In addition, research regarding the involvement of gut microbiota in IBD pathogenesis is still evolving and even though dysbiosis has been pointed out, the "hen and the egg" question still holds and hence, much awaits to be done. Furthermore, apart from the general dysbiosis, specific bacteria have been associated with IBD pathogenesis but the same question of the cause or consequence effect still applies. There are also challenges of studying the role of microbiota given the effect of other factors including diet and living conditions on the overall composition, distribution and functional activities of the microbiota, as well as the limitations of looking at a specific microbe whose activities/impact might be largely depended on its interactions with the rest of the bacterial community.

Therefore, in addition to the hen and egg question regarding the role of microbiota dysbiosis in IBD pathogenesis, there is need to establish the behavior of pathobionts in the gut, with or without the presence of inflammation. This will help to shed more light on the role played by these bacteria with respect to initiation or exacerbation of inflammation, and the connection between the bacteria with mucosal barrier integrity.

Search into several alternative therapeutic measures has been an active area and a number of therapeutic measures have been investigated. However, some of the results although promising in attenuating microbiota associated IBD complications, the studies have yielded conflicting or less convincing results making it difficult to arrive at a unified conclusion about their use in the management of IBD. As a result, the use of these measures such as fecal microbiota transplantation, prebiotics, probiotics and synbiotics is more confined to research, a last resort alternative, or personal choice, but they are not officially recognized as therapeutic

measures. Therefore, more research is needed in this area in order to maximally investigate the best mode of application, dosage, timing and the length of use so as to facilitate advancement towards a concrete conclusion.

Investigating some disease conditions in IBD, and possible therapeutic measures calls for invasive procedures and is also limited by ethical considerations in the use of human subjects to test products. This therefore calls for use of alternative models that can be extrapolated to humans. Different animal models have thus been developed to combat these limitations and although they are not perfect, they have greatly contributed to the advancement in the IBD research as most of the developments made so far in the diagnosis, treatment and management of IBD may have started from the bench before moving to the bedside.

Therefore, the aim of this thesis was to investigate the impact of adherent invasive *E. coli (*AIEC) strain UM146, and antepartum use of antibiotics on gut microbiota profile and immune responses in mice and pig models of inflammatory bowel disease, and therapeutic roles of prebiotics and probiotics. The main objectives were: a) to investigate the impact of degraded carrageenan gum on gut microbiota profile, and role of AIEC strain UM146 in a pig model of IBD; b) to investigate therapeutic role of Probiotics (*E. coli* strains UM2 and UM7), prebiotics (resistant starch/MSPrebiotic), or their combinations in piglet model of experimental colitis; and c) to investigate the impact of antepartum use of antibiotics on the offspring gut microbial profile and future susceptibility to experimental colitis using a mice model.

CHAPTER THREE

MANUSCRIPT 1

Degraded carrageenan gum and adherent invasive *Escherichia coli* in a piglet model of experimental inflammatory bowel disease: impact on intestinal mucosa-associated microbiota

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3.1. ABSTRACT

Inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC), are chronic conditions characterized by chronic intestinal inflammation. Adherent invasive *Escherichia coli* (AIEC) pathotype has been increasingly implicated in the etiopathogenesis of IBD. In a 21-day study, we investigated the effects of AIEC strain UM146 inoculation on the microbiota profile of the ileal, cecal, ascending and descending colon in a pig model of experimental colitis. Degraded carrageenan gum (CG) was used to induce colitis in weaner piglets whereas AIEC strain UM146 previously isolated from a CD patient was included to investigate a cause or consequence effect in IBD. Treatments were: 1) control; 2) CG; 3) AIEC strain UM146; and 4) CG+UM146. Pigs in groups 2 and 4 received 1% CG in drinking water from day one while pigs in groups 3 and 4 were infected with UM146 on day 8. Following euthanization on day 21, tissue mucosal scrapings were collected and used for DNA extraction. The V4 region of bacterial 16S rRNA was then subjected to Illumina sequencing. Microbial diversity, composition and the predicted functional genome were determined in addition to short chain fatty acids profiles in the digesta and inflammatory cytokines in the intestinal tissue. CG-induced colitis decreased bacterial species richness and shifted community composition. At the phylum level, an increase in Proteobacteria and Deferribacteres and a decrease in Firmicutes, Actinobacteria and Bacteroidetes were observed in CG and CGUM146 compared to control and UM146. The metabolic capacity of the microbiome was also altered in CG and CGUM146 compared to UM146 and control in the colon. We demonstrated that CG resulted in bacterial dysbiosis and shifted community composition similar to what has been previously observed in IBD patients. However, AIEC strain UM146 alone did not cause any clear changes compared to CG or control in our experimental IBD pig model.

Key words: pigs, carrageenan gum, adherent invasive *Escherichia coli*, ulcerative colitis, Crohn's disease, 16S rRNA gene sequencing, microbiome.

3.2. INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are a group of inflammatory conditions related to the colon and small intestines and characterized by chronic inflammation. The etiopathology includes genetic susceptibility, environmental factors, deregulation of the immune system and host relationship with commensal microbiota, as well as abnormal interactions between the intestinal microbiota and immune system (Abraham and Medzhitov, 2011; Khor et al., 2011; Maloy and Powrie, 2011; Manichanh et al., 2012). The intestinal microbiota plays a major role in gut development and in host homeostasis through development of immune regulation via inhibition of responses to inappropriate targets, such as gut contents and allergens, and by turning off inappropriate background inflammation (Hooper et al., 2002; Guarner, 2005). Therefore, abnormal shifts in the intestinal microbiota, termed dysbiosis, may lead to adverse health effects in the host, and could be critical in the pathogenesis and severity of IBD (Manichanh et al., 2006; Frank et al., 2007; Honda and Littman, 2012; Morgan et al., 2012; Fite et al., 2013). In this context, mice genetically modified for IBD do not develop colitis under germ-free conditions (Horwitz, 2007; Vijay-Kumar et al., 2007; Vijay-Kumar et al., 2010), and the therapeutic effect of antibiotics further supports bacterial contribution in the pathogenesis of IBD (Chapman et al., 1986; van Kruiningen, 1995; Vijay-Kumar et al., 2010).

A significant reduction in gut microbial diversity, which is characterized by a marked increase in the phylum Proteobacteria especially within Enterobacteriaceae family, or a decrease in the phylum Firmicutes (*e.g. Faecalibacterium prausnitzii*) has been observed in patients with

IBD (Ott et al., 2004; Lupp et al., 2007; Sepehri et al., 2007; Sokol et al., 2008; Sokol et al., 2009; Friswell et al., 2010; Qiu et al., 2013; Cao et al., 2014; Walters et al., 2014; Wright et al., 2015). Generally, the breakdown in the balance between "mutualistic and commensal" versus "opportunistic and pathogenic" intestinal bacteria, largely characterized by reduced abundance of members of Firmicutes, Actinobacteria and Bacteroidetes, and an increase in Proteobacteria and in some cases Bacteroidetes, is suggested to promote chronic intestinal inflammation (Sokol et al., 2008; Man et al., 2011; Walters et al., 2014; Wright et al., 2015).

Increased abundance of members of the Enterobacteriaceae family, especially *Escherichia coli*, have been observed in humans and dogs with IBD and also in experimental animal models of IBD (Sellon et al., 1998; Darfeuille-Michaud et al., 2004; Schuppler et al., 2004; Mylonaki et al., 2005; Kotlowski et al., 2007; Lupp et al., 2007; Xenoulis et al., 2008; Sepehri et al., 2009; Sepehri et al., 2011; Wright et al., 2015). Although the adherent-invasive *E. coli* (AIEC) pathotype has been repeatedly identified in the intestinal mucosa of patients with CD (Darfeuille-Michaud et al., 1998; Darfeuille-Michaud et al., 2004; Agus et al., 2014b), it is still difficult to pinpoint whether AIEC triggers intestinal inflammation leading to the disease, or if they colonize the gut mucosa as a consequence of pre-existing inflammation (Martin et al., 2004; Agus et al., 2014b). Therefore, a cause and consequence relationship between *E. coli* strains and IBD is yet to be determined. To further investigate the role of AIEC in IBD, several AIEC strains, including LF82 and UM146, have been isolated from IBD patients and characterized (Miquel et al., 2010; Krause et al., 2011; Sepehri et al., 2011; Desilets et al., 2015). Strain UM146 was previously isolated in our lab from a CD patient and shown to be able to invade and replicate within macrophages, which is characteristic of an AIEC (Krause et al., 2011; Sepehri et al., 2011).

In the present study, we used a pig model of CG-induced colitis to investigate the possible role of AIEC strain UM146 in IBD. Pigs share a similar gastrointestinal morphology and physiology with humans (Miller and Ullrey, 1987; Heinritz et al., 2013), which makes them more suitable model for human studies. Degraded carrageenan has been used in different animal models (mouse, guinea pig, rat, rabbit, rhesus monkey) to chemically induce intestinal ulceration or inflammation (Watt et al., 1979; Tobacman, 2001b). The focus of these studies has been on the dynamic and profile of mucosal response in relation to CG-induced colitis and its similarity to that observed in IBD patients. However, there is no detailed and clear understanding of the structural and functional alterations of the intestinal microbiota in response to CG- induced colitis.

Here, we used Illumina high throughput sequencing of the 16SrRNA and inferred metagenomics by PICRUSt to investigate differences in microbial composition and function in the ileum, cecum, ascending and descending colon tissue samples of piglets with CG-induced colitis and infected with AIEC strain UM146. We demonstrated that CG-induced colitis influenced bacterial diversity and caused community composition changes at the phylum and lower taxonomical levels that are comparable to microbial changes observed in IBD patients. However, these changes were not observed in AIEC strain UM146 treatment. Similarly, the combination of CG and UM146 did not significantly influence the extent of the observed changes during CG-induced colitis.

3.3. MATERIALS AND METHODS

3.3.1. Animals and housing

A total of 24 male piglets [Duroc × (Yorkshire × Landrace)] weaned at 17 ± 2 d were obtained from Sunnyside Colony (Newton Siding, Manitoba, Canada). The pigs were housed in a

temperature-controlled room within the T. K. Cheung Center for Animal Science Research, University of Manitoba (Winnipeg, MB, Canada). Room temperatures were maintained at 30°C during week (wk) 1 and 29°C during wk 2 and 3, with a 16 h lighting system. All pigs had *ad libitum* access to water and a basal diet in mash form formulated to meet or exceed the National Research Council (NRC, 2012) recommendations for a 7 to 11 kg pig. The experiment lasted for 21 days and the pigs were allowed to acclimate for 3 days before the start of experimental treatments.

3.3.2. Ethical considerations

The procedures were approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee, and the pigs were cared for according to the guidelines of the Canadian Council of Animal Care (CCAC, 1993).

3.3.3. Degraded carrageenan gum (CG)

To induce mild ulcers on the intestinal tract of pigs, a 1% CG solution prepared from carrageenan powder (CarboMer, Inc. San Diego, CA, USA), a sulphated polysaccharide that induces predominantly mucosal and sub mucosal lesions with histological similarity to UC (Elson et al., 1995b), was administered. The CG was administered via drinking water using elevated jugs connected to normal drinking nipples. The 1% concentration was chosen based on a pilot study conducted prior to this experiment (data not shown) where 0, 1, 2 and 4% CG concentrations were tested. Administration of 1% CG in drinking water only induced a mild injury in the pigs' gastrointestinal (GI) tract with varying degree of mucosa and sub mucosal edema, but without any granulomatous inflammation which is similar to what is observed in UC.

3.3.4. Preparation of degraded carrageenan gum

Un-degraded carrageenan has molecular weights of 1.5×10^6 to 2×10^7 (Tong et al., 1980b; Tobacman, 2001a) and it is generally considered safe; however, its degradation to low molecular weights is associated with ulcerations and cancer-promoting effects (Watt et al., 1979; Tobacman, 2001b). The solution used in this study was prepared by acid hydrolysis according to the procedure described previously (Watt et al., 1979), and is expected to yield a degraded carrageenan of average molecular weight of 20,000 to 30,000 (Weiner, 1991; Marcus et al., 1992). Briefly, to each gram of the dry powder, one mL of concentrated HCl was added in a glass beaker and thoroughly mixed at room temperature (22°C) with a glass rod for 1 h. After 1 h, distilled water was added while stirring the mixture. The acidified solution was neutralized with 2M sodium hydroxide to pH of 7-8 and the volume was adjusted to give a 1% concentration.

3.3.5. Preparation of AIEC strain UM146

Adherent invasive *E. coli* (AIEC) strain UM146 previously isolated from a human subject with CD (Sepehri et al., 2011) was used in this study. The strain was cultured aerobically in LB broth and incubated at 37°C overnight to achieve a concentration of 10^8-10^{10} CFU/mL, and 100 mL of the overnight culture was used for dosing as described below.

3.3.6. Induction of colitis and infection with AIEC strain UM146

Pigs were weighed and randomly assigned to 4 treatment groups with 3 pigs per pen and 2 replicate pens per treatment. The treatments were as follows: 1) control; 2) pigs receiving 1% CG in drinking water; 3) pigs infected with AIEC strain UM146; and 4) as in 2 and 3; CGUM146. From day 1 of the study, pigs that were assigned to receive CG were given a 1% CG solution in overhead plastic jugs connected to normal drinking water nipples. Administration of CG was

done on a daily basis until end of the study (21 d). The CG solution level was monitored and jugs were refilled with freshly prepared CG every morning (at 9: 30 am) and in the evening (at 4:30 pm), if needed. On day 8 of the study, pigs that were assigned to be inoculated with UM146 were given an overnight culture of the AIEC strain UM146 (**Table 3.1**). In this case, for each pig, 100 mL of the overnight culture was mixed with small amount of feed and the animals were allowed to consume the freshly mixed feed first.

3.3.7. Fecal score

Severity of diarrhea was characterized in a treatment-blinded manner by two trained individuals using a fecal consistency scoring system, as described previously (Marquardt et al., 1999). The scoring system was as follows: 0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea. Scoring was done at 48 h, 96 h, 7 d, and 14 d after inoculation with AIEC strain UM146.

3.3.8. Tissue and digesta sampling

On days 21 and 22 of the study, all pigs were sedated by intramuscular injection of Ketamine:Xylazine (20:2 mg/kg BW) and euthanized by an intracardiac injection of 110 mg/kg BW sodium pentobarbital (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). The abdominal cavity was opened from sternum to pubis to expose the gastrointestinal tract without damaging the wall of the digestive tract. The small intestine was stripped free of its mesentery and ileal, cecal and colon digesta samples were obtained, divided into two sub-samples and transferred to sterile sample bags. One sub-sample of the digesta was used for determination of pH, whereas the second sub-sample was kept on ice and later transferred to -20°C for later analysis of volatile fatty acids (VFA). Tissue samples (two 5 cm long segments) were collected from the ileum, cecum, ascending and descending colon, flushed with sterile saline to remove

Table 3.1. Schedule of CG and adherent invasive *Escherichia coli* (AIEC) strain UM146 administration[1]

Treatment administration	Study day																				
	Adaptation period (3 days)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CG (1% in drinking water; ad lib)		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AIEC strain UM146									+													

[1]The first 3 d served as adaptation period, CG was administered from d 1 of the study until the end of the study, whilst administration of UM146 was done on d 8 of the study only.

excess lumen contents, immediately frozen in liquid nitrogen and transferred to -80°C until used for DNA extraction and further molecular/microbial analyses.

3.3.9. Analysis of pH, ammonia N and volatile fatty acids

pH was measured immediately after digesta collection using an Accumet Basic 15 pH meter (Fisher Scientific, Fairlawn, NJ) equipped with a Sensorex 450C Flat Surface Combination pH/Reference Electrode (Sensorex, Stanton, CA), which was standardized with certified pH 4 and 7 buffer solutions. Ammonia nitrogen concentration was measured using a calorimetric technique as described previously (Novozamsky et al., 1974), while volatile fatty acids (VFA) were determined using gas chromatography (Bhandari et al., 2007).

3.3.10. Characterization of inflammatory responses

Tissue samples were homogenized (50 mg/mL) in Tris lysis buffer (Meso scale discovery diagnostics, Rockvile, MD, USA) containing protease inhibitors (Roche, Mississauga, ON, Canada). Samples were centrifuged at 3000 × g for 10 min and the supernatant was recovered and stored at -80°C until analyzed. Cytokine levels [interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrotic factor (TNF)-α] were determined using a custom meso scale porcine kit (Meso scale discovery diagnostics, Rockville, MD, USA), according to the manufacturer's instructions.

3.3.11. Correlation coefficients

Associations between bacterial taxa with an abundance ≥ 0.5% of community in the ileum, cecum, and ascending colon and short chain fatty acids (acetate, propionate, butyrate), or inflammatory markers (IL-1b, IL-6, IL-8, IL-10, TNF-a) were explored using non-parametric Spearman's rank correlation implemented in PAST software (Hammer et al., 2001). For each correlation, correlation coefficient (Spearman's Rho) and *P*-values were obtained and the

resulting correlation matrix was visualized in a heat map 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.

3.3.12. DNA extraction

Tissue samples were thawed at room temperature. The inner wall was then gently scrapped with a blunt blade to obtain 200-300 mg of mucosa, of which, approximately 50 mg was used for DNA extraction. DNA was extracted using a ZR Tissue and Insect DNA kit (Zymo Research Corp., Orange, CA), which included a bead-beating step for the mechanical lysis of the microbial cells. DNA concentration was determined using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the DNA quality was evaluated by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3'), as previously described (Khafipour et al., 2009). Amplicons were verified using agarose gel electrophoresis.

3.3.13. Library construction and Illumina sequencing

The V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al., 2012), as previously described (Derakhshani et al., 2015). 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 Master Mix (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 Master cycler 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 Pico green (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 Qubit 2.0 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´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-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). The sequencing data were uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed through accession number SRR 2601043.

3.3.14. Bioinformatics analyses

The PANDAseq assembler (Masella et al., 2012) was used to merge overlapping paired-

end Illumina fastq files. All the sequences with low quality base calling scores as well as those containing uncalled bases in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipeline of the open source software package QIIME (Caporaso et al., 2010b). Assembled reads were demultiplexed according to the barcode sequences and 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 RDP classifier (Wang et al., 2007) and aligned with the Greengenes (v. 13.5) reference database (DeSantis et al., 2006) using PyNAST algorithms (Caporaso et al., 2010a). Phylogenetic tree was built with FastTree 2.1.3. (Price et al., 2010).

3.3.15. Alpha- and beta-diversities

Within-community diversity (α-diversity) was calculated by different indices of species richness and evenness including observed number of species, Chao1, abundance-based coverage estimator (ACE), Shannon, Simpson, Inverse Simpson (InvSimpson), and Fisher using the open source R software (3.1.0). The *P* values were calculated using the SAS Proc mixed procedure (SAS 9.3). An even depth of 5000, 4000, 12000 and 10000 sequences per sample was used to calculate the richness and diversity indices for the ileum, cecum, ascending and descending colon, respectively. To assess the beta-diversity (β-diversity) differences among bacterial communities from different treatments within each compartment, non-metric multidimensional scaling (nMDS) ordination plots were generated using R software (3.1.0) by employing Bray-Curtis similarity matrices with a conventional cut-off of < 0.2 for the stress value. 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 (PERMANOVA) (Anderson, 2005) was performed and *P* values were calculated.

3.3.16. Partial least square discriminant analysis

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.02, Umetrics, Umea, Sweden) was performed on the lower taxonomic data to identify the effects of CG and UM146 (Li et al., 2012). The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the variables on X. In this case, X variables were bacterial genera and Y was observations of different treatments compared together. To avoid over-parameterization of the model, variable influence on projection value (VIP) was estimated for each taxa, and taxa with VIP < 0.5 were removed from the final model. R^2 estimates were used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. Data are presented in loading scatter plots. The PLS-DA regression coefficients were used to identify taxa that were positively or negatively correlated with each treatment group. The significant shifts of taxa were determined when the error bars of each component was above or below x axis of coefficient plot.

3.3.17. 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 on the Greengene picked OTUs 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., 2010). To determine the functional KEGG pathways that could be associated with the microbial changes observed, we compared the functional pathways for the mucosal microbiota of samples from CG and CGUM146 to control or UM146 at all the intestinal sites (ileum, cecum, ascending and descending colon).

3.3.18. Other statistical analysis

For the fecal score, cytokines, VFAs, pH, ammonia and the phylum data, the treatment effect was evaluated using a completely randomized design and the data was subjected to ANOVA using the SAS PROC MIXED procedure (SAS 9.3). Differences between means were determined using Tukey's test. SAS UNIVARIATE procedure was used to test the normality of residuals. For non-normally distributed data, Poisson and negative binomial distributions were fitted in the GLIMMIX procedure of SAS and the goodness of fit for different distributions was determined using Pearson chi-square /DF (closer to 1 is better). In both MIXED and GLIMMIX models, the effect of treatment was considered fixed and pig were treated as random factor. The differences between treatments were considered significant at $P < 0.05$.

3.4. RESULTS

3.4.1. Diarrhea post-infection with AIEC strain UM146

At 48 h post infection, diarrhea was observed in colitic pigs that were also infected with *E. coli* (CGUM146 treatment) but not in the other treatment groups (**Figure 3.1 a**). At 96 h post

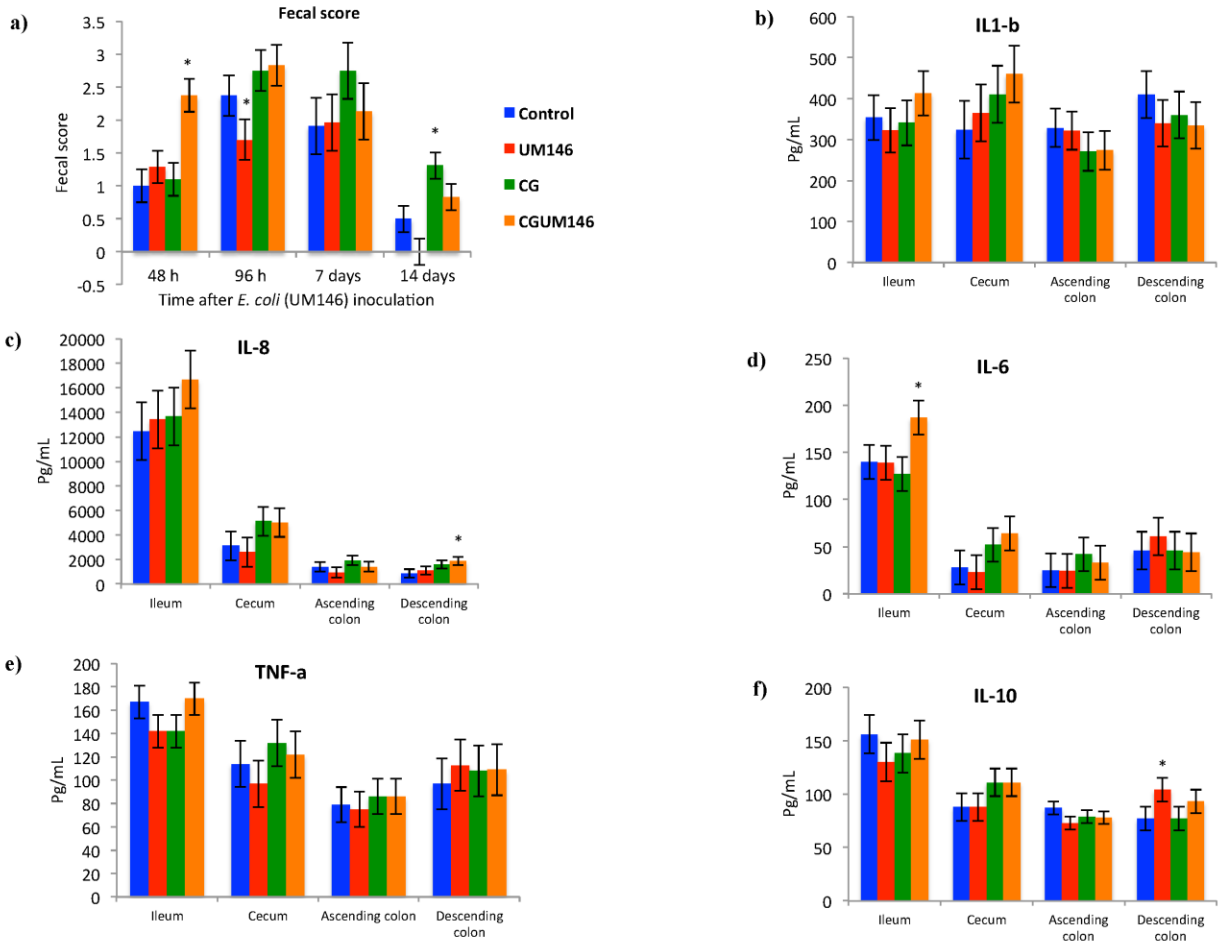


Figure 3.1 (a-f). The effect of degraded carrageenan gum (CG) and adherent invasive *Escherichia coli* (AIEC) strain UM146 inoculation on fecal score, as determined by stool consistency, and on inflammatory markers (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in weaned pigs. CG was administered to the designated groups from day 1 of the experiment. CG: pigs received 1% CG only in drinking water daily; UM146: pigs were inoculated with AIEC UM146 on day 8; CGUM146: pigs received CG from day 1 and were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed. Severity of diarrhea was characterized using an established fecal consistency (FC) score system in pigs (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea). *Designates a significant difference compared to other groups; $P \leq 0.05$.

inoculation, pigs in UM146 treatment had soft feces (an average fecal score of 1.5) compared to pigs in other treatments that experienced mild diarrhea (an average fecal score of 2.5).

3.4.2. Volatile fatty acids, ammonium N, and pH in the ileum, cecum and colon

As shown in **Table 3.2**, ammonium N and pH were similar among treatments in the ileum, cecum and colon except for a lower ammonium N in the cecum that was observed in the UM146 treatment compared to the control. VFA concentrations were not different among treatment groups in the ileum. In both cecum and colon, butyrate was significantly lower in the CG treatment compared to UM146, but the difference wasn't significant compared to other treatments. In addition, colonic acetate was significantly lower in both CG and CGUM146 treatments compared to the control, but there was no significant difference compared to the UM146 treatment.

3.4.3. Inflammatory cytokines in the ileum, cecum, ascending and descending colon

Among the cytokines analyzed in the four intestinal segments on day 21, CGUM146 treatment up-regulated IL-8 and IL-6 in the descending colon and ileum, respectively, whereas UM146 up-regulated IL-10 in the descending colon. IL-1β and TNF-α did not differ among treatment groups in all intestinal segments examined (**Figure 3.1b-f**).

3.4.4. Correlation coefficient

As shown in **Table 3.3** and **Figures 3.2 a-c:** several taxa were positively or negatively correlated with various short chain fatty acids and/or inflammatory markers in the ileum, cecum and ascending colon.

3.4.5. Alpha-diversity in different intestinal segments

As shown in **Table 3.4**, no significant difference was observed among treatment groups in the ileum. Also, according to most of the diversity indices calculated, lower bacterial diversity

Table 3.2. Effect of degraded carrageenan gum (CG)[1] and adhered invasive *Escherichia coli* (AIEC) strain UM146[2] inoculation on microbial activities in the ileum, cecum and colon digesta of weaned pigs, as determined by changes in the level of pH, ammonium nitrogen (N), and volatile fatty acids (VFA).

Item	Treatment[3]				SED[4]	*P value*[5]
	Control	CG	UM146	CGUM146		
pH						
Ileum	6.00	6.06	5.62	6.20	0.37	0.4667
Cecum	5.63	5.59	5.55	5.68	0.17	0.9044
Colon	5.84	6.01	5.82	5.84	0.15	0.6118
Ammonium N, mg/dL						
Ileum	23.86	23.62	19.13	19.97	3.4	0.4165
Cecum	17.01^{a}	14.43^{ab}	10.51^{b}	14.61^{ab}	2.3	0.0729
Colon	35.95	30.83	30.75	27.83	4.7	0.4113
Ileum VFA, mmol/mL						
Acetate	8.17	6.47	4.33	6.57	1.48	0.1590
Propionate	0.25	0.23	0.11	0.30	0.11	0.4697
Butyrate	0.39	0.04	0.69	0.37	0.35	0.3823
Valerate	0.29	0.21	0.22	0.27	0.09	0.7313
Iso-butyrate	0.49	0.37	0.28	0.28	0.4	0.9473
Iso-valerate	0.48	0.33	0.42	0.38	0.16	0.8369
Cecal VFA, mmol/mL						
Acetate	48.6	38	56	45.54	6.8	0.1017
Propionate	24.86	28	32.37	26.12	4.4	0.3785
Butyrate	13.94^{ab}	8.9^{b}	21.42^{a}	18.58^{ab}	4.1	**0.0352**
Valerate	3.52	2.39	6.09	6.55	1.7	0.0695
Iso-butyrate	0.12	0.20	0.32	0.23	0.09	0.2715
Iso-valerate	0.31	0.35	0.55	0.51	0.15	0.3391
Colon VFA, mmol/mL						
Acetate	54.88^{a}	42.12^{b}	50.34^{ab}	42.35^{b}	4.3	**0.0185**
Propionate	24.39	24.69	27.04	20	3.3	0.2390
Butyrate	14.91^{ab}	9.75^{b}	17.06^{a}	13.79^{ab}	1.9	**0.0114**
Valerate	4.37	3.36	5.17	4.86	1.02	0.3377

Iso-butyrate	0.3	0.14	0.34	0.21	0.08	0.1023
Iso-valerate	0.81	0.67	0.80	0.64	0.18	0.7186

[ab] Means with different superscripts within the same row differs significantly; $P < 0.05$.

[1]CG was administered to the designated groups from day 1 of the experiment. Pigs received 1% CG in drinking water on daily basis.

[2]*AIEC* UM146 inoculation was administered on day 8. Pigs in designated groups received 100 mL of an overnight *E. coli* UM146 culture (10^8 CFU/mL) in feed.

[3]CG=pigs received 1% CG only in drinking water daily; UM146=pigs were inoculated with *E. coli* UM146 on day 8; CGUM146= pigs received 1 % CG from day 1 of the study and were inoculated with *AIEC* UM146 on day 8.

[4]SED: standard error of difference between treatment means.

[5]Significant *P*-values are highlighted with bold font.

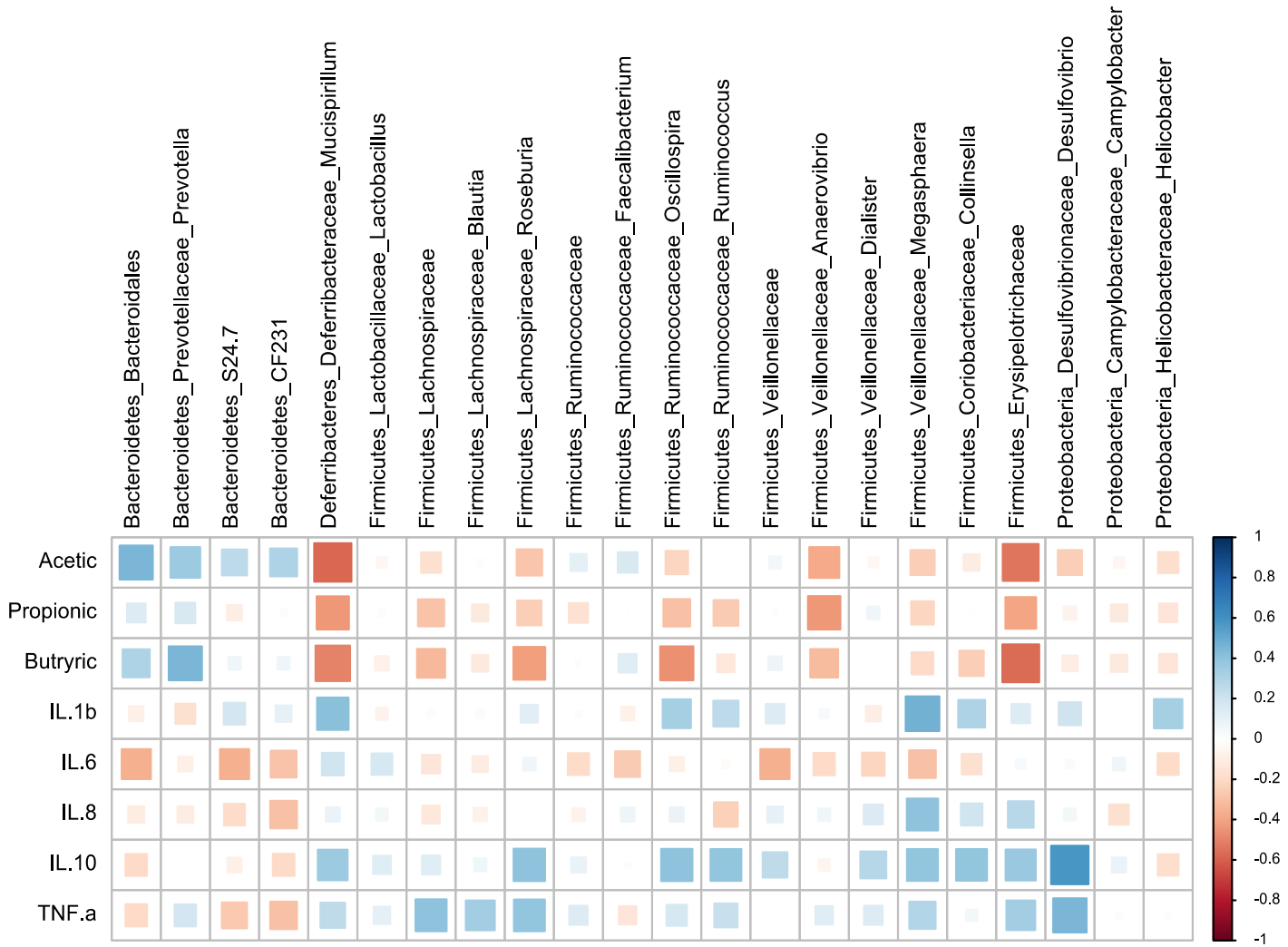
Table 3.3. Correlation coefficient between selected taxa[1] and short chain fatty acids (acetic, propionic, butyric acid) and inflammatory markers in the ileum, cecum and ascending colon.

GI segment	Taxa	Cytokine or SCFA	Rho (ρ)	*P value*
Ileum	g. *Ruminococcus*	IL-1β	0.4714	0.026
		IL-6	0.4987	0.018
		IL-10	0.4498	0.035
	f. Enterobacteriaceae	IL-1β	0.4254	0.048
		IL-8	0.5594	0.006
	g. *Prevotella*	IL-6	0.4411	0.039
		IL-10	0.4509	0.035
	f. Clostridiaceae	IL-6	0.6862	< 0.001
	g. *Escherichia*	IL-8	0.4226	0.050
	g. *Faecalibacterium*	IL-10	0.5439	0.008
		Butyrate	0.4696	0.027
	g. *Lactobacillus*	IL-10	-0.4617	0.030
	g. *Helicobacter*	Acetate	0.4229	0.049
	g. *Streptococcus*	Propionate	0.4336	0.043
Cecum				
	g. *Megasphaera*	IL-1β	0.4743	0.022
	g. *Desulfovibrio*	IL-10	0.58	0.003
		TNF-α	0.4599	0.027
	o. Bacteroidales	Acetate	0.4555	0.028
	g. *Mucispirillum*	Acetate	-0.579	0.003
		Propionate	-0.4328	0.039
		Butyrate	-0.499	0.015
	f. Erysipelotrichaceae	Acetate	-0.5365	0.008
		Butyrate	-0.5622	0.005
	g. *Anaerovibrio*	Propionate	-0.4367	0.037
	g. *Prevotella*	Butyrate	0.4555	0.028
	g. *Roseburia*	Butyrate	-0.4189	0.046
	g. *Oscillospira*	Butyrate	-0.4555	0.028
Ascending colon				
	o. Clostridiales	IL-6	0.5474	0.005
		IL-8	-0.54	0.006
		Acetate	0.6139	0.001
	f. Ruminococcaceae	IL-6	0.5062	0.011
		IL-8	-0.4426	0.030
		Acetate	0.6573	< 0.001
	g. *Oscillospira*	IL-6	0.5092	0.011
		IL-8	-0.4773	0.018
		Acetate	0.5391	0.006
	g. *Ruminococcus*	IL-6	0.5728	0.003
		IL-8	-0.4547	0.025
		Acetate	0.5426	0.006
		Butyrate	0.4539	0.025

GI segment	Taxa	Cytokine or SCFA	Rho (ρ)	*P value*
	g. *Desulfovibrio*	IL-6	0.6121	0.001
		IL-8	0.6121	0.001
		Acetate	-0.4287	0.036
		Butyrate	-0.5887	0.002
	g. *Mucispirillum*	IL-6	-0.4274	0.037
		IL-8	0.5634	0.004
		Acetate	-0.7356	< 0.001
		Butyrate	-0.6173	0.001
	o. RF39	IL-6	-0.4134	0.044
		IL-8	-0.4134	0.044
		Acetate	0.4569	0.024
	g. *Bacteroides*	IL-8	0.5649	0.004
		Acetate	-0.5231	0.008
	g. *Prevotella*	IL-8	-0.433	0.034
	f. S24-7	IL-6	0.4835	0.016
		IL-8	-0.4791	0.017
		IL-10	-0.414	0.044
		Acetate	0.7452	< 0.001
	g. *CF231*	IL-8	-0.4596	0.023
	g. *Helicobacter*	IL-10	-0.4066	0.048
	f. Veillonellaceae	TNF-α	-0.5579	0.004
	g. *Blautia*	Acetate	0.4321	0.034
	g. *Treponema*	Propionate	0.4217	0.040
	g. *Lactobacillus*	Propionate	-0.4939	0.014
	f. Streptococcaceae	Propionate	-0.452	0.026
		Butyrate	-0.4295	0.036
	g. *Campylobacter*	Acetate	-0.5188	0.009
		Butyrate	-0.5366	0.006
	g. *Campylobacter*	Acetate	-0.5188	0.009
		Butyrate	-0.5366	0.006

***** Taxa with relative abundance ≥ 0.5% of the population were used for the correlation analysis and only the significant correlations are shown in the Table. SCFA, short chain fatty acid

b) Cecum


Bacteroidetes_Bacteroidales
Bacteroidetes_Prevotellaceae_Prevotella
Bacteroidetes_S24.7
Bacteroidetes_CF231
Deferribacteres_Deferribacteraceae_Mucispirillum
Firmicutes_Lactobacillaceae_Lactobacillus
Firmicutes_Lachnospiraceae
Firmicutes_Lachnospiraceae_Blautia
Firmicutes_Lachnospiraceae_Roseburia
Firmicutes_Ruminococcaceae
Firmicutes_Ruminococcaceae_Faecalibacterium
Firmicutes_Ruminococcaceae_Oscillospira
Firmicutes_Ruminococcaceae_Ruminococcus
Firmicutes_Veillonellaceae
Firmicutes_Veillonellaceae_Anaerovibrio
Firmicutes_Veillonellaceae_Dialister
Firmicutes_Veillonellaceae_Megasphaera
Firmicutes_Coriobacteriaceae_Collinsella
Firmicutes_Erysipelotrichaceae
Proteobacteria_Desulfovibrionaceae_Desulfovibrio
Proteobacteria_Campylobacteraceae_Campylobacter
Proteobacteria_Helicobacteraceae_Helicobacter
Acetic
Propionic
Butryric
IL.1b
IL.6
IL.8
IL.10
TNF.a
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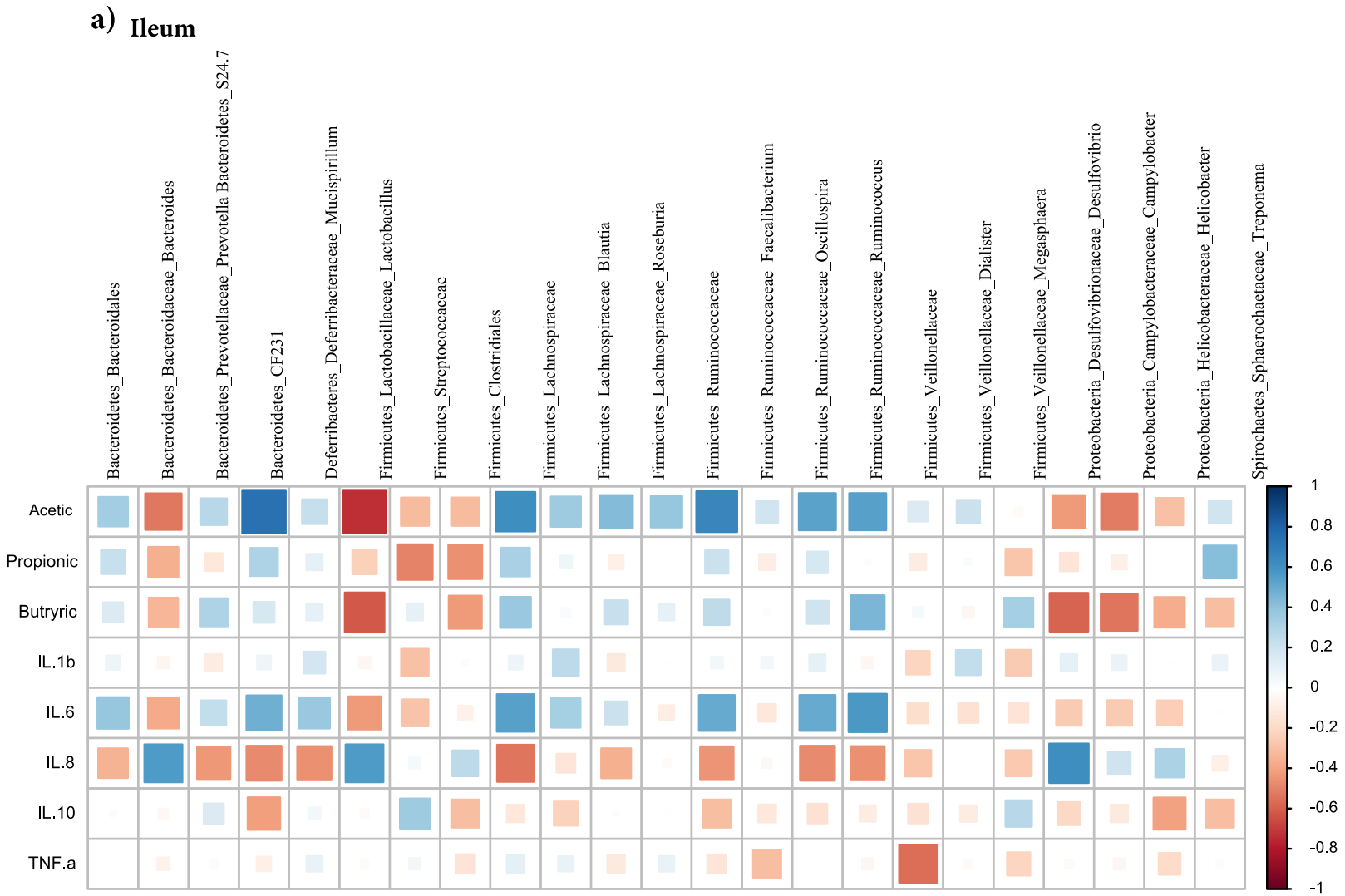
-0.4
-0.6
-0.8
-1


a) Ileum
Bacteroidetes_Bacteroidales
Bacteroidetes_Bacteroidaceae_Bacteroides
Bacteroidetes_Prevotellaceae_Prevotella Bacteroidetes_S24.7
Bacteroidetes_CF231
Deferribacteres_Deferribacteraceae_Mucispirillum
Firmicutes_Lactobacillaceae_Lactobacillus
Firmicutes_Streptococcaceae
Firmicutes_Clostridiales
Firmicutes_Lachnospiraceae
Firmicutes_Lachnospiraceae_Blautia
Firmicutes_Lachnospiraceae_Roseburia
Firmicutes_Ruminococcaceae
Firmicutes_Ruminococcaceae_Faecalibacterium
Firmicutes_Ruminococcaceae_Oscillospira
Firmicutes_Ruminococcaceae_Ruminococcus
Firmicutes_Veillonellaceae
Firmicutes_Veillonellaceae_Dialister
Firmicutes_Veillonellaceae_Megasphaera
Proteobacteria_Desulfovibrionaceae_Desulfovibrio
Proteobacteria_Campylobacteraceae_Campylobacter
Proteobacteria_Helicobacteraceae_Helicobacter
Spirochaetes_Sphaerochaetaceae_Treponema
Acetic
Propionic
Butyric
IL.1b
IL.6
IL.8
IL.10
TNF.a
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0.4
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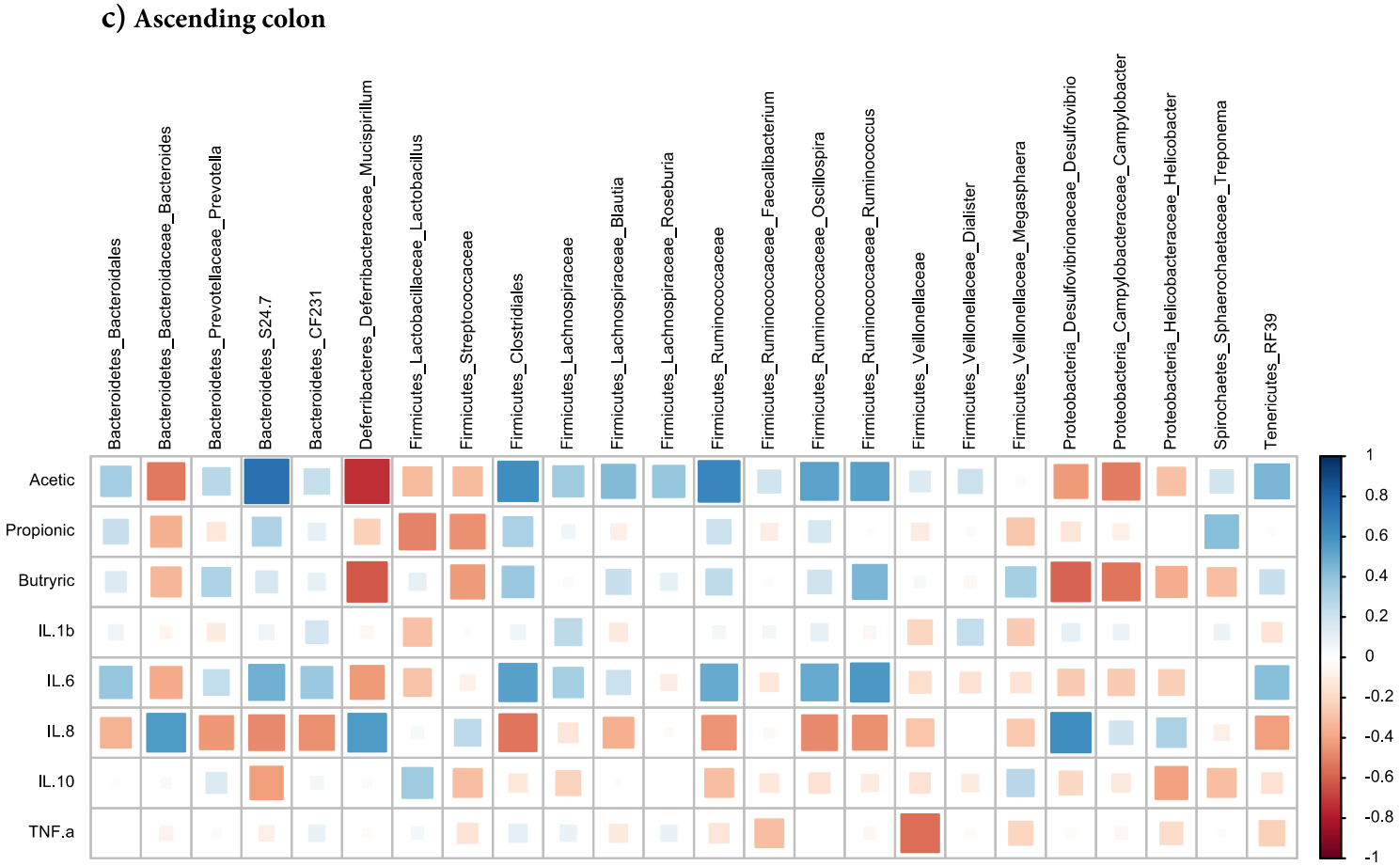


Figure 3.2. Correlation coefficient of selected taxa with short chain fatty acids (acetic, propionic and butyric acid) and inflammatory markers in the a) ileum, b) cecum, and c) ascending colon. The blue color shows a positive correlation while the coral color shows a negative correlation. The intensity of the color depicts the strength of the correlation.

Table 3.4. Alpha-diversity indices of the ileal, cecal, ascending, and descending colon mucosa-associated microbiota of pigs treated with degraded carrageenan gum (CG)[1] and inoculated with adhered invasive *Escherichia coli* (AIEC) strain UM146[2]

Items	Treatment[3]				SED[4]	*P value*[5]
	Control	UM146	CG	CGUM146		
Ileum						
Observed species	95.167	100.25	105.7	147.17	36.2	0.4343
Chao1	176.75	163.61	179.67	245.36	57.4	0.5419
ACE	191.2	170.51	202.32	263.2	68.3	0.5625
Shannon	1.714	1.767	1.986	2.641	0.6	0.4542
Simpson	0.588	0.484	0.608	0.768	0.15	0.4030
InvSimpson	2.933	2.633	2.596	5.357	1.1	0.0647
Fisher	22.148	25.93	24.13	37.806	10.2	0.4701
Cecum						
Observed species	442.29^{a}	422.33^{a}	255.71^{b}	337.5^{ab}	68.5	**0.0503**
Chao1	765.11^{a}	736.57^{a}	439.83^{b}	623.27^{ab}	124	**0.0701**
ACE	845.64^{a}	790.69^{a}	482.55^{b}	576.43^{ab}	115	**0.0140**
Shannon	3.503	3.478	2.422	2.918	0.47	0.1190
Simpson	0.906^{a}	0.928^{a}	0.701^{b}	0.805^{ab}	0.07	**0.0230**
InvSimpson	13.185^{a}	13.548^{a}	4.004^{b}	5.409^{b}	3.4	**0.0175**
Fisher	120.14^{a}	122.11^{a}	58.258^{b}	84.919^{ab}	20.8	**0.0216**
Ascending colon						
Observed species	912.43^{a}	807.33^{ab}	574^{b}	537.22^{b}	126	**0.0188**
Chao1	1597.16^{a}	1329.93^{a}	985.36^{ab}	783.04^{b}	268	**0.0272**
ACE	1737.07^{a}	1425.97^{ab}	1049.47^{ab}	826.98^{b}	296	**0.0250**
Shannon	4.256^{a}	4.205^{ab}	3.126^{b}	3.591^{ab}	0.04	0.0552
Simpson	0.9326^{a}	0.9254^{a}	0.7749^{b}	0.8821^{ab}	0.06	**0.0445**
InvSimpson	21.4548^{a}	18.2106^{a}	5.1508^{b}	10.1446^{ab}	4.7	**0.0074**
Fisher	242.25^{a}	201.67^{ab}	137.39^{b}	121.39^{b}	27.8	**0.0221**
Descending colon						
Observed species	1012.29^{a}	989.67^{a}	652.14^{b}	680.29^{b}	143	**0.0298**

Chao1	1800.9	1726.7	1195.66	1180.2	332	0.1390
ACE	1942.05	1928.19	1280.03	1331.88	360	0.1465
Shannon	0.9733[a]	0.9591[a]	0.8623[b]	0.9033[ab]	0.03	**0.0058**
Simpson	289.74	280.52	165.38	176.3	47.9	**0.0259**
InvSimpson	40.7524[a]	28.5449[ab]	13.3929[b]	12.6113[b]	6.0	**0.0002**
Fisher	289.74[a]	280.52[ab]	165.38[b]	176.3[ab]	47.8	**0.0259**

[ab] Means with different superscripts within the same row differs significantly; $P < 0.05$.

[1]CG was administered to the designated groups from day 1 of the experiment. Pigs received 1% CG in drinking water on daily basis.

[2]*AIEC* UM146 inoculation was administered on day 8. Pigs in designated groups received 100 mL of an overnight *E. coli* UM146 culture (108 CFU/mL) in feed.

[3]CG=pigs received 1% CG only in drinking water daily; UM146=pigs were inoculated with *E. coli* UM146 on day 8; CGUM146= pigs received 1 % CG from day 1 of the study and were inoculated with *AIEC* UM146 on day 8.

[4]SED: standard error of difference between treatment means.

[5]Significant *P*-values are highlighted with bold font.

was observed in both CG and CGUM146 treatments compared to control and UM146 in the cecum, ascending and descending colon.

3.4.6. Beta-diversity differences among treatment groups in the ileum, cecum, ascending and descending colon

As shown in **Figures 3.3 a-d**, PERMANOVA analysis of beta-diversity data showed significant difference between treatment groups in the cecum (P = 0.013) and descending colon (P = 0.0014). In this context, cecal and descending colon samples clustered separately according to treatment status of the pig, suggesting that the samples are composed of distinct bacterial communities. In the ileum and ascending colon although overall P values were not significant (ileum, P = 0.0612; ascending colon, P = 0.0545) pair-wise comparisons between control vs. CGUM146 (P = 0.0327) and UM146 vs. CG (P = 0.0287) in the ileum, and UM146 vs. CGUM146 (P = 0.0145) in the ascending colon were significant.

3.4.7. Microbiota composition at phylum and lower taxonomic levels in different intestinal segments

Ileum: A total of 266,412 quality-filtered sequences were obtained from the samples with an average of 11,583 sequences per sample. Fourteen phyla were identified in all the samples. Among the most abundant phyla, Firmicutes was the most dominant phylum but did not differ significantly between treatment groups **(Figure 3.4 a).** Classification of OTUs at lower taxonomical levels resulted in the identification of 191 taxa of which 95 were ≥ 0.01% of the community, while 96 taxa were < 0.01% of the community. Bacterial taxa with relative abundance of greater than 0.01% of community were analyzed using PLS-DA to identify taxa that were most characteristic of different treatment groups and the results are shown in **Figure 3.5** (**a-b**)**.**

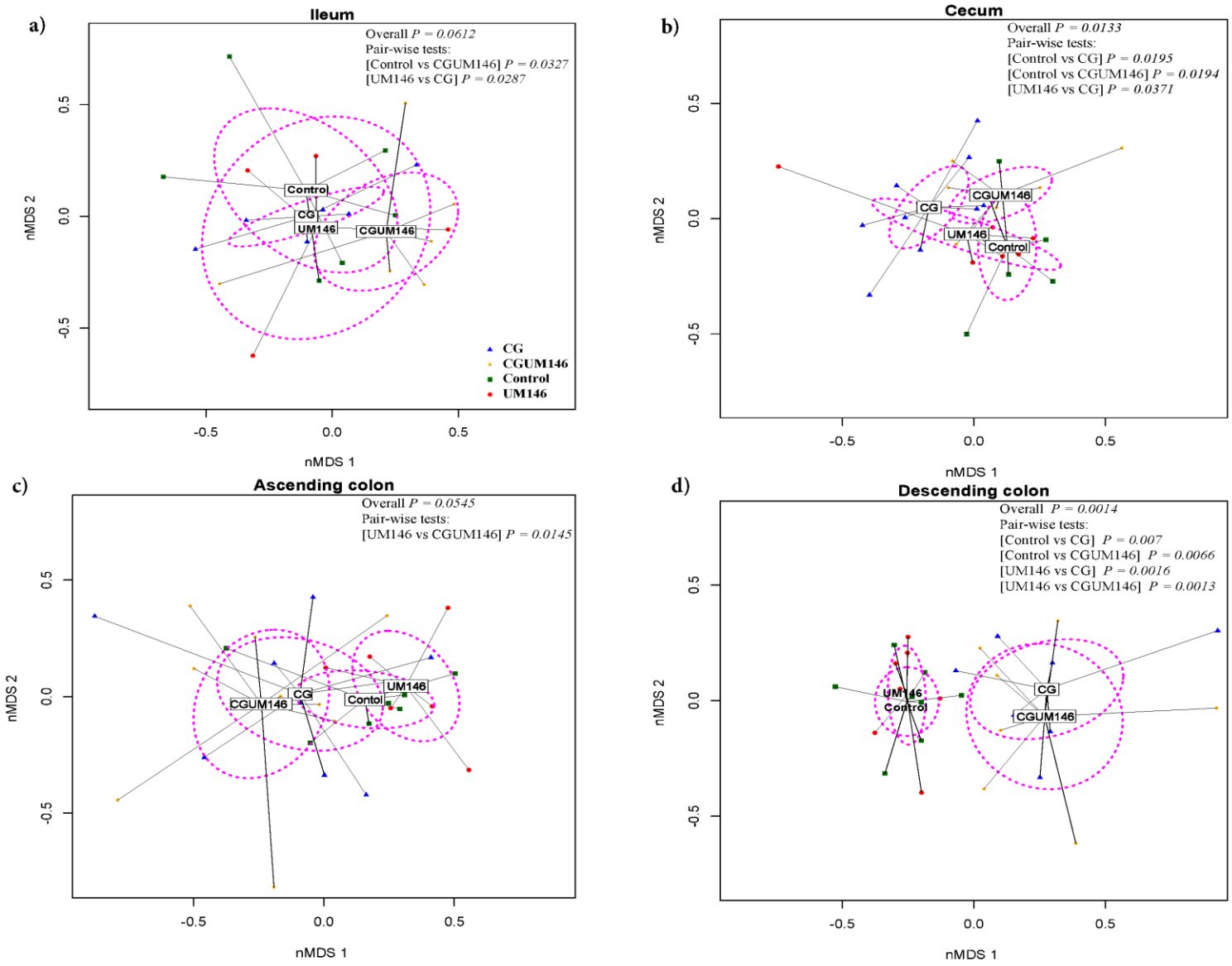

a)
Ileum
Overall P = 0.0612
Pair-wise tests:
[Control vs CGUM146] P = 0.0327
[UM146 vs CG] P = 0.0287
Control
CG
UM146
CGUM146
CG
CGUM146
Control
UM146
nMDS 1
nMDS 2
b)
Cecum
Overall P = 0.0133
Pair-wise tests:
[Control vs CG] P = 0.0195
[Control vs CGUM146] P = 0.0194
[UM146 vs CG] P = 0.0371
CGUM146
CG
UM146
Control
nMDS 1
nMDS 2
c)
Ascending colon
Overall P = 0.0545
Pair-wise tests:
[UM146 vs CGUM146] P = 0.0145
UM146
CG
CGUM146
Contol
nMDS 1
nMDS 2
d)
Descending colon
Overall P = 0.0014
Pair-wise tests:
[Control vs CG] P = 0.007
[Control vs CGUM146] P = 0.0066
[UM146 vs CG] P = 0.0016
[UM146 vs CGUM146] P = 0.0013
UM146
Control
CG
CGUM146
nMDS 1
nMDS 2

Figure 3.3. Non-metric multidimensional scaling (nMDS) ordination plot, a measure of relative difference in the bacterial community composition in the: **a**) ileum; **b**) cecum; **c**) ascending colon; and **d**) descending colon of pigs treated with degraded carrageenan gum (CG) and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146. The colored points are shaded according to different treatment groups. CG: pigs received 1% CG only in drinking water daily; UM146: pigs were inoculated with AIEC UM146 on day 8 of the study; CGUM146: pigs received 1 % CG from day 1 of the study and were inoculated with AIEC UM146 on day 8 of the study. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed. The *P* values were calculated using PERMANOVA. For the pair-wise tests, only the significant *P* values are included.

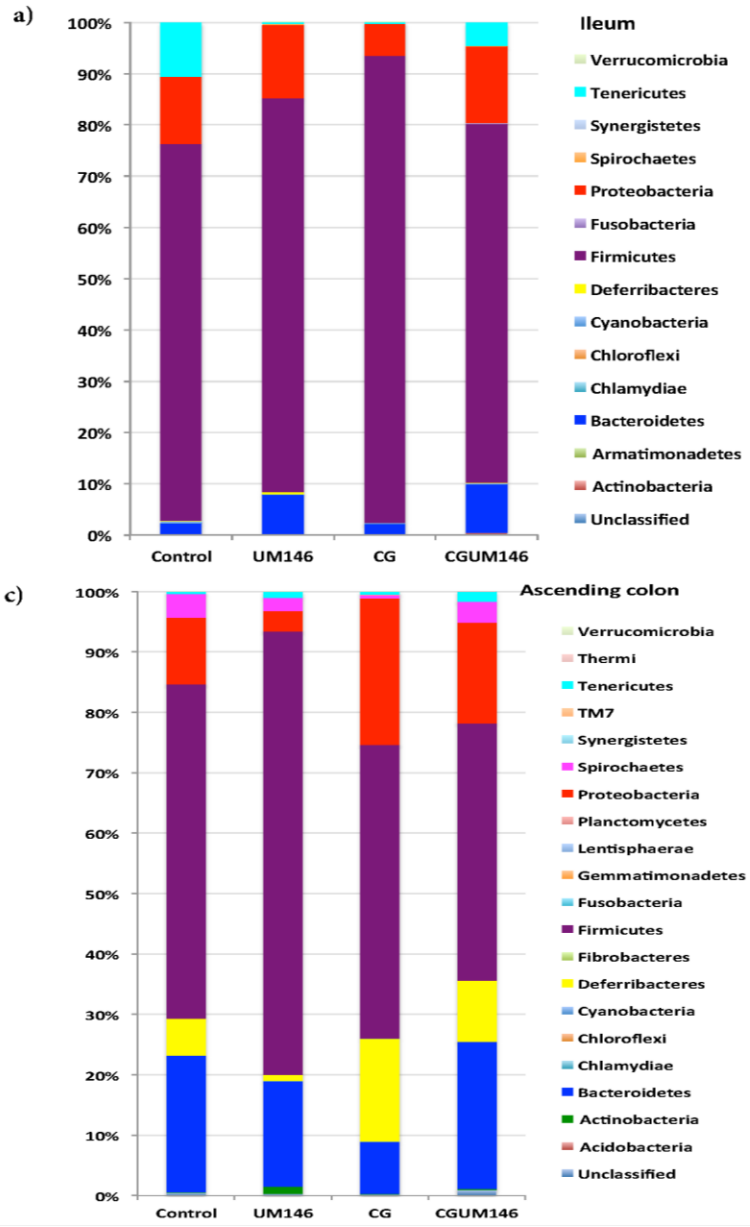
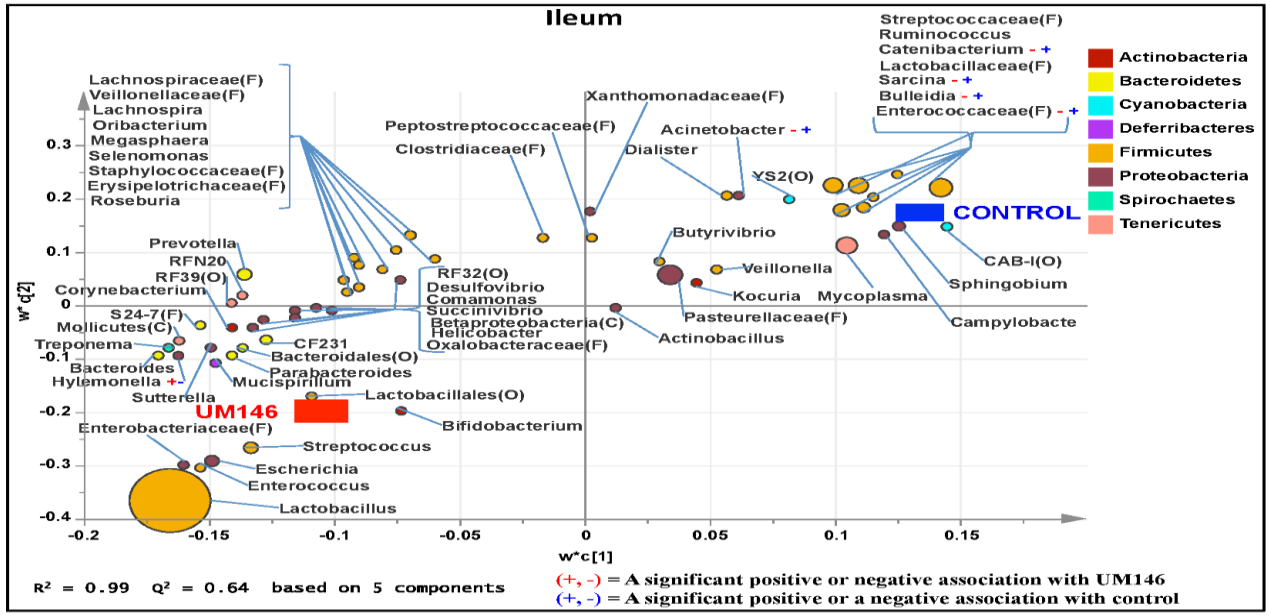

a)
Ileum
Verrucomicrobia
Tenericutes
Synergistetes
Spirochaetes
Proteobacteria
Fusobacteria
Firmicutes
Deferribacteres
Cyanobacteria
Chloroflexi
Chlamydiae
Bacteroidetes
Armatimonadetes
Actinobacteria
Unclassified
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Control
UM146
CG
CGUM146
b)
Cecum
Verrucomicrobia
Tenericutes
Synergistetes
Spirochaetes
Proteobacteria
OP8
Lentisphaerae
Fusobacteria
Firmicutes
Elusimicrobia
Deferribacteres
Cyanobacteria
Chlamydiae
Bacteroidetes
Actinobacteria
Acidobacteria
Unclassified
c)
Ascending colon
Verrucomicrobia
Thermi
Tenericutes
TM7
Synergistetes
Spirochaetes
Proteobacteria
Planctomycetes
Lentisphaerae
Gemmatimonadetes
Fusobacteria
Firmicutes
Fibrobacteres
Deferribacteres
Cyanobacteria
Chloroflexi
Chlamydiae
Bacteroidetes
Actinobacteria
Acidobacteria
Unclassified
d)
Descending colon
Verrucomicrobia
Tenericutes
TM7
Synergistetes
Spirochaetes
Proteobacteria
Planctomycetes
Lentisphaerae
Fusobacteria
Firmicutes
Fibrobacteres
Elusimicrobia
Deferribacteres
Cyanobacteria
Chlamydiae
Bacteroidetes
Actinobacteria
Unclassified

Figure 3.4. Percentage of relative abundance of bacterial phyla in the: **a**) ileum; **b**) cecum; **c**) ascending colon; and **d**) descending colon of pigs treated with degraded carrageenan gum (CG) and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146. Ileum: **a)** A total of 14 phyla were identified in all the samples, of which 4 were considered to be more abundant within the community (≥ 1%), including Firmicutes, Bacteroidetes, Proteobacteria and Tenericutes. Among the most abundant phyla, Firmicutes were the most dominant but the phyla did not differ between treatment groups ($P > 0.05$). Cecum: **b)** A total of 16 phyla were identified in all the samples, of which 4 were considered to be more abundant within the community (≥1%), including Firmicutes, Bacteroidetes, Proteobacteria and Deferribacteres. Among the most abundant phyla, the proportion of Proteobacteria were slightly higher ($P < 0.05$) in the CG and CGUM146 groups compared to the control and UM146 treatments, whereas Bacteroidetes were significantly lower in the CG and CGUM146 groups compared to the control and UM146 treatments. Ascending colon: **c**) A total of 20 phyla were identified in all the samples, of which 5 were considered to be more abundant within the community (≥1%), including Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres and Spirochaetes. The proportion of Firmicutes was significantly lower in CG and CGUM146 groups ($P = 0.007$), while Proteobacteria ($P = 0.001$) and Deferribacteres ($P = 0.039$) were significantly higher in these groups compared to control and UM146 treatments. Bacteroidetes abundance was lower ($P < 0.0001$) in CG treatment compared to other groups whereas Spirochaetes did not differ significantly among groups ($P > 0.05$). Descending colon: **d)** A total of 17 phyla were identified in all the samples, of which 5 were considered to be more abundant within the community (≥1%), including Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres and Tenericutes. Firmicutes were significantly lower ($P = 0.0055$) in the CG and CGUM146 treatments while Proteobacteria were higher ($P < 0.0001$) in these groups compared to the control and UM146 treatments. The Bacteroidetes proportion was lower ($P < 0.0001$) in CG treatment compared to other treatment groups. CG: pigs received 1% CG only in drinking water daily; UM146: pigs were inoculated with AIEC UM146 on day 8; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

a)


Ileum
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
CONTROL
UM146
w*c[2]
w*c[1]
R² = 0.99 Q² = 0.64 based on 5 components
(+, -) = A significant positive or negative association with UM146
(+, -) = A significant positive or a negative association with control

b)

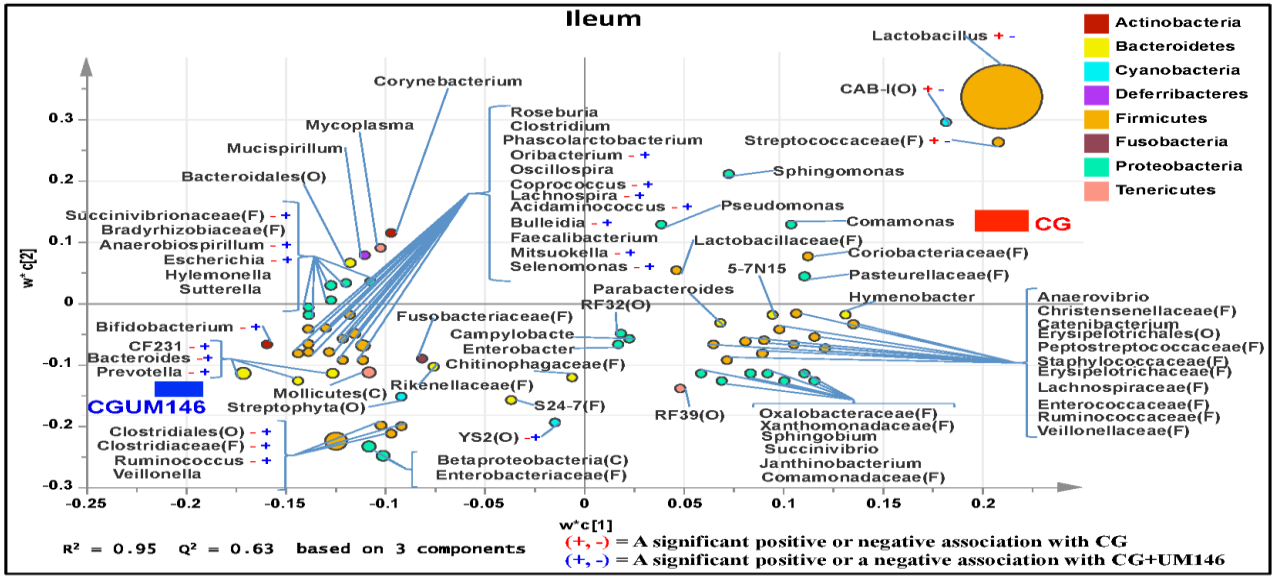

Ileum
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Fusobacteria
Proteobacteria
Tenericutes
CG
CGUM146
w*c[2]
w*c[1]
R² = 0.95 Q² = 0.63 based on 3 components
(+, -) = A significant positive or negative association with CG
(+, -) = A significant positive or a negative association with CG+UM146

Figure 3.5. Taxa that were positively or negatively associated with: **a**) control or UM146 in the ileum of pigs inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146, **b**) CG or CGUM146 in the ileum of pigs treated with CG and inoculated with AIEC UM146. UM146: pigs were inoculated with AIEC UM146 on day 8; CG: pigs received 1% CG only in drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

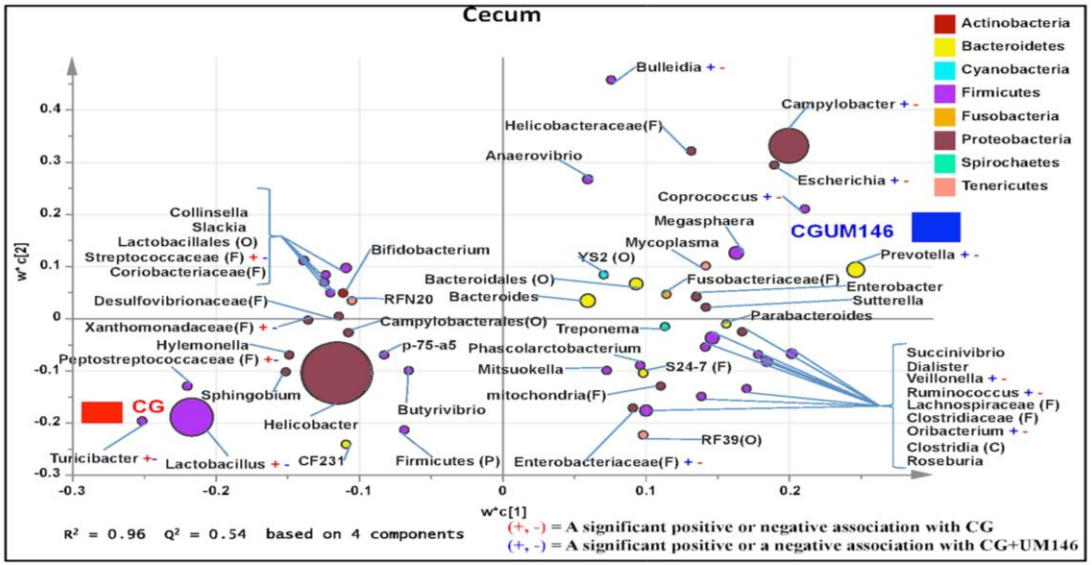
Cecum: A total of 571,706 quality-filtered sequences were obtained from the samples with a mean of 19,056 sequences per sample. Sixteen phyla were identified in all the samples. Among the most abundant phyla, the Bacteroidetes population was lower (P = 0.002) in the CG compared to the control and UM146 treatments, but it was not different from CGUM146 **(Figure 3.4 b).** Classification of OTUs at lower taxonomical levels resulted in identification of 177 taxa, of which 76 were ≥ 0.01% of the community, and the rest were < 0.01% of the community. Bacterial taxa with a relative abundance of ≥ 0.01% of the community were analyzed using PLS-DA to identify bacteria that were most characteristic of different treatment groups and the results are shown in **Figure 3.6** (**a-b**).

Ascending colon: A total of 1,293,477 quality-filtered sequences were obtained from the samples with an average of 41,725 sequences per sample. Twenty phyla were identified in all the samples. Among the most abundant phyla, the population of Firmicutes was significantly lower (P = 0.007) in CG and CGUM146 groups, while Proteobacteria (P = 0.001) and Deferribacteres (P = 0.039) were significantly higher in these groups compared to control and UM146 treatments. In addition, Bacteroidetes population was lower (P < 0.0001) in the CG group but not different from UM146 compared to the other treatments **(Figure 3.4 c)**. Classification of OTUs at lower taxonomical levels resulted in the identification of 232 taxa of

which 81 were ≥ 0.01% of the community, while 151 were < 0.01% of the community. Bacterial taxa with a relative abundance of ≥ 0.01% of the community were analyzed using PLS-DA to identify bacteria that were most characteristic of different treatment groups. The results are shown in **Figure 3.7** (**a-b**).

Descending colon: A total of 1,148,413 quality-filtered sequences were obtained from the samples with a mean of 39,600 sequences per sample. Seventeen phyla were identified in all

a)


Cecum
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Fusobacteria
Proteobacteria
Spirochaetes
Tenericutes
CGUM146
CG
Bulleidia + -
Campylobacter + -
Helicobacteraceae(F)
Anaerovibrio
Escherichia + -
Coprococcus + -
Megasphaera
Mycoplasma
YS2 (O)
Prevotella + -
Fusobacteriaceae(F)
Enterobacter
Sutterella
Parabacteroides
Collinsella
Slackia
Lactobacillales (O)
Streptococcaceae (F) + -
Coriobacteriaceae(F)
Bifidobacterium
Bacteroidales (O)
Bacteroides
RFN20
Desulfovibrionaceae(F)
Campylobacterales(O)
Xanthomonadaceae(F) + -
Treponema
Hylemonella
Peptostreptococcaceae (F) +-
p-75-a5
Phascolarctobacterium
Mitsuokella
S24-7 (F)
Succinivibrio
Dialister
Veillonella + -
Ruminococcus + -
Lachnospiraceae (F)
Clostridiaceae (F)
Oribacterium + -
Clostridia (C)
Roseburia
Sphingobium
mitochondria(F)
Butyrivibrio
Helicobacter
RF39(O)
Turicibacter +-
Lactobacillus + -
CF231
Firmicutes (P)
Enterobacteriaceae(F) + -
w*c[2]
w*c[1]
R² = 0.96 Q² = 0.54 based on 4 components
(+, -) = A significant positive or negative association with CG
(+, -) = A significant positive or a negative association with CG+UM146

b)

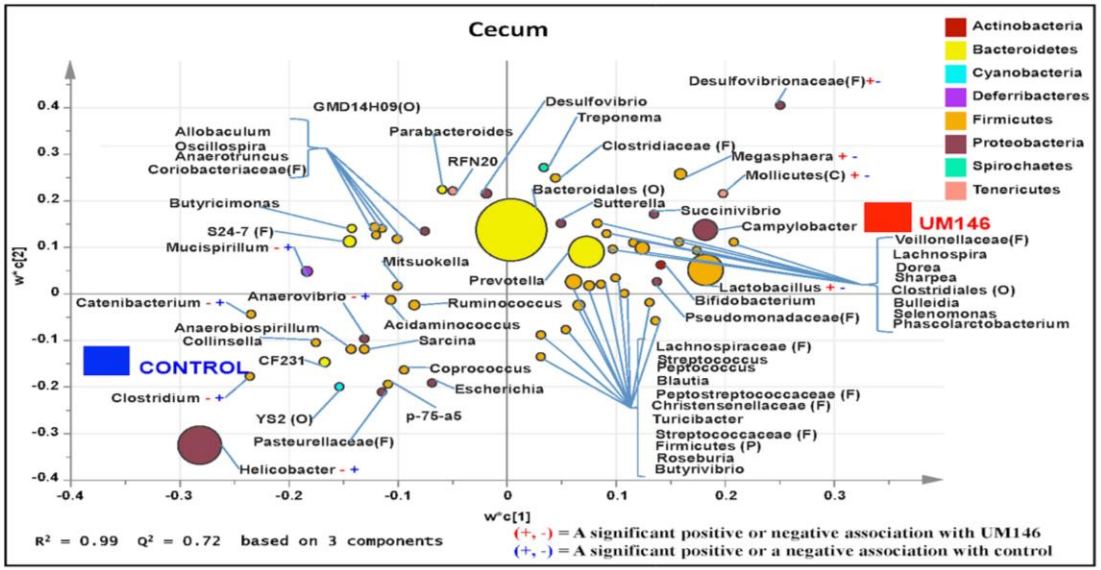
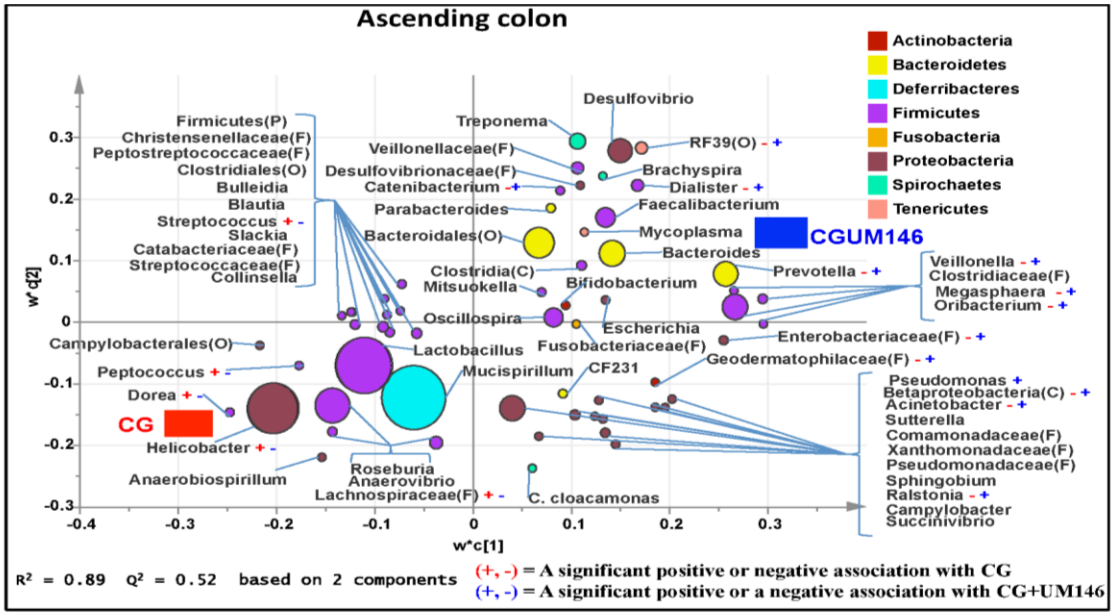

Cecum
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
UM146
CONTROL
Desulfovibrionaceae(F)+-
GMD14H09(O)
Desulfovibrio
Treponema
Allobaculum
Oscillospira
Anaerotruncus
Coriobacteriaceae(F)
Parabacteroides
RFN20
Clostridiaceae (F)
Megasphaera + -
Mollicutes(C) + -
Bacteroidales (O)
Sutterella
Succinivibrio
Butyricimonas
Campylobacter
S24-7 (F)
Mucispirillum - +
Veillonellaceae(F)
Lachnospira
Dorea
Sharpea
Clostridiales (O)
Bulleidia
Selenomonas
Phascolarctobacterium
Mitsuokella
Prevotella
Lactobacillus + -
Bifidobacterium
Pseudomonadaceae(F)
Catenibacterium - +
Anaerovibrio - +
Ruminococcus
Acidaminococcus
Anaerobiospirillum
Collinsella
Sarcina
Lachnospiraceae (F)
Streptococcus
Peptococcus
Blautia
Peptostreptococcaceae (F)
Christensenellaceae (F)
Turicibacter
Streptococcaceae (F)
Firmicutes (P)
Roseburia
Butyrivibrio
CF231
Coprococcus
Escherichia
Clostridium - +
YS2 (O)
p-75-a5
Pasteurellaceae(F)
Helicobacter - +
w*c[2]
w*c[1]
R² = 0.99 Q² = 0.72 based on 3 components
(+, -) = A significant positive or negative association with UM146
(+, -) = A significant positive or a negative association with control

Figure 3.6. Taxa that were positively or negatively associated with: **a**) CG or CGUM146 in the cecum of pigs treated with CG and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146; **b**) control or UM146 in the cecum of pigs inoculated with AIEC UM146. CG: pigs received 1% CG only in the drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8; UM146: pigs were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

a)


Ascending colon
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Fusobacteria
Proteobacteria
Spirochaetes
Tenericutes
Firmicutes(P)
Christensenellaceae(F)
Peptostreptococcaceae(F)
Clostridiales(O)
Bulleidia
Blautia
Streptococcus + -
Slackia
Catabacteriaceae(F)
Streptococcaceae(F)
Collinsella
Treponema
Desulfovibrio
Veillonellaceae(F)
RF39(O) - +
Desulfovibrionaceae(F)
Brachyspira
Catenibacterium - +
Dialister - +
Parabacteroides
Faecalibacterium
Bacteroidales(O)
Mycoplasma
CGUM146
Bacteroides
Clostridia(C)
Prevotella - +
Mitsuokella
Bifidobacterium
Veillonella - +
Clostridiaceae(F)
Megasphaera - +
Oribacterium - +
Oscillospira
Escherichia
Enterobacteriaceae(F) - +
Campylobacterales(O)
Lactobacillus
Fusobacteriaceae(F)
Geodermatophilaceae(F) - +
Peptococcus + -
Mucispirillum
CF231
Dorea + -
Pseudomonas +
Betaproteobacteria(C) - +
Acinetobacter - +
Sutterella
CG
Comamonadaceae(F)
Xanthomonadaceae(F)
Helicobacter + -
Pseudomonadaceae(F)
Sphingobium
Roseburia
Anaerobiospirillum
Anaerovibrio
Ralstonia - +
Lachnospiraceae(F) + -
C. cloacamonas
Campylobacter
Succinivibrio
w*c[2]
w*c[1]
R² = 0.89 Q² = 0.52 based on 2 components
(+, -) = A significant positive or negative association with CG
(+, -) = A significant positive or a negative association with CG+UM146

b)

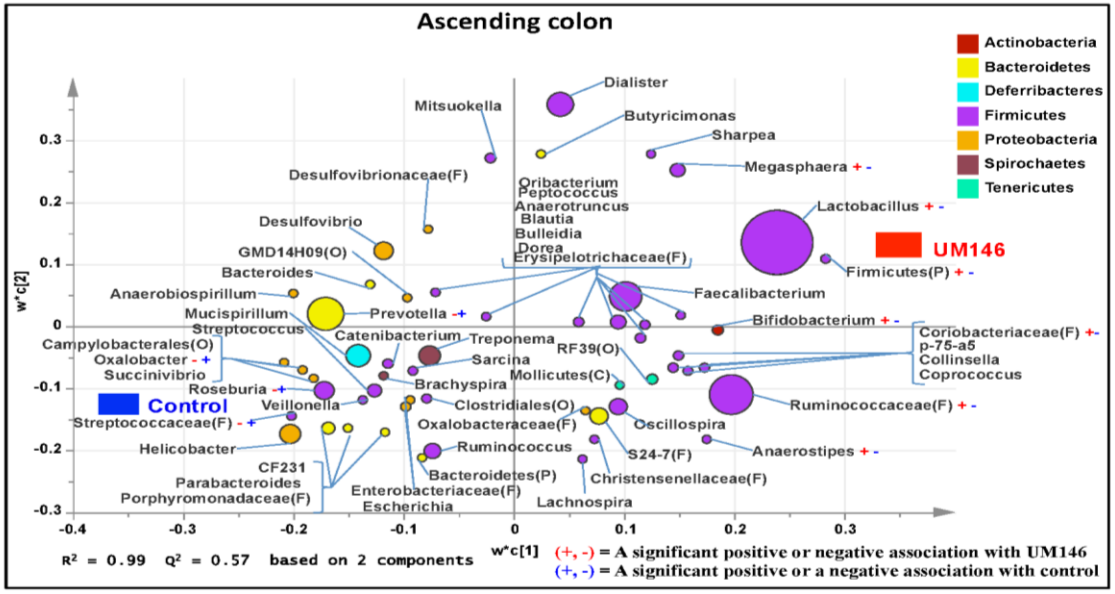

Ascending colon
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
Dialister
Mitsuokella
Butyricimonas
Sharpea
Megasphaera + -
Desulfovibrionaceae(F)
Oribacterium
Peptococcus
Anaerotruncus
Blautia
Bulleidia
Dorea
Erysipelotrichaceae(F)
Lactobacillus + -
UM146
Firmicutes(P) + -
Desulfovibrio
GMD14H09(O)
Bacteroides
Faecalibacterium
Anaerobiospirillum
Mucispirillum
Prevotella - +
Bifidobacterium + -
Streptococcus
Catenibacterium
Treponema
RF39(O)
Coriobacteriaceae(F) + -
Campylobacterales(O)
Oxalobacter - +
Sarcina
p-75-a5
Collinsella
Succinivibrio
Mollicutes(C)
Coprococcus
Roseburia - +
Brachyspira
Control
Veillonella
Clostridiales(O)
Ruminococcaceae(F) + -
Streptococcaceae(F) - +
Oxalobacteraceae(F)
Oscillospira
Helicobacter
Ruminococcus
S24-7(F)
Anaerostipes + -
CF231
Bacteroidetes(P)
Christensenellaceae(F)
Parabacteroides
Porphyromonadaceae(F)
Enterobacteriaceae(F)
Escherichia
Lachnospira
w*c[2]
w*c[1]
R² = 0.99 Q² = 0.57 based on 2 components
(+, -) = A significant positive or negative association with UM146
(+, -) = A significant positive or a negative association with control

Figure 3.7. Taxa that were positively or negatively associated with: **a**) CG or CGUM146 in the ascending colon of pigs treated with CG and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146; **b**) control or UM146 in the ascending colon of pigs inoculated with AIEC UM146. CG: pigs received 1% CG only in the drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8; UM146: pigs were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

the samples. Among the most abundant phyla, the population of Firmicutes and Tenericutes were significantly lower (P = 0.0055 and P = 0.0047 respectively) in the CG and CGUM146 treatments compared to the control, but wasn't different compared to UM146. Proteobacteria population was higher (P < .0001) in CG treatment compared to other treatments. Conversely, the population of Bacteroidetes was lower (P < 0.0001) in CG treatment compared to other treatment groups **(Figure 3.4 d).**

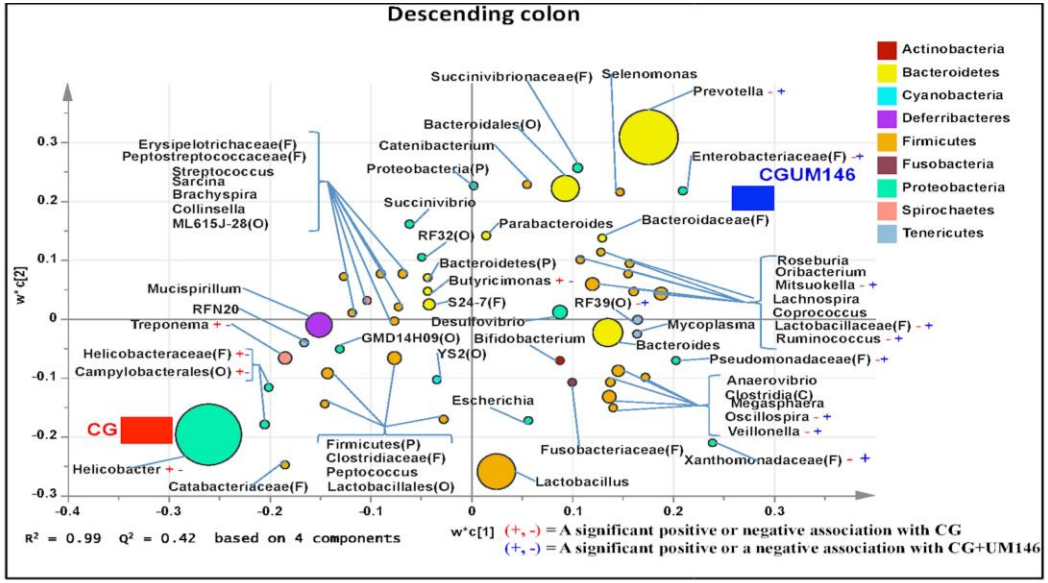
Classification of OTUs at lower taxonomical levels resulted in the identification of 181 taxa, of which 84 were ≥ 0.01% of the community, while 97 were < 0.01% of the community. Bacterial taxa with a relative abundance of ≥ 0.01% of the community were analyzed using PLS-DA to identify bacteria that were most characteristic of different treatment groups. The results are shown in **Figure 3.8** (**a-b**).

Appendix 1 Supplementary Table 3.1 Shows the analyzed data for the most abundant phyla in the ileum, cecum, ascending and descending colon, whereas **Supplementary Tables 3.2, 3.3, 3.4 and 3.5** shows a summary of mean abundance of all the taxa in the ileum, cecum, ascending and descending colon, respectively.

3.4.8. Functional metagenome of microbiomes

A closed-reference based OUT picking step was employed for the PICRUST (v. 1.0.0-dev) analysis using the Greengenes (v.13.5) database. The proportion of reads that mapped to reference during OTU picking was 96.7 % for the ileum, 91.4% for the cecum, 88.1% for the ascending colon, and 85.7 % for the descending colon (an average of 90.5% for all the tissues). The data was normalized by copy numbers before metagenomes prediction, and the Nearest Sequenced Taxon Index (NSTI) for each sample which reflects the availability of reference genomes that are closely related to the abundant microorganisms in the samples were

a)


Descending colon
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Fusobacteria
Proteobacteria
Spirochaetes
Tenericutes
Succinivibrionaceae(F)
Selenomonas
Prevotella - +
Bacteroidales(O)
Erysipelotrichaceae(F)
Peptostreptococcaceae(F)
Streptococcus
Sarcina
Brachyspira
Collinsella
ML615J-28(O)
Catenibacterium
Proteobacteria(P)
Succinivibrio
Enterobacteriaceae(F) - +
CGUM146
Parabacteroides
Bacteroidaceae(F)
RF32(O)
Bacteroidetes(P)
Butyricimonas + -
S24-7(F)
RF39(O) - +
Roseburia
Oribacterium
Mitsuokella - +
Lachnospira
Coprococcus
Lactobacillaceae(F) - +
Ruminococcus - +
Mucispirillum
RFN20
Treponema + -
Desulfovibrio
GMD14H09(O)
Bifidobacterium
Mycoplasma
Bacteroides
YS2(O)
Pseudomonadaceae(F) - +
Helicobacteraceae(F) + -
Campylobacterales(O) + -
Anaerovibrio
Clostridia(C)
Megasphaera
Oscillospira - +
Veillonella - +
Escherichia
CG
Fusobacteriaceae(F)
Xanthomonadaceae(F) - +
Firmicutes(P)
Clostridiaceae(F)
Peptococcus
Lactobacillales(O)
Helicobacter + -
Catabacteriaceae(F)
Lactobacillus
w*c[2]
w*c[1]
R² = 0.99 Q² = 0.42 based on 4 components
(+, -) = A significant positive or negative association with CG
(+, -) = A significant positive or a negative association with CG+UM146

b)

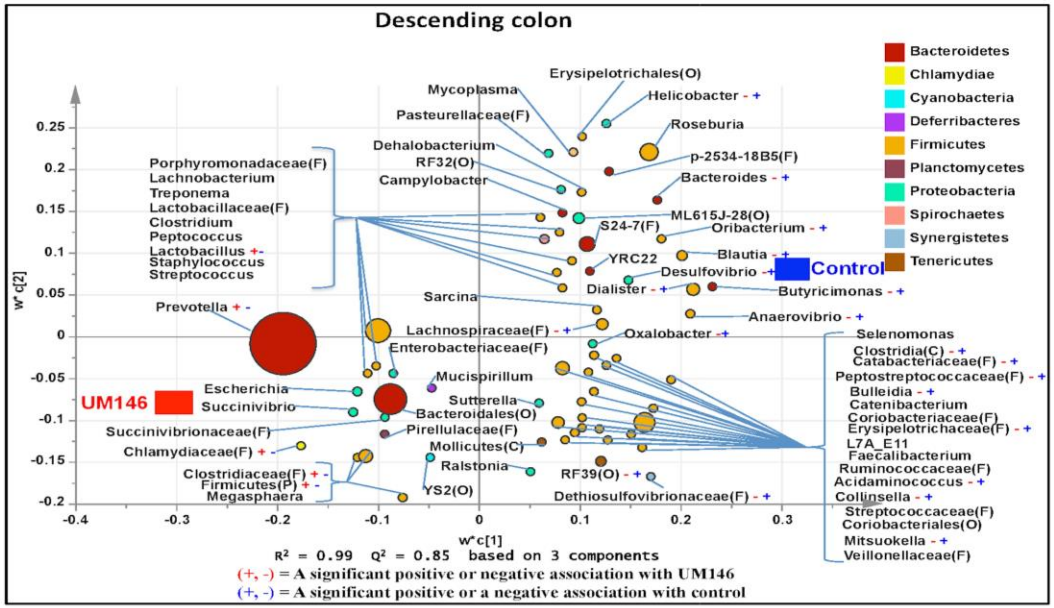

Descending colon
Bacteroidetes
Chlamydiae
Cyanobacteria
Deferribacteres
Firmicutes
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Erysipelotrichales(O)
Mycoplasma
Helicobacter - +
Pasteurellaceae(F)
Roseburia
Dehalobacterium
RF32(O)
p-2534-18B5(F)
Campylobacter
Bacteroides - +
Porphyromonadaceae(F)
Lachnobacterium
Treponema
Lactobacillaceae(F)
Clostridium
Peptococcus
Lactobacillus + -
Staphylococcus
Streptococcus
ML615J-28(O)
S24-7(F)
Oribacterium - +
YRC22
Blautia - +
Desulfovibrio - +
Control
Dialister - +
Butyricimonas - +
Sarcina
Anaerovibrio - +
Prevotella + -
Lachnospiraceae(F) - +
Oxalobacter - +
Selenomonas
Enterobacteriaceae(F)
Clostridia(C) - +
Catabacteriaceae(F) - +
Peptostreptococcaceae(F) - +
Bulleidia - +
Catenibacterium
Coriobacteriaceae(F)
Erysipelotrichaceae(F) - +
L7A_E11
Faecalibacterium
Ruminococcaceae(F)
Acidaminococcus - +
Collinsella - +
Streptococcaceae(F)
Coriobacteriales(O)
Mitsuokella - +
Veillonellaceae(F)
Escherichia
UM146
Succinivibrio
Mucispirillum
Sutterella
Bacteroidales(O)
Succinivibrionaceae(F)
Pirellulaceae(F)
Chlamydiaceae(F) + -
Mollicutes(C)
Ralstonia
RF39(O) - +
Clostridiaceae(F) + -
Firmicutes(P) + -
Megasphaera
YS2(O)
Dethiosulfovibrionaceae(F) - +
w*c[2]
w*c[1]
R² = 0.99 Q² = 0.85 based on 3 components
(+, -) = A significant positive or negative association with UM146
(+, -) = A significant positive or a negative association with control

Figure 3.8. Taxa that were positively or negatively associated with: **a**) CG or CGUM146 in the descending colon of pigs treated with CG and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146; **b**) control or UM146 in the descending colon of pigs inoculated with AIEC UM146. CG: pigs received 1% CG only in the drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8; UM146: pigs were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

determined during metagenome prediction. On average, the NSTIs were: 0.06 for the ileum, 0.06 for the cecum, 0.10 for ascending colon, and 0.10 for descending colon. High NSTI scores (> 0.15) are indicative that fewer related references are available and predictions were of low quality whereas low scores (< 0.06) are reflective of availability of closely related reference genomes.

As shown in **Figure 3.9**, different metabolic pathways were enriched in the mucosal microbiota of the ascending colon tissue in UM146 including fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, ribosome biogenesis, DNA replication proteins, and DNA repair and recombination proteins, among others. Functional pathways enriched in the ascending colon mucosal microbiota of CG- or CGUM146-treated pigs include, but are not limited to, bacterial chemotaxis, flagellar assembly and lipopolysaccharide biosynthesis proteins. As shown in **Figure 3.10**, different functional pathways were enriched in the mucosal microbiota of the descending colon tissue in the control group including, but not limited to, transporters, transcription factors, DNA repair and recombination proteins, and starch and sucrose metabolism. In addition, several functional pathways were also enriched in the descending colon mucosal microbiota of CG- or CGUM146-treated pigs including secretion system, flagellar assembly, bacterial secretion system, and lipopolysaccharide biosynthesis proteins. Comparisons with the control or UM146 in the ascending and descending colon, respectively, were not significant after correcting the *P* values and therefore, they were not included. Also, no comparisons were included for the ileum and the cecum as there were no significant differences in pathways after correcting for the *P* values.

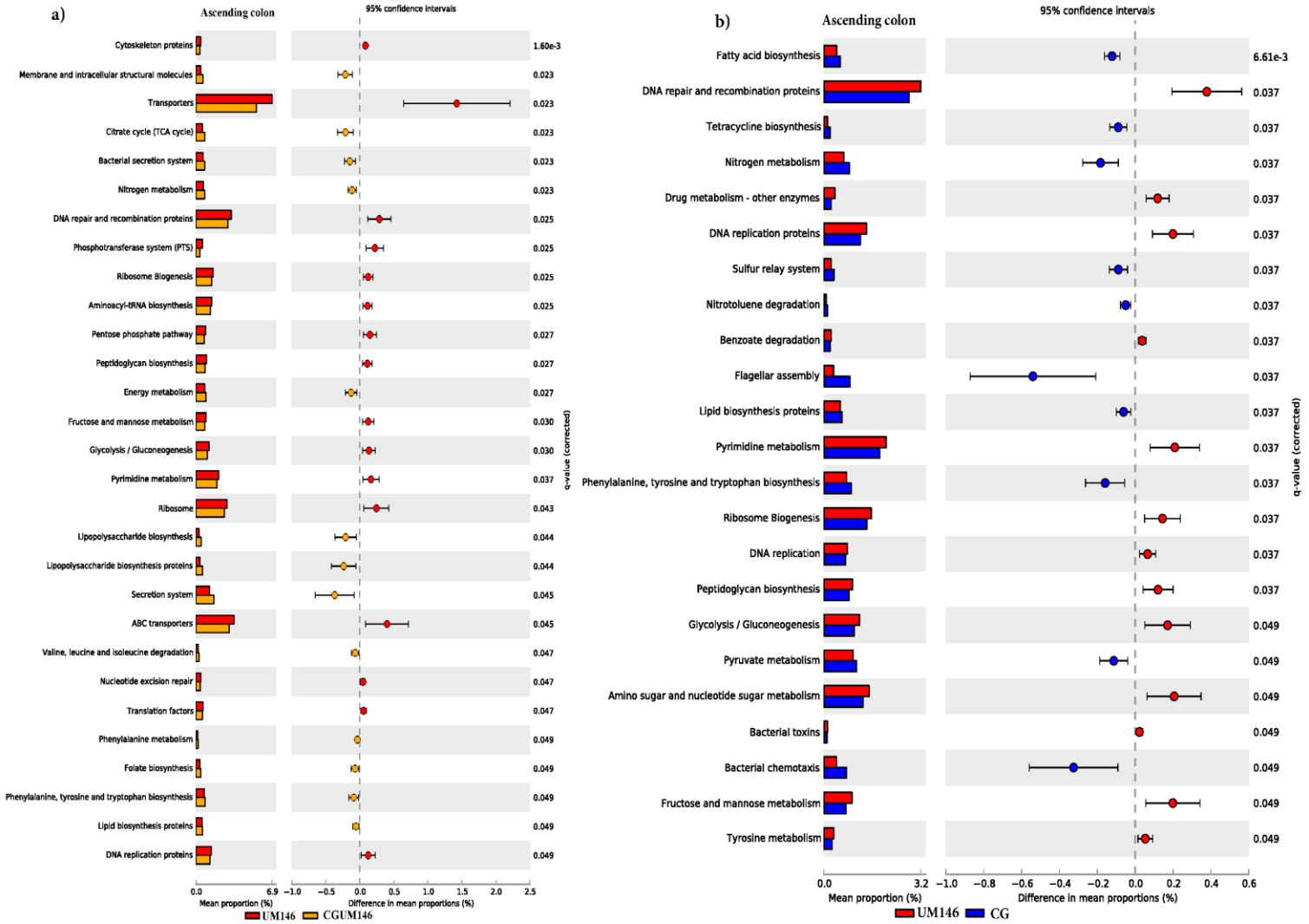
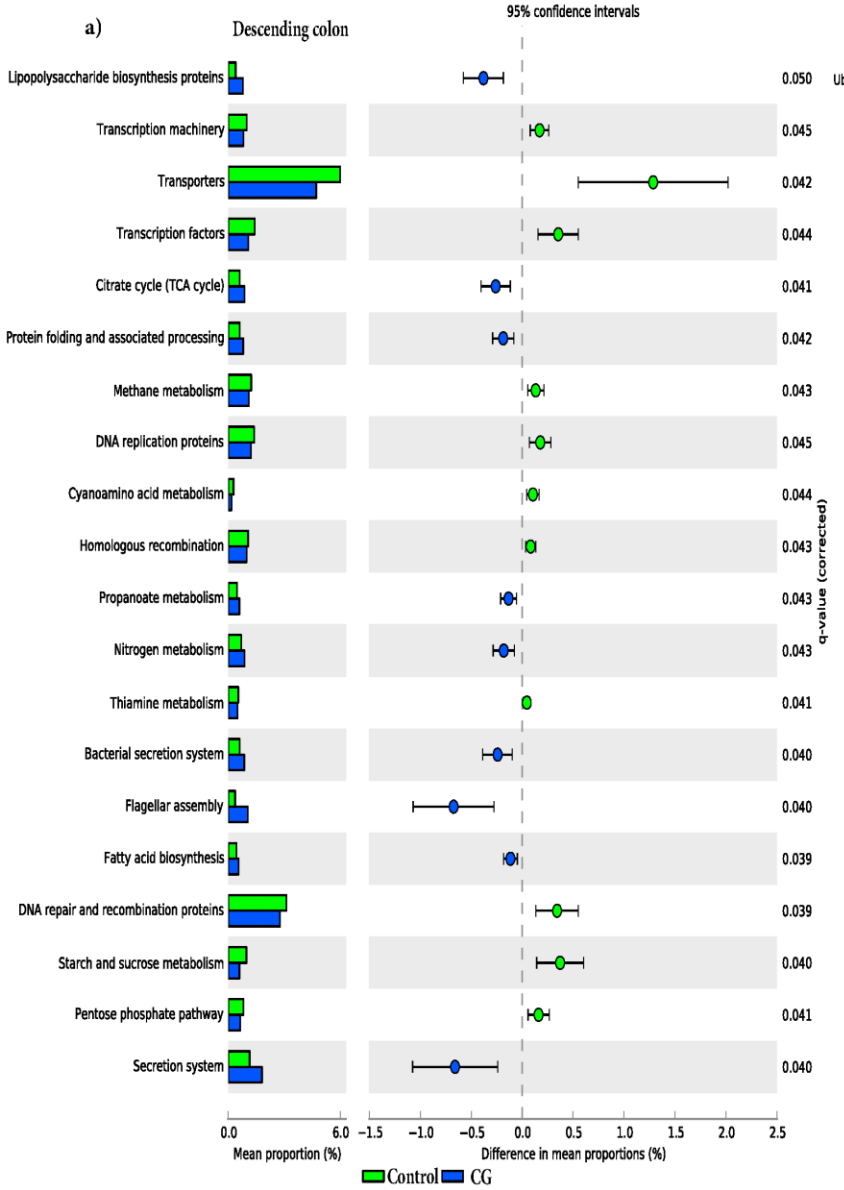

a)
Ascending colon
95% confidence intervals
Cytoskeleton proteins
Membrane and intracellular structural molecules
Transporters
Citrate cycle (TCA cycle)
Bacterial secretion system
Nitrogen metabolism
DNA repair and recombination proteins
Phosphotransferase system (PTS)
Ribosome Biogenesis
Aminoacyl-tRNA biosynthesis
Pentose phosphate pathway
Peptidoglycan biosynthesis
Energy metabolism
Fructose and mannose metabolism
Glycolysis / Gluconeogenesis
Pyrimidine metabolism
Ribosome
Lipopolysaccharide biosynthesis
Lipopolysaccharide biosynthesis proteins
Secretion system
ABC transporters
Valine, leucine and isoleucine degradation
Nucleotide excision repair
Translation factors
Phenylalanine metabolism
Folate biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Lipid biosynthesis proteins
DNA replication proteins
1.60e-3
0.023
0.023
0.023
0.023
0.023
0.025
0.025
0.025
0.025
0.027
0.027
0.027
0.030
0.030
0.037
0.043
0.044
0.044
0.045
0.045
0.047
0.047
0.047
0.049
0.049
0.049
0.049
0.049
q-value (corrected)
0.0
6.9
Mean proportion (%)
−1.0
−0.5
0.0
0.5
1.0
1.5
2.0
2.5
Difference in mean proportions (%)
UM146
CGUM146
b)
Ascending colon
95% confidence intervals
Fatty acid biosynthesis
DNA repair and recombination proteins
Tetracycline biosynthesis
Nitrogen metabolism
Drug metabolism - other enzymes
DNA replication proteins
Sulfur relay system
Nitrotoluene degradation
Benzoate degradation
Flagellar assembly
Lipid biosynthesis proteins
Pyrimidine metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Ribosome Biogenesis
DNA replication
Peptidoglycan biosynthesis
Glycolysis / Gluconeogenesis
Pyruvate metabolism
Amino sugar and nucleotide sugar metabolism
Bacterial toxins
Bacterial chemotaxis
Fructose and mannose metabolism
Tyrosine metabolism
6.61e-3
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.037
0.049
0.049
0.049
0.049
0.049
0.049
q-value (corrected)
0.0
3.2
Mean proportion (%)
−1.0
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6
Difference in mean proportions (%)
UM146
CG

Figure 3.9. Subsystems and pathways enriched or decreased in: **a**) UM146 vs. CG; and **b**) UM146 vs. CGUM146 of the **ascending colon** samples from pigs treated with CG or inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146. Corrected *P* values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the (UM146) or CG samples have a (positive) or negative differences between mean proportions and are indicated by (red) and blue coloring, respectively. Subsystems or pathways overrepresented in the (UM146) or CGUM146 samples have a (positive), or negative difference between mean proportions and are indicated by (red) and orange coloring respectively. CG: pigs received 1% CG only in the drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8; UM146: pigs were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.


a)
Descending colon
95% confidence intervals
Lipopolysaccharide biosynthesis proteins
Transcription machinery
Transporters
Transcription factors
Citrate cycle (TCA cycle)
Protein folding and associated processing
Methane metabolism
DNA replication proteins
Cyanoamino acid metabolism
Homologous recombination
Propanoate metabolism
Nitrogen metabolism
Thiamine metabolism
Bacterial secretion system
Flagellar assembly
Fatty acid biosynthesis
DNA repair and recombination proteins
Starch and sucrose metabolism
Pentose phosphate pathway
Secretion system
0.050
0.045
0.042
0.044
0.041
0.042
0.043
0.045
0.044
0.043
0.043
0.043
0.041
0.040
0.040
0.039
0.039
0.040
0.041
0.040
q-value (corrected)
0.0
6.0
Mean proportion (%)
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
2.0
2.5
Difference in mean proportions (%)
Control
CG

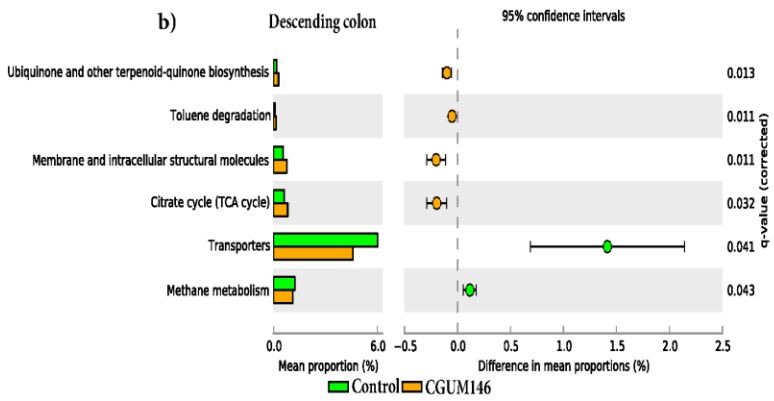

b)
Descending colon
95% confidence intervals
Ubiquinone and other terpenoid-quinone biosynthesis
Toluene degradation
Membrane and intracellular structural molecules
Citrate cycle (TCA cycle)
Transporters
Methane metabolism
0.013
0.011
0.011
0.032
0.041
0.043
q-value (corrected)
0.0
6.0
Mean proportion (%)
−0.5
0.0
0.5
1.0
1.5
2.0
2.5
Difference in mean proportions (%)
Control
CGUM146

Figure 3.10. Subsystems and pathways enriched or decreased in: **a**) control vs. CG; and **b**) control vs. CGUM146 of the **descending colon** samples from pigs treated with CG and inoculated with adherent invasive *Escherichia coli* (AIEC) strain UM146. Corrected *P* values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the (control) or CG pig descending colon samples have a (positive) or a negative difference between mean proportions and are indicated by (light green) and blue coloring, respectively. Subsystems or pathways overrepresented in the (control) or CGUM146 pig samples have a (positive) or a negative difference between mean proportions and are indicated by (light green) and orange coloring, respectively. CG: pigs received 1% CG only in the drinking water daily; CGUM146: pigs received 1 % CG from day 1 and were inoculated with AIEC UM146 on day 8; UM146: pigs were inoculated with AIEC UM146 on day 8. Pigs in designated groups received 100 mL of AIEC UM146 culture (10^8 CFU/mL) in feed.

3.5. DISCUSSION

Changes in microbial composition and its interaction with the immune system play an important role in the pathogenesis of IBD (Abraham and Medzhitov, 2011; Khor et al., 2011; Manichanh et al., 2012; Sartor and Mazmanian, 2012; Hansen, 2015). In this study, we used 16S rRNA gene Illumina sequencing to investigate differences in the composition and function of mucosa associated microbial communities of different GI tract segments in a piglet model of experimental IBD. In agreement with previous studies in IBD patients (Sokol et al., 2008; Man et al., 2011; Walters et al., 2014; Wright et al., 2015), our results showed that the relative abundance of Proteobacteria was higher in pigs with CG-induced colitis whilst Firmicutes and Bacteroides were lower compared to control in the large intestine. Also, pigs with CG-induced colitis clustered separately from the control and UM146 in the cecum and descending colon indicating a distinct shift in bacterial composition, which is in line with previous observations in microbiota profiles of patients with IBD (Ott et al., 2004; Sokol et al., 2008). Metabolic capacity of the microbiome in the colon of colitic pigs was also altered but no significant changes were observed in UM146 treatment alone compared to the control, and neither did the combination of CG and UM146 further impact the extent of observed changes.

This indicates that either *E. coli* strain UM146 did not successfully colonize the GI tract and therefore did not have much impact on microbial equilibrium and inflammation, or that its effects were transient. In addition, by combining CG-induced colitis and UM146 inoculation, it was expected that the pigs would experience adverse effects and manifest more dysbiosis and inflammation compared to the pigs with colitis alone; however, this was not the case in our study as the observed changes could only be mainly associated with the colitis and not UM146 inoculation. The lack of clear effects of UM146 could be due to the one time

inoculation that was used in our study and the length of our study after inoculation. However, AIEC UM146 is well adapted to colonize human intestines rather than pigs and, therefore, a one-time inoculation may not be sufficient for AIEC UM146 to effectively overrun a plethora of indigenous microbes that colonize pig's digestive tract. Repeated dosing over a relatively longer period of time may, therefore, provide a better insight on the cause or consequence role of this AIEC strain in experimental colitis/IBD.

The impact of experimental treatments on the microbiota of the ileum was minor compared to other tested intestinal segments. This suggests that CG-induced colitis has less effect on the ileum, which indicates that CG may exhibit different impacts on mucosal microbiota in different intestinal segments. One point worth noting is that the proportion of Bacteroidetes was consistently lower in the large intestine of pigs with CG-induced colitis compared to the colitic pigs that were also inoculated with UM146. This might suggest that UM146 induced or enhanced proliferation of Bacteroidetes in pigs with colitis, which is in agreement with some previous studies in patients with CD that have reported increase in Bacteroidetes, based on a recent systematic review (Wright et al., 2015). The proliferation of Proteobacteria, especially AIEC, has been consistently reported in IBD patients and in experimental models of IBD, but it is still not clear if members of this phylum cause inflammation or if their proliferation is a result of inflammatory status in the gut (Mukhopadhya et al., 2012). A recent study (Chassaing et al., 2014b) found that transient colonization with AIEC is sufficient to trigger chronic intestinal inflammation in genetically susceptible hosts – mice lacking Toll-like receptor 5 (TLR5), the sensor for bacterial flagellin. Such chronic inflammation lasted even when AIEC was no longer detectable and was also accompanied by alterations in fecal microbial composition, loss of species

diversity and higher levels of LPS and flagellin. None of these alterations were observed in wild-type mice/non- susceptible healthy hosts. This supports a causal role of AIEC in IBD in genetically susceptible hosts, but a consequent role in non-susceptible hosts, and could partially explain our results in which AIEC strain UM146 alone did not have much effect on inflammation or microbial dysbiosis. However, because intestinal inflammation may still persist even after AIEC is undetectable in the susceptible host, AIEC may be an intestinal arsonist pathobiont that ignites IBD and flees the scene in genetically susceptible hosts (Jellbauer and Raffatellu, 2014). Still, it is not clear whether the same process happens in healthy hosts that are not genetically susceptible to IBD, which could also be the reason why the pigs in our study did not have diarrhea beyond 48 h after inoculation.

We identified bacterial taxa that were consistently associated with particular treatments in all the four intestinal segments examined, or shared in between two or three intestinal sections. For example, *Lactobacillus* and *Megasphaera* were positively associated with UM146 while *Lactobacillus, Helicobacter* and unclassified members of Streptococcaceae were positively associated with CG treatment. On the other hand, *Prevotella*, *Ruminococcus*, *Oribacterium*, *Escherichia*, *Coprococcus, Bulleidia*, *Veillonella*, *Mitsuokella* and unclassified members of Enterobacteriaceae and RF39 were positively associated with CGUM146. The mechanism by which UM146 and CG treatments were associated with *Lactobacillus* growth is difficult to elucidate. It could be speculated that the changes induced by the presence of CG or UM146 may favor specific bacterial colonization. Moreover, the increased presence of *Lactobacillus* in pigs exposed to UM146 and CG could be relevant since these bacteria are protective against infections including inflammatory and infectious diseases (Lara-Villoslada et al., 2007). *Lactobacillus* may also produce lactate that can be utilized by other bacteria to produce short

chain fatty acids (SCFA), which are metabolites that are well known for their health benefits (Tsukahara et al., 2002), although our correlation analysis did not reveal a strong association between some of these taxa and various SCFA. *Helicobacter pylori* is commonly found in the gut and under normal gut conditions, it does not cause adverse effects; however, it is a major reason for the genesis of gastritis and peptic ulcers in humans. Colonization of gnotobiotic piglets with *H. pylori* is also associated with gastritis and gastric ulcers (Heinritz et al., 2013). Interestingly, *H. pylori* has been found to be negatively correlated with IBD, which may be explained by the "hygiene hypothesis", and only non- *pylori Helicobacters* including *Helicobacter hepaticus* have been shown to induce colitis in rodent models of experimental colitis (Hold et al., 2014). It is therefore reassuring that perhaps *H. hepaticus* was the major species associated with the CG treatment in the colon in our study, even though our data was only classified to the genus level and therefore it was not possible to definitively discriminate between different species of the g. *Helicobacter*.

Some of the taxa were shared/positively associated with specific treatments across different intestinal compartments, which may imply strong relations between the taxa and treatment conditions. Certain taxa were positively correlated with certain inflammatory cytokines/SCFAs, reinforcing that different taxa exhibit redundancy and pleiotropy, which makes elucidation on their association with specific treatments difficult. However, increased levels of Enterobacteriaceae have been repeatedly reported in IBD patients, especially in CD, although results related, but not limited, to Enterobacteriaceae, *E. coli*, *Bacteroides* and *Lactobacillus* species are not consistent between studies (Takaishi et al., 2008; Andoh et al., 2011; Wright et al., 2015). Such differences may be explained by variations in sample sources, sampling locations, analytical methodologies and disease activity among other

confounding factors in separate studies. Moreover, we only analyzed samples collected at one time point, which is a limitation of our study and possibly many other studies as it is not possible to discriminate between "normal" changes and pathological conditions, and between microbes that are transient and those that are permanent residents of the gut.

Microbial activities in the gastrointestinal tract can result in production of different metabolites, such as SCFA and butyrate, substances with known immunoregulatory properties that greatly contribute to gut health (Sartor, 2008; Canani et al., 2011; Smith et al., 2013). We observed a lower level of butyrate in the cecum and colon digesta of pigs receiving CG, a condition that may be associated with poor colonocyte development and an increase in inflammatory markers. However, we did not observe much elevation in the inflammatory markers in CG-treated pigs although the analysis was only performed once and at the end of the experiment, and therefore, some changes in cytokine levels during the course of the study may have been missed. Since perturbations of the gut microbiota that lead to pathological conditions are still not fully understood, determining what aspects of the gut microbiota structurally and functionally change in IBD conditions remains an important part of research. Based on our functional metagenome prediction results, most of the categories associated with the mucosal microbiota in control pigs that did not differ from the pigs in the UM146 treatment included sugars, starch and sucrose metabolism, DNA replication, repair and recombination proteins, and transcription factors. Conversely, the large intestine's microbiota in CG-induced colitis (both CG and CGUM146) was enriched with capacities associated with secretion systems, lipopolysaccharide biosynthesis proteins, membrane and structural molecules, and flagellar assembly. Therefore, gut microbiota that were associated with CG-induced colitis appear to have a reduced capacity for energy

acquisition and a dysregulated microbial signaling and repair pathways. Lipopolysaccharide bacterial structures are known drivers of inflammation whilst flagellar bacterial antigens have been implicated as disease drivers in both mice models of colitis and in IBD patients (Steiner, 2007).

In conclusion, our study has demonstrated that induction of colitis using 1% CG caused intestinal bacterial dysbiosis. Certain bacterial shifts observed in the distal GI tract as a result of CG treatment are consistent with previous findings in IBD patients. In this context, CG significantly influenced bacterial diversity and induced notable microbial changes in the percentages of the major phyla Firmicutes, Bacteroidetes and Proteobacteria, especially in the cecum, ascending and descending colon. No significant difference was observed between UM146 and the control group, suggesting that the ability of UM146 to cause bacterial dysbiosis may be limited in healthy subjects – a known characteristic of opportunistic pathogens. Overall, CG was shown to be an acceptable model for mimicking human colitis in pigs, but the role of UM146 in IBD needs further investigation.

BRIDGE TO CHAPTER 4

In the experiment reported in manuscript I, we have demonstraed that an administration of 1 % CG for 21 days caused bacterial dysbiosis. Therefore, CG could be an acceptable model for mimicking human colitis. Having established that CG can induce colitis, the next step would be to test therapeutic products that can attenuate or modulate the effects of CG-induced colitis. In this context, our second specific objective of the research described in this thesis was to investigate therapeutic role of probiotics (*E. coli* UM 2 &7) and prebiotics (resistant starch) in a pig model of experimental colitis. Therefore, the next chapter describes a study, which was designed to achieve this objective.

Rationale: Current medical treatment of IBD involves use of antibiotics and compounds with immunomodulation or anti-inflammatory properties (Bernstein, 2015; Chandel et al., 2015; Shahidi et al., 2016); however, not all patients respond well to these therapies and IBD continues to cause significant morbidity. This has led to research into alternative therapeutic measures including use of different types of probiotics, prebiotics and or synbiotics but the results are inconsistent or inconclusive (Steed et al., 2008; Sinagra et al., 2013; Ghouri et al., 2014; Bernstein, 2015; Peterson et al., 2015; Wasilewski et al., 2015) hence, the need for further investigations.

CHAPTER 4

MANUSCRIPT II

Mucosa associated microbiota dysbiosis in a pig model of experimental colitis: impact of Prebiotics (resistant starch) and *Escherichia coli* (UM2 and UM7) probiotics.

A version of the material presented in chapter four of this thesis will be submitted to Beneficial Microbes journal for publication. The authors are: Peris M Munyaka, Jean-Eric Ghia, Ehsan Khafipour.

4.1. ABSTRACT

Current medical treatment of IBD involve use of antibiotics and compounds with anti-inflammatory properties; however, not all patients respond well to these therapies calling for alternative measures. We investigated therapeutic role of potato resistant starch (RS; prebiotics) and *Escherichia coli* probiotics (UM2& UM7) in a pig model of colitis. Degraded carageenan gum (CG) was used to induce mild ulcers/colitis in the gastrointestinal tract. Thirty piglets received basal diet and were divided into 5 treatments: 1) control 2) CG only 3) CG+Prebiotics 4) CG+Probiotics, and 5) CG+Prebiotics+Probiotics. Pigs in treatments 2 - 5 recived 1% CG in drinking water from day (d)1 of experiment, while probiotics and prebiotics were administered from d 8 of the study and all pigs were euthanized on d 21. Tissue mucosal scrapings from ileum, cecum, ascending and descending colon were used for DNA extraction and the V4 region of bacterial 16SrRNA was amplified and sequenced. RS down-regulated IL-1β, IL-6 and TNF-α in the descending colon, cecum and ileum, respectively. Alpha-diversity was lower in CG relative to control in the cecum and ascending colon whereas bacterial structure differed among some treatment groups in various intestinal segments. In addition, few differences were observed at phylum level and specific taxa (e.g *Blautia, Helicobacter*, Bacteroidaceae, *Bacteroides*, *Lactobacillus*) were enriched in CG, whereas other taxa, such as *Prevotella, Escherichia*, Succinivibrionaceae, *Desulfovibrio, Oscillospira,* and *Ruminococcus*, were enriched in therapeutic groups and control. Also, various functional pathways including folate biosynthesis, metabolism of vitamin B6 and metabolism/ biosynthesis of various amino acids associated with the therapeutic treatments while glycolysis/gluconeogenesis and peptidases were enriched in CG. The results indicate that CG-induced colitis caused dysbiosis, but inclusion of RS and the *E. coli* probiotics or their combination showed minimal effects in modulation of bacterial dysbiosis.

Key words: IBD, pig model, prebiotic, probiotic, colitis, microbiota, and dysbiosis.

4.2. INTRODUCTION

Dysbiosis of gut microbiota has been associated with pathogenesis of inflammatory bowel disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC) (Seksik et al., 2006). The dysbiosis is mostly characterized by a decrease in microbial diversity and reduced abudance of members of Firmicutes and Bacteroides, and an increase in Proteobacteria (Prideaux et al., 2013; Thorkildsen et al., 2013; de Souza and Fiocchi, 2016). Current medical treatment of IBD involves use of antibiotics and compounds with immunomodulation or anti-inflammatory properties (Bernstein, 2015; Chandel et al., 2015; Shahidi et al., 2016); however, not all patients respond well to these therapies and IBD continues to cause significant morbidity. This has led to research into alternative therapeutic measures including use of different types of probiotics, prebiotics and or synbiotics but the results are inconsistent or inconclusive (Steed et al., 2008; Sinagra et al., 2013; Ghouri et al., 2014; Bernstein, 2015; Peterson et al., 2015; Wasilewski et al., 2015) hence, the need for further investigations.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). On the other hand, prebiotics represents a diverse range of indigestible dietary carbohydrates that are not digested and absorbed in the upper digestive tract and, so, pass into the large bowel where they undergo microbial fermentation (Higgins and Brown, 2013; Giuberti et al., 2015). Prebiotics have the potential to selectively stimulate growth of beneficial gut bacteria, for example *Lactobacilli* and *Bifidobacterium* (Patel and DuPont, 2015), and evidence suggests that resistant starch (RS) act as substrate for fermentation in humans and pigs (Giuberti et al., 2015). *Escherichia coli* UM2 and UM7 probiotics were previously isolated and characterized in our lab, and they were shown to be able

to inhibit growth of pathogenic *E. coli* through production of colicin, an antimicrobial peptide known to suppress growth of other pathogenic *E. coli* without being harmful to the host (Amit, 2007). The probiotics can also weakly utilize starch to gain a competitive advantage over pathogenic *E. coli* that lack the ability to utilize starch in the hindgut (Amit, 2007). Synbiotics are products that contain both probiotics and prebiotics.

The gut microbiota has a significant role in human health and disease, and dysbiosis of the intestinal ecosystem contributes to the development of certain illnesses that can be reversed by favourable alteration of the microbiota. In this context, use of animal models is an important tool in understanding the interactions of different dietary inclusions with the gut microbiota and consequently the immune system, and how this might influence the course of diseases such as IBD. Therefore the aim of this study was to test the hypothesis that prebiotics (resistant starch), probiotics (*E. coli* UM2 nd UM7), or their combination can attenuate dysbiosis associated with UC in a pig model of ulcerative colitis.

4.3. MATERIALS AND METHODS

4.3.1. Animals, housing and ethical considerations.

A total of 30 piglets [Duroc x (Yorkshire x Landrace)] weaned at 17±2 d were obtained from Sunnyside Colony (Newton Siding, Manitoba, Canada). The Pigs were housed in a temperature-controlled room within the T. K. Cheung Center for Animal Science Research, University of Manitoba (Winnipeg, MB, Canada). Room temperatures were maintained at 31°C during week (wk) 1 and 29°C during wk 2 and 3, with a 16 h lighting system. All pigs had *ad libitum* access to water and basal diet in mash form formulated to meet or exceed the national research council (NRC, 2012) recommendations for a 7 to 11 kg pig. The experiment lasted for 21 days and the pigs were allowed to acclimate for 3 days before the start of experimental treatments. The

procedures were approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee, and the pigs were cared for according to the guidelines of the Canadian Council of Animal Care (CCAC, 1993).

4.3.2. Degraded carrageenan gum (CG).

To induce mild ulcers on the intestinal tract of pigs, a 1% CG solution prepared from carrageenan powder (CarboMer, Inc. San Diego, CA, USA), a sulphated polysaccharide that induces predominantly mucosal and submucosal lesions with histological similarity to UC (Elson et al., 1995b), was administered. The CG was administered via drinking water using elevated jugs connected to normal drinking nipples. The 1% concentration was chosen based on a pilot study conducted prior to this experiment (data not shown) where 0, 1, 2 and 4% CG concentrations were tested. Administration of 1% CG in drinking water only induced a mild injury in the pigs' gastrointestinal tract (GIT) with varying degree of mucosa and sub mucosal edema but without any granulomatous inflammation which is similar to what is observed in UC.

4.3.3. Preparation of degraded carrageenan gum.

Un-degraded carrageenan has molecular weights of 1.5×10^6 to 2×10^7 (Tong et al., 1980b; Tobacman, 2001a) and it is generally considered safe; however, its degradation to low molecular weights is associated with ulcerations and cancer-promoting effects (Watt et al., 1979; Tobacman, 2001a). The solution used in this study was prepared by acid hydrolysis according to the procedure described previously (Watt et al., 1979), and is expected to yield a degraded carrageenan of average molecular weight of 20,000 to 30,000 (Weiner, 1991; Marcus et al., 1992). Briefly, to each gram of the dry powder, one mL of concentrated HCl was added in a glass beaker and thoroughly mixed at room temperature (22°C) with a glass rod for 1 h. After 1 h, distilled water was added while stirring the mixture. The acidified solution was neutralized

with 2 M sodium hydroxide to pH of 7-8 and the volume was adjusted to give a 1% concentration.

4.3.4. Induction of colitis and administration of probiotics and prebiotics.

Pigs were weighed and randomly assigned to 5 treatment groups with 3 pigs per pen and 2 replicate pens per treatment. The treatments were: 1) Control; 2) Pigs receiving 1% CG in drinking water, CG; 3) CG+Prebiotics (potato resistant starch), CGPre; 4) CG+Probiotics *E. coli* strains UM2 and UM7, CGPro; and 5) CG+Prebiotics+Probiotics, CGPP. Pigs in treatments 2 - 5 recived 1% CG in drinking water from d1 of experiment whereas pigs in treatments 3 received a basal diet supplimented with 5 % resisant starch (prebiotics) from d 8 until end of the study. Pigs in treatment 4 received an overnight cocktail culture of probiotics *E. coli* UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with a small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. Pigs in treatment 5 were treated as in 2, 3 and 4 and all pigs were euthanized on d 21 (**Table 4.1)**.

4.3.5. Tissue and Digesta Sampling

On d 21 of the study, all pigs were sedated by intramuscular injection of Ketamine: Xylazine (20:2 mg/kg BW) and euthanized by an intracardiac injection of 110 mg/kg BW sodium pentobarbital (Bimeda-MTC Animal Health, Inc., Cambridge, On, Canada). The abdominal cavity was opened from sternum to pubis to expose the gastrointestinal tract without damaging the wall of the digestive tract. The small intestine was stripped free of its mesentery and ileal, cecal and colon digesta samples were obtained, divided into two sub-samples and

Table 4.1 Schedule showing dates when CG, prebiotics (potato starch), and probiotics (*Escherichia coli* UM2 & UM7) were administered

Treatment administration	Day of the study																				
	Adaptation period (3 days)[1]	d	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CG (1% in drinking water; ad lib)[2]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potato starch (Prebiotics)[3]									+	+	+	+	+	+	+	+	+	+	+	+	+	+
Probiotics (*E. coli* UM2 & UM7)[4]									+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1]The first 3 d served as adaptation period.

[2]CG was administered from d 1 of the study until the end of the study, whilst administration of the prebiotics (potato starch), and probiotics (*E. coli* UM2 & UM7) was started on d 8 until the end of the study.

[3]Potato starch was offered as a dietary suppliment at 5 % of diet inclusion rate.

[4]An overnight cocktail culture of probiotics *E. coli* UM2 and UM7 (50 mL per pig; 10^8 cfu/mL); the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first

transferred to sterile sample bags. One sub-sample of the digesta was used for determination of pH, whereas the second sub-sample was kept on ice and later transferred to -20 °C for later analysis of volatile fatty acids (VFA). Tissue samples (two 5 cm long segments) were collected from the ileum, cecum, ascending, and descending colon, flushed with sterile saline to remove excess lumen contents, immediately frozen in liquid nitrogen and later stored at - 80° C for DNA extraction and further molecular/microbial analyses.

4.3.6. Analysis of pH, ammonia N and volatile fatty acids (VFA).

pH was measured immediately after digesta collection using an Accumet Basic 15 pH meter (Fisher Scientific, Fairlawn, NJ) equipped with a Sensorex 450C Flat Surface Combination pH/Reference Electrode (Sensorex, Stanton, CA), which was standardized with certified pH 4 and 7 buffer solutions. Ammonia nitrogen concentration was measured using a calorimetric technique as described previously (Novozamsky et al., 1974), whilst volatile fatty acids were determined using gas chromatography (Bhandari et al., 2007).

4.3.7. Characterization of inflammatory responses.

Tissue samples were homogenized (50mg/mL) in Tris lysis buffer (Meso scale discovery diagnostics, Rockvile, MD, USA) containing protease inhibitors (Roche, Mississauga, ON, Canada). Samples were centrifuged at 3000 xg for 10 minutes and the supernatant was recovered and stored at – 80 °C until analyzed. Levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) and anti-inflammatory mediator (IL-10,) were determined using a custom meso scale porcine kit (Meso scale discovery diagnostics, Rockvile, MD, USA), according to the manufacturer's instructions.

4.3.8. Correlation coefficients

Associations between bacterial taxa with an abundance ≥ 0.01% of community in the ileum, cecum and ascending colon and short chain fatty acids (acetic, propionic and butyric) were determined using non-parametric Spearman's rank correlation implemented in PAST software (Hammer et al., 2001). For each correlation, correlation coefficient (Spearman's Rho) and *P*-value were obtained. 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.

4.3.9. DNA extraction.

Tissue samples were thawed at room temperature. The inner wall was then gently scrapped with a blunt blade to obtain 200-300 mg of mucosa, of which, approximately 50 mg of sample was used for DNA extraction. DNA was extracted using a ZR Tissue and Insect DNA kit (Zymo Research Corp., Orange, CA), which included a bead-beating step for the mechanical lysis of the microbial cells. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the DNA quality was evaluated by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3'). Amplicons were verified by agarose gel electrophoresis.

4.3.10. Library construction and Illumina sequencing

The V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al., 2012), as previously described (Derakhshani et al., 2015). Briefly, the reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. PCR reaction for each sample was performed in duplicates and contained 1.0 µl of pre-normalized DNA, 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 by 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 Qubit 2.0 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´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-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. The sequencing data were uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed through accession number SRR2919071.

4.3.11. Bioinformatic analyses.

The PANDAseq assembler (Masella et al., 2012) was used to merge overlapping paired-end Illumina fastq files. All the sequences with low quality base calling scores as well as those containing uncalled bases in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME (Caporaso et al., 2010b). Assembled reads were demultiplexed according to the barcode sequences and 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 OUT picking process (Rideout et al., 2014). Taxonomies were assigned to the representative sequence of each OTU using RDP classifier (Wang et al., 2007) and aligned with the Greengenes (v. 13.5) reference database (DeSantis et al., 2006) using PyNAST algorithms (Caporaso et al., 2010a). Phylogenetic tree was built with FastTree 2.1.3. (Price et al., 2010).

4.3.12. Alpha- and beta-diversities.

Within sample diversity (α-diversity) was calculated using Chao 1 (Chao, 1984), a measure of species richness, and the *P* values were determined using the Proc mixed procedure of SAS (SAS 9.3). An even depth of 5000, 5000, 14000, and 10000 sequences per sample was used to calculate richness and diversity indices for ileum, cecum, ascending and descending colon respectively. To assess the beta-diversity (β-diversity) differences among bacterial communities from different treatments within each intestinal compartment, non-metric multidimensional scaling (nMDS) ordination plots were generated using the open source R software (3.1.0) by employing Bray-Curtis similarity matrices with a conventional cut-off of < 0.2 for the stress value. 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 may be interpreted as the relative difference in the bacterial community composition; thus, points that are closer are more similar than points that are more distant. To assess the statistical differences in β-diversity of bacterial communities among treatment groups, permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2005) was performed and *P*-values were calculated.

4.3.13. Linear discriminant analysis with effect size (LEfSe) analysis

LEfSe (Segata et al., 2011)was used to identify overrepresented taxa between pigs with CG-induced colitis and pigs receiving probiotics and prebiotics therapy.

4.3.14. 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 and Beiko, 2010). To determine the functional KEGG pathways that could be associated with the microbial changes observed, we compared the functional pathways for the mucosal microbiota of samples from CG and CGPRE or CGPRO at all the intestinal sites (ileum, cecum, ascending and descending colon).

4.3.15. Other statistical analysis.

For the cytokines, volatile fatty acids, pH, ammonia, alpha-diversity and phylum data, the effect of CG and the additives was evaluated in a completely randomized design and the data was subjected to ANOVA using the PROC MIXED procedure of SAS (SAS 9.3). Means were separated using Tukey's test, and the UNIVARIATE procedure of SAS was used to test the normality of residuals. For non-normally distributed data, negative binomial distributions were fitted in the GLIMMIX procedure of SAS and the goodness of fit for different distributions was determined using Pearson chi-square/DF (closer to 1 is better). Normalized data were used to assess the effect of treatment using MIXED procedure of SAS (SAS 9.3), with treatment as the fixed and pig as the random factor. The differences between treatments were considered significant at $P < 0.05$.

4.4. RESULTS

4.4.1. Short chain fatty acids, ammonium N, and pH in the ileum, cecum and colon.

As shown in **Table 4.2**, ammonium N did not differ among treatment groups in the ileum, cecum and colon, while pH was higher in the CGPP treatment compared to other treatments in all the intestinal segments. VFA concentrations were not different among treatment groups in the ileum and cecum. In the colon, acetate and butyrate were significantly higher in both the control and CGPP, although CGPP's acetate level wasn't significantly different from the other treatment groups.

4.4.2. Inflammatory cytokines in the ileum, cecum, ascending and descending colon.

CGPRE treatment down-regulated pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the descending colon, cecum and ileum respectively, but no significant differences were observed in the ascending colon. IL-8 and IL-10 did not differ among treatment groups in all intestinal segments (**Figure 4.1**).

Table 4.2. Effect of degraded carrageenan gum (CG), prebiotics (potato starch), and probiotics (*Escherichia coli* UM2 & UM7) microbial activities in the ileum, cecum and colon digesta in weaned pigs[1], as determined by changes in the level of pH, ammonium nitrogen (N), and volatile fatty acids (VFA).

	Treatments[2]						
Variable	**Control**	**CG**	**CGPre**	**CGPro**	**CGPP**	**SED[3]**	***P Value***
pH							
Ileum	6.01[b]	6.06[b]	5.891[b]	6.49[ab]	7.23[a]	0.43	**0.0331**
Cecum	5.63[b]	5.59[b]	5.66[b]	6.05[b]	6.584[a]	0.19	**0.0001**
Colon	5.84[b]	6.01[b]	5.82[b]	6.02[b]	7.01[a]	0.2	**<. 0001**
Ammonium N, mg/dL							
Ileum	17.01	14.43	14.16	16.13	13.84	2.6	0.7046
Cecum	23.86	23.62	27.52	25.38	20.75	3.7	0.4946
Colon	35.95	30.83	36.22	35.81	32.09	4.1	0.8328
Ileum VFA, mmol/mL							
Acetate	9.97	5.59	8.68	6.33	7.48	1.9	0.2024
Propionate	0.428	0.372	0.807	0.152	0.327	0.2	0.1756
Butyrate	0.555	0.668	0.355	0.418	0.436	0.3	0.8726
Valerate	0.237	0.448	0.255	0.229	0.247	0.1	0.2234
Iso-butyrate	0.286	0.389	0.359	0.23	0.184	0.2	0.9084
Iso-valerate	0.422	0.706	0.463	0.577	0.53	0.2	0.6775
Cecal VFA, mmol/mL							
Acetate	48.60	38.05	38.14	31.65	43.23	5.7	0.0753
Propionate	24.87	28.00	22.44	17.32	25.8	3.8	0.0975
Butyrate	13.94	8.9	10.19	8.18	12.73	2.1	**0.0493**
Valerate	3.52	2.39	3.37	2.60	3.12	1.0	0.7599
Iso-butyrate	0.12	0.20	0.207	0.192	0.148	0.09	0.8731
Iso-valerate	0.312	0.348	0.467	0.458	0.416	0.17	0.8770
Colon VFA, mmol/mL							
Acetate	54.89[a]	42.12[b]	43.90[b]	41.64[b]	49.06[ab]	3.8	**0.0098**
Propionate	24.39	24.69	21.03	20.49	23.31	2.7	0.4097
Butyrate	14.91[a]	9.75[b]	8.67[b]	8.56[b]	14.68[a]	1.8	**0.0009**
Valerate	4.37	3.36	2.52	2.87	3.69	0.7	0.1655

Iso-butyrate	0.303	0.138	0.23	0.201	0.202	0.09	0.5906
Iso-valerate	0.81	0.67	0.79	0.69	0.57	0.2	0.8643

[ab]Means within the same row with different superscripts are significantly different.

[1]CG Pigs received CG from d 1 of the study until the end of the study, whilst administration of the prebiotics (potato starch), and probiotics (*E. coli* UM2 & UM7) was started on d 8 until the end of the study.

[2]CG= pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

[3]SED= Standard error of difference between treatment means.

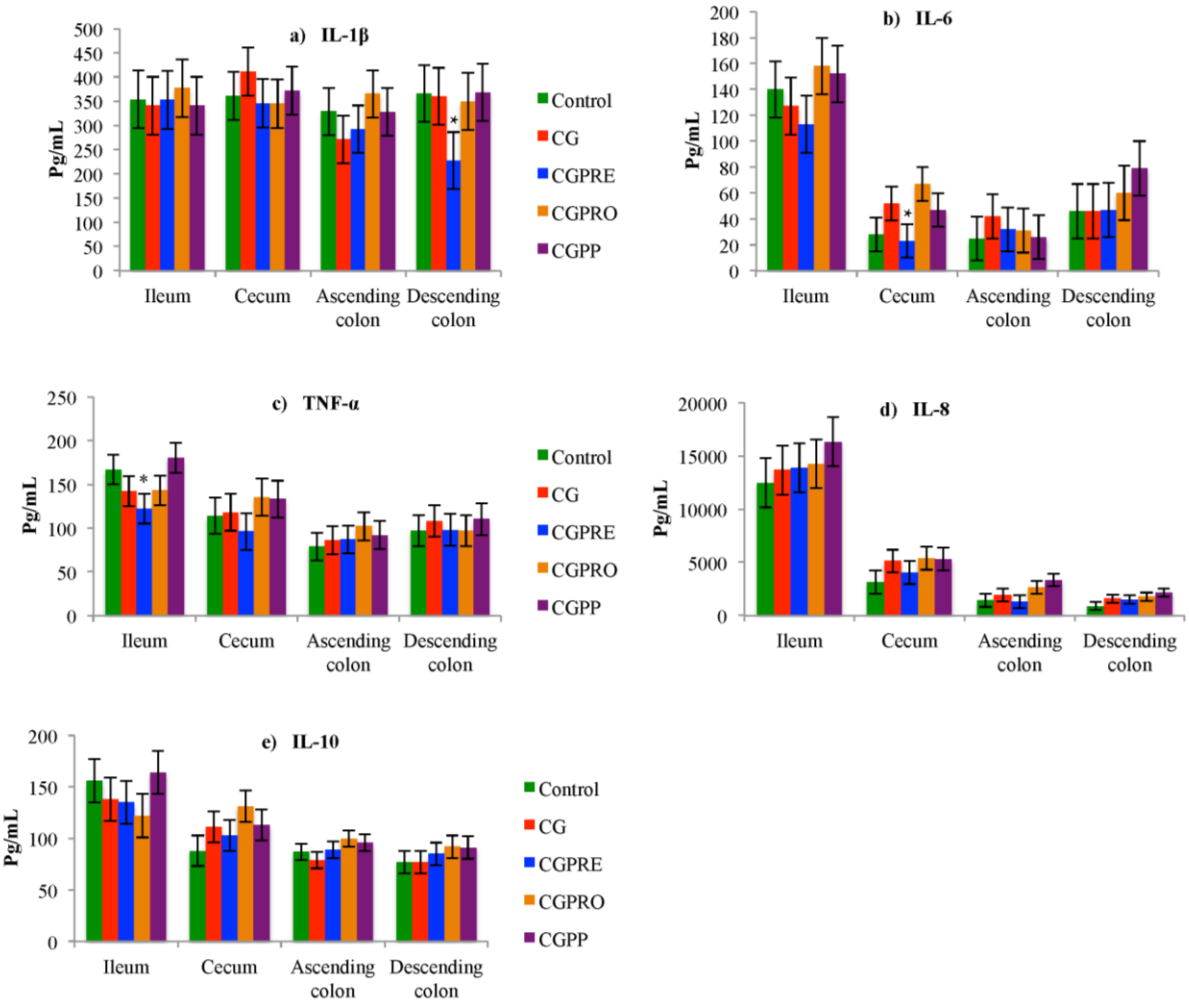


Figure 4.1. a, **b**, **c**, **d**, and **e:** Effect of prebiotics (resistant starch) and *E. coli* probiotics on inflammatory markers (IL-1β, IL-6, TNF-α IL-8 and IL-10) in a pig model of experimental colitis. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO. *Designates a significant difference compared to other treatment groups within the specific intestinal tissue; $P \leq 0.05$

4.4.3. Correlation coefficient

As shown in **Tables 4.3, 4.4 and 4.5**, several taxa were positively or negatively correlated with various short chain fatty acids (SCFA) and inflammatory cytokines in the ileum, cecum and ascending colon.

4.4.4. Alpha-diversity in different intestinal segments.

Based on the Chao 1 analysis, the trend for all intestinal segments was that we observed the highest alpha diversity in the control and the lowest alpha-diversity in the CG, but the inclusion of RS and or probiotics did not have significant impact on alpha diversity. As shown in **Figure 4.2 a**, statistical analysis revealed no significant difference in species richness among treatment groups in the ileum (P = 0.3320). However, species richness in the cecum was significantly different (P = 0.0149), which was due to a lower diversity in both CG and CGPRO compared to the control (**Figure 4.2 b**). Also, as shown in **Figure 4.2 c**, overall species richness was not statistically different among treatments in the ascending colon (P = 0.1006), however, Tukey's comparisons between Lsmeans showed significant difference between CG and the control. No significant differences were observed in the descending colon (P = 0.1597; **Figure 4.2 d**).

4.4.5. Beta-diversity differences across the intestinal segments.

Ileum: As shown in the nMDS plot (**Figure 4.3 a**), some samples clustered separately according to treatment groups. PERMANOVA analysis showed significant differences in both weighted (P = 0.0088) and unweighted (P = 0.0324) UniFrac distances. In this context, pair-wise analysis revealed significant differences between specific groups including CG vs CGPRE; P = 0.0235, CG vs CGPRO; P = 0.0138, and CG vs CGPP; P = 0.0324. Other pair-wise comparisons were not significantly different.

Table 4.3. Correlation coefficient between selected taxa[1], short chain fatty acids (SCFA; acetate, propionate, butyrate) and inflammatory cytokines in the ileum.

Ileum	SCFA/Cytokine	Taxa	Rho (ρ)	*P value*
	Acetate	Unclassified members of Pseudomonadaceae	-0.4690	0.0089
		Unclassified members of Oxalobacteraceae	-0.4096	0.0246
		Lactobacillus	-0.5582	0.0013
		Unclassified members of Lactobacillales	0.4003	0.0284
	Propionate	*Desulfovibrio*	-0.4523	0.0121
		Campylobacter	-0.6075	0.0004
		Unclassified members of Succinivibrionaceae	-0.4355	0.0162
		Unclassified members of Erysipelotrichaceae	-0.4277	0.0184
		Veillonella	0.3749	0.0412
		Dialister	-0.4035	0.0270
		Unclassified members of Streptococcaceae	0.5286	0.0027
		Streptococcus	0.5481	0.0017
		Turicibacter	0.4286	0.0181
		Unclassified members of Lactobacillales	0.4952	0.0054
		Unclassified members of Bacteroidales	-0.4374	0.0156
		Pedobacter	-0.4213	0.0204
		Actinobacillus	0.4945	0.0055
	Butyrate	*Catenibacterium*	0.4514	0.0123
		Phascolarctobacterium	0.4659	0.0095
		Bacteroides	0.3952	0.0307
		Parabacteroides	0.3839	0.0362
		Unclassified members of S24-7	0.4001	0.0285
		CF231	0.3925	0.0319
	IL-1β	Unclassified members of Streptophyta	-0.4696	0.0088
		Unclassified members of Staphylococcaceae	-0.4348	0.0163
		Unclassified members of Lactobacillales	-0.3702	0.0441
		Bacteroides	0.4030	0.0272
		Bifidobacterium	-0.3703	0.0440
	IL-6	*Anaerovibrio*	0.3976	0.0296
		Unclassified members of Veillonellaceae	0.3851	0.0356
		Unclassified members of Clostridiaceae	0.4524	0.0121
		CF231	0.4175	0.0217
	IL-8	*Acinetobacter*	-0.5550	0.0015
		Unclassified members of Pseudomonadaceae	-0.4826	0.0069
		Escherichia	0.3784	0.0392
		Unclassified members of Betaproteobacteria	-0.4976	0.0051
	IL-10	*Enterobacter*	-0.4528	0.0120
		Unclassified members of Oxalobacteraceae	-0.0703	0.0507

	Unclassified members of Coriobacteriaceae	-0.3974	0.0297
	Lactobacillus	-0.3625	0.0490
	Unclassified members of Firmicutes	0.4199	0.0209
	Bifidobacterium	-0.4117	0.0238
TNF-α	Unclassified members of Enterobacteriaceae	-0.4869	0.0064
	Citrobacter	-0.4166	0.0220
	Enterobacter	-0.6382	0.0001

[1]Taxa with relative abundance ≥ 0.01% of the population were used for the correlation analysis and only the significant correlations are shown in the Table.

Table 4.4. Correlation coefficient between selected taxa[1], short chain fatty acids (SCFA; acetate, propionate, butyrate) and inflammatory cytokines in the cecum.

Cecum	SCFA/Cytokine	Taxa	Rho (ρ)	*P value*
	Acetate	*Prevotella*	0.5119	0.0038
		CF231	0.4391	0.0152
		Roseburia	-0.5288	0.0027
		Anaerotruncus	-0.5295	0.0026
	Propionate	*Parabacteroides*	-0.3705	0.0439
		Unclassified members of Clostridiales	-0.4349	0.0163
		Anaerotruncus	-0.3754	0.0409
		Dialister	0.4518	0.0122
		Unclassified members of Erysipelotrichaceae	0.4154	0.0225
	Butyrate	Unclassified members of Lactobacillaceae	-0.4799	0.0073
		Roseburia	-0.3749	0.0412
		Anaerotruncus	-0.5457	0.0018
		Sutterella	0.3760	0.0406
	IL-1β	*Parabacteroides*	0.5648	0.0011
		Treponema	0.3995	0.0287
	IL-6	*Butyricimonas*	-0.4154	0.0224
		Ruminococcus	-0.4354	0.0162
		Unclassified members of Campylobacterales	0.3674	0.0458
		Treponema	-0.3823	0.0371
		Lactobacillus	0.4624	0.0101
	IL-8	*Streptococcus*	0.4372	0.0157
		Unclassified members of Clostridiales	-0.3887	0.0338
		Unclassified members of Clostridiaceae	-0.4118	0.0238
		Coprococcus	-0.3666	0.0463
		Unclassified members of Proteobacteria	-0.3888	0.0337
		Unclassified members of Desulfovibrionaceae	-0.4653	0.0096
		Desulfovibrio	-0.3993	0.0288
	IL-10	*Lachnospira*	0.3729	0.0424
		Oscillospira	0.3725	0.0427
		Hylemonella	0.4031	0.0272
		Campylobacter	0.3968	0.0300
	TNF-α	*Catenibacterium*	-0.4480	0.0130
		Unclassified members of Campylobacterales	0.6191	0.0003
		Campylobacter	0.5342	0.0024

[1]Taxa with relative abundance ≥ 0.01% of the population were used for the correlation analysis and only the significant correlations are shown in the Table.

Table 4.5 Correlation coefficient between selected taxa[1], short chain fatty acids (SCFA; acetate, propionate, butyrate) and inflammatory cytokines in the Ascending colon.

Ascending colon	SCFA/Cytokine	Taxa	Rho (ρ)	*P value*
	Acetate	Unclassified members of Bacteroidales	0.4741	0.0081
		Parabacteroides	0.3922	0.0321
		Prevotella	0.3780	0.0395
		Butyricimonas	0.4849	0.0066
		CF231	0.3900	0.0332
		Unclassified members of Catabacteriaceae	0.4111	0.0240
		Lachnospira	0.4481	0.0130
		Oribacterium	0.4809	0.0071
		Roseburia	-0.4750	0.0080
		Peptococcus	0.4693	0.0089
		Unclassified members of Ruminococcaceae	0.5399	0.0021
		Faecalibacterium	0.5346	0.0023
		Unclassified members of GMD14H09	0.5230	0.0030
		Unclassified members of Campylobacterales	-0.3777	0.0396
		Unclassified members of Helicobacteraceae	-0.4300	0.0177
		Helicobacter	-0.4047	0.0265
		Unclassified members of Succinivibrionaceae	-0.4488	0.0129
		Treponema	0.3661	0.0466
	Propionate	*Butyricimonas*	0.4329	0.0169
		Ruminococcus	-0.3713	0.0434
		Unclassified members of mitochondria	-0.3875	0.0344
	Butyrate	Unclassified members of BS11	-0.4426	0.0143
		Ruminococcus	-0.3845	0.0359
		Acinetobacter	-0.3788	0.0390
	IL-1β	Unclassified members of Streptococcaceae	-0.3660	0.0467
		Unclassified members of Fusobacteriaceae	-0.4390	0.0152
	IL-6	*Anaerobiospirillum*	0.4167	0.0220
	IL-8			
	IL-10	Unclassified members of S24-7	0.4537	0.0118
		Unclassified members of Catabacteriaceae	0.4228	0.0199
		Slackia	0.3899	0.0332
		RFN20	0.3694	0.0445
	TNF-a	*Oscillospira*	0.4073	0.0255
		Ruminococcus	0.4567	0.0112
		Brachyspira	0.3960	0.0303

[1]Taxa with relative abundance ≥ 0.01% of the population were used for the correlation analysis and only the significant correlations are shown in the Table.

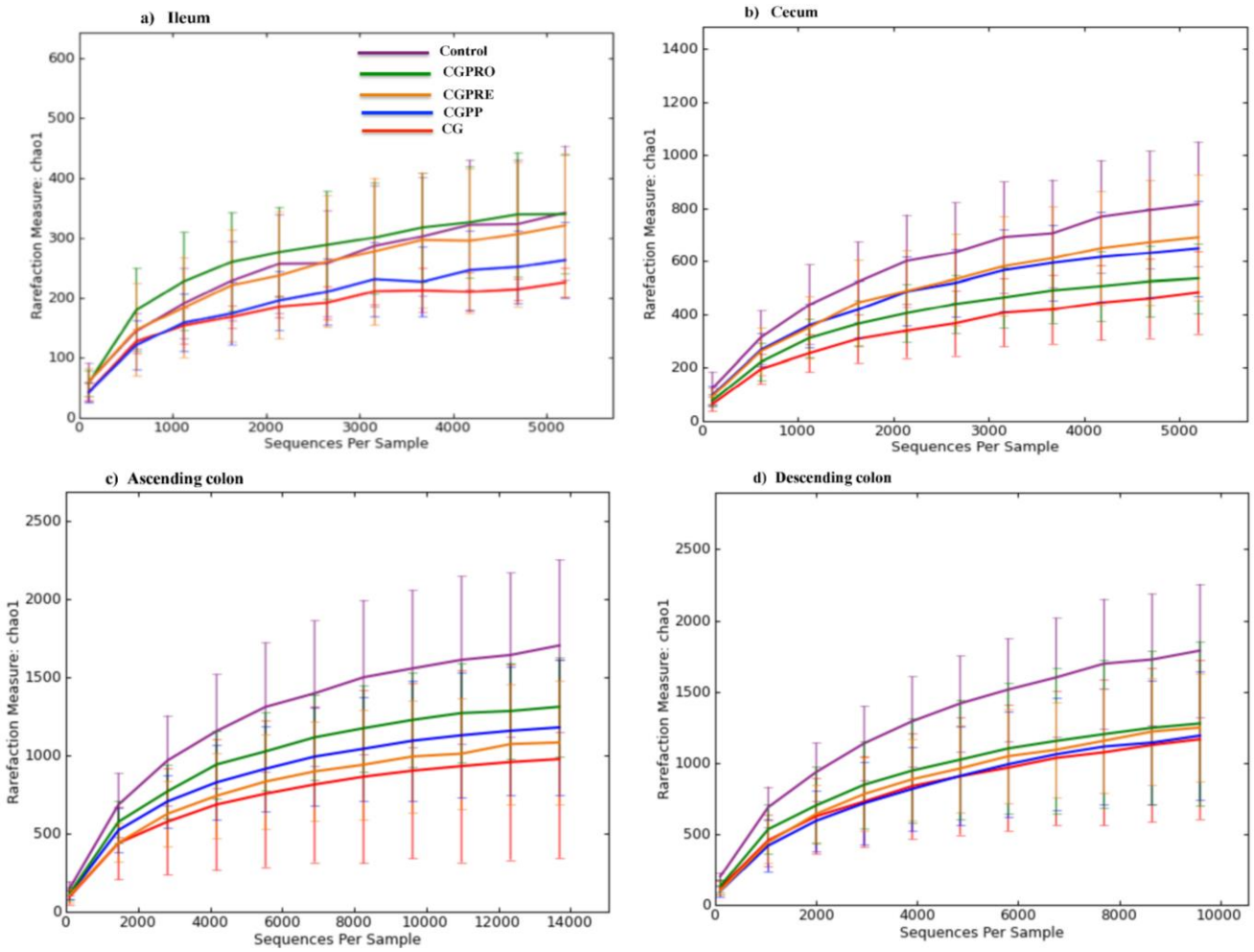


Figure 4.2. Alpha-diversity analysis based on Chao1, a measure of species richness in the: **a**) ileum; **b**) cecum; **c**) ascending colon; and **d**) descending colon of pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

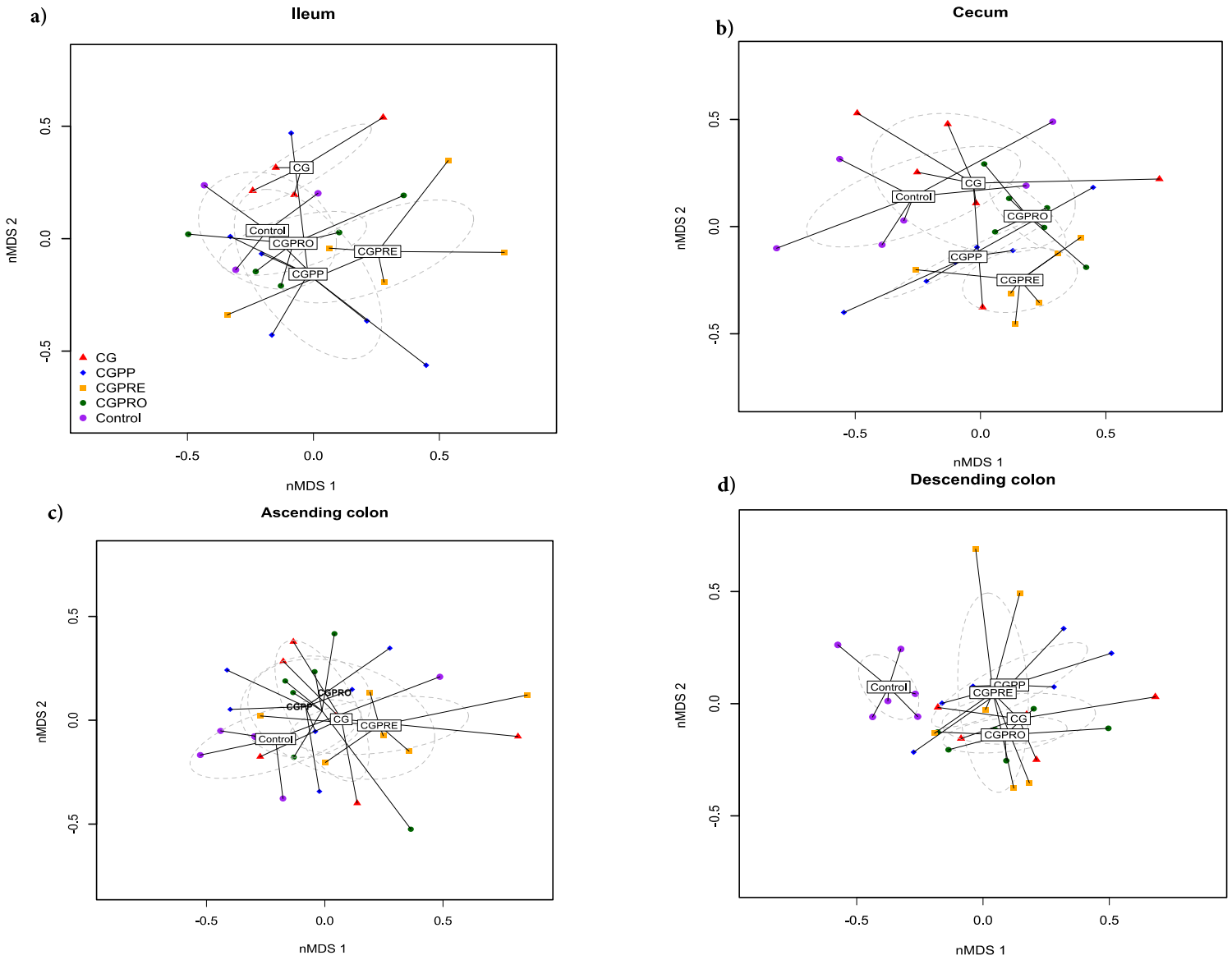

a)
Ileum
nMDS 2
nMDS 1
CG
Control
CGPRO
CGPRE
CGPP
CG
CGPP
CGPRE
CGPRO
Control
b)
Cecum
c)
Ascending colon
d)
Descending colon

Figure 4.3. Non-metric multidimensional scaling (nMDS) ordination plot, a measure of relative difference in the bacterial community composition in the: **a**) ileum; **b**) cecum; **c**) ascending colon; and **d**) descending colon of pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

Cecum: As shown in the nMDS plot (**Figure 4.3 b**), some samples clustered relatively separately according to treatment groups, which was confirmed by PERMANOVA analysis in both weighted and unweight UniFrac distances (P = 0.0001). Further pair-wise analysis revealed significant differences between specific groups in most of the comparisons ($P < 0.01$) except for the CGPRO vs CG, which was not significantly different (P = 0.0954).

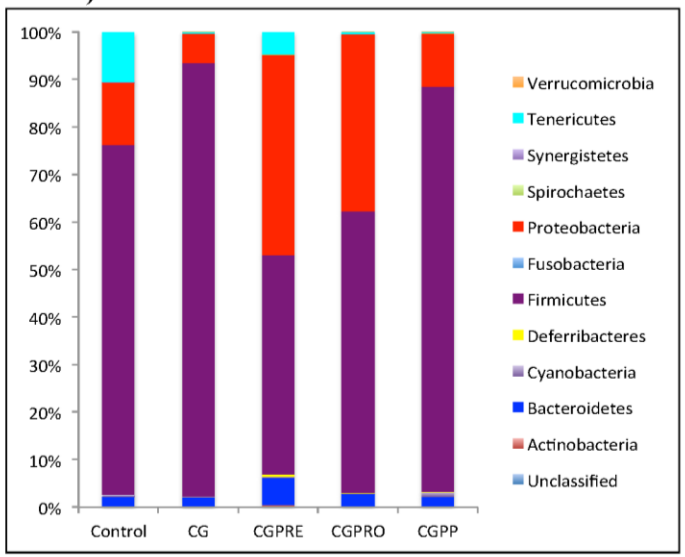
Ascending colon: As shown in the nMDS plot (**Figure 4.3 c**), although samples clustering was not very distinct, PERMANOVA analysis showed overall significant difference among treatments for both weighted and unweight UniFrac distances (P = 0.0242 and P = 0.0028), which was due to significant differences between specific groups as confirmed by pair-wise analysis including control vs CGPRO (P = 0.0332), control vs CGPRE (P = 0.0209), and control vs CG (P = 0.0145).

Descending colon: As shown in the nMDS plot (**Figure 4.3 d**), control samples clustered separately from other treatments, and PERMANOVA analysis for both weighted and unweighted UniFrac distances revealed significant differences among treatment groups (P = 0.0001). Pair-wise analysis between specific groups revealed that only comparisons between each treatment group with the control were significantly different ($P < 0.001$).

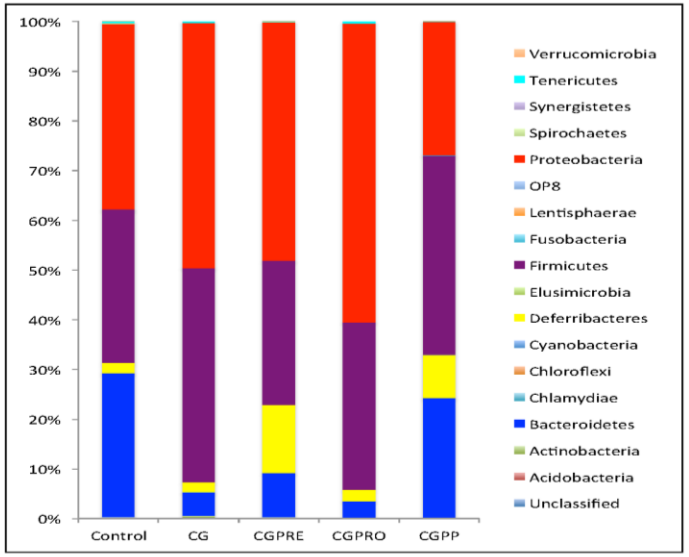
4.4.6. Effect of RS and probiotics E. coli UM2 & 7 on microbiota composition at phylum level.

As shown in **Figure 4.4 a**, the proportion of Firmicutes and Proteobacteria in the ileum was lower (P = 0.0044) and higher (P = 0.0016), respectively in the CGPRO and CGPRE groups. Also, compared to other treatment groups, the proportion of Proteobacteria in the cecum was highest (P = 0.0491) in CGPRO, while phylum Bacteroidetes was lowest (P = 0.0006) in the CG and CGPRO treatments compared to other treatments (**Figure 4.4 b**. No significant difference was observed among treatment groups in the ascending colon (**Figure 4.4 c)**.

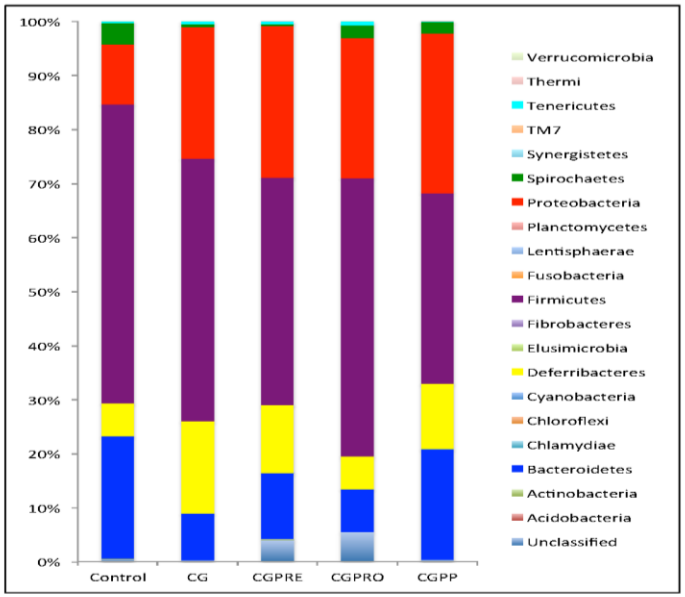
a) Ileum



b) Cecum



c) Ascending colon



d) Descending colon

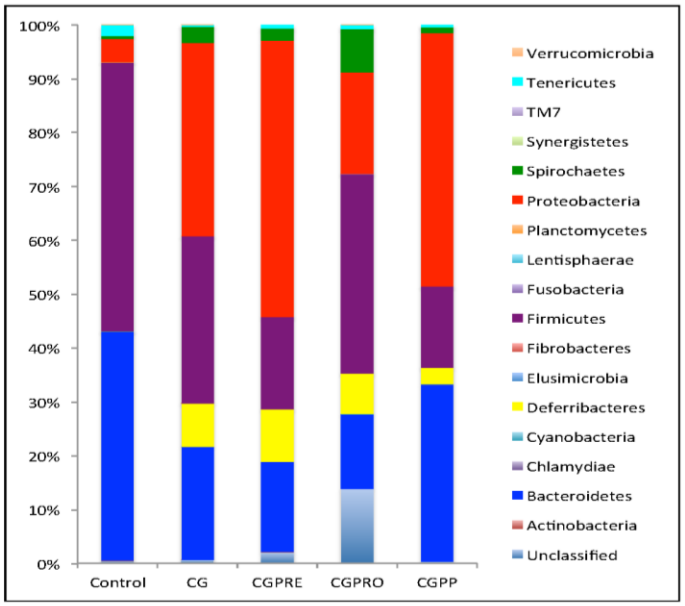


Figure 4.4. Percentage of relative abundance of bacterial phyla in the: **a**) ileum; **b**) cecum; **c**) ascending colon; and **d**) descending colon of pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 of the study until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

However, the proportion of Bacteroidetes in the descending colon was significantly reduced (P = 0.0011) in CG, CGPRO, and CGPRE groups compared to the control, whereas the proportion of Firmicutes and Proteobacteria was higher (P = 0.0001) and lower (P = 0.001) respectively, in the control and CGPRO compared to other groups (**Figure 4.4 d**). **Table 4.6** shows the analyzed phylum data.

4.4.7. Taxa characterizing treatment groups within tissue sites

Figures 4.5, 4.6, 4.7 and **4.8**, shows different bacterial clades that were significantly associated with various treatment groups in each intestinal segment, as revealed by Lefse analysis. The results are also summarized in **Table 4.7**.

4.4.8. Functional metagenome of microbiomes

The proportion of reads that mapped to reference database during OTU picking was 96.26 % for the Ileum, 91.52% for the cecum, 88.03% for the ascending colon, and 84.61 % for the descending colon (an average of 90.11% for all the tissues). The data was normalized by copy numbers before metagenome prediction, and the Nearest Sequenced Taxon Index (NSTI) for each sample which reflects the availability of reference genomes that are closely related to the abundant microorganisms in the samples were determined during metagenome prediction. On average, the NSTIs were: 0.057 for ileum, 0.062 for cecum, 0.097 for ascending colon, and 0.096 for descending colon. High scores (>0.15) generally mean few related references are available and predictions will be of low quality whereas low scores (< 0.06) indicate availability of closely related reference genomes (PICRUST 1.0.0-dev documentation tutorials for quality control). As shown in **Figures 4.9**, **4.10**, 4.**11** and **4.12**, different metabolic pathways were enriched in the mucosal microbiota of the ileum, cecum, ascending and descending colon in CG, CGPRE and CGPRO.

Table 4.6. Analysis of the most abundant phyla in each intestinal segment of pigs treated with degraded carrageenan gum (CG), prebiotics (resistant starch), and probiotics (*Escherichia coli* UM2 & UM7)[1]

	Treatments[2]						
	Control	**CG**	**CGPro**	**CGPre**	**CGPP**	**SED**[3]	***P value***
Ileum							
Firmicutes	73.62^{ab}	91.18^{a}	59.15^{b}	46.11^{b}	85.32^{a}	11.8	**0.0044**
Bacteroidetes	2.033	2.029	2.575	5.73	2.026	3.1	0.7427
Proteobacteria	13.09^{b}	6.23^{b}	37.38^{a}	42.19^{a}	11.12^{b}	9.5	**0.0016**
Tenericutes	0.316	0.224	0.347	0.14	0.2	1.2	0.8283
Cecum							
Firmicutes	30.82	43.04	33.69	29.07	40.15	9.2	0.5425
Bacteroidetes	28.962^{a}	4.702^{c}	3.332^{c}	8.736^{bc}	24.00^{ab}	6.7	**0.0006**
Proteobacteria	37.33^{ab}	49.31^{ab}	60.14^{a}	47.95^{ab}	26.82^{b}	11.8	**0.0491**
Deferribacteres	2.067	2.059	2.243	13.67	8.659	4.8	0.0631
Ascending colon							
Firmicutes	55.33	48.67	51.52	42.07	35.28	9.9	0.3416
Bacteroidetes	22.59	8.59	7.91	12.22	20.39	6.3	0.0775
Proteobacteria	11.048	24.289	25.994	28.115	29.527	7.7	0.1901
Deferribacteres	6.1	17.01	6.06	12.54	12.09	9.3	0.7273
Spirochaetes	1.12	0.569	2.288	0.326	2.109	1.0	0.2368
Descending colon							
Firmicutes	49.93^{a}	30.99^{ab}	37.01^{a}	17.1^{b}	15.08^{b}	7.3	**0.0001**
Bacteroidetes	42.38^{a}	20.99^{b}	13.86^{b}	16.74^{b}	32.79^{ab}	7.2	**0.0011**
Proteobacteria	4.26^{b}	35.88^{a}	18.92^{b}	51.36^{a}	47.00^{a}	10.3	**0.0001**
Deferribacteres	0.039	8.05	7.515	9.75	3.059	4.7	0.2060
Spirochaetes	0.629^{b}	3.071^{ab}	8.038^{a}	2.222^{ab}	1.184^{ab}	2.6	0.0566

[1]CG Pigs received CG from d 1 of the study until the end of the study, whilst administration of the prebiotics (potato starch), and probiotics (*E. coli* UM2 & UM7) was started on d 8 until the end of the study.

[2]CG= pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the

study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

[3]SED= Standard error of difference between treatment means.

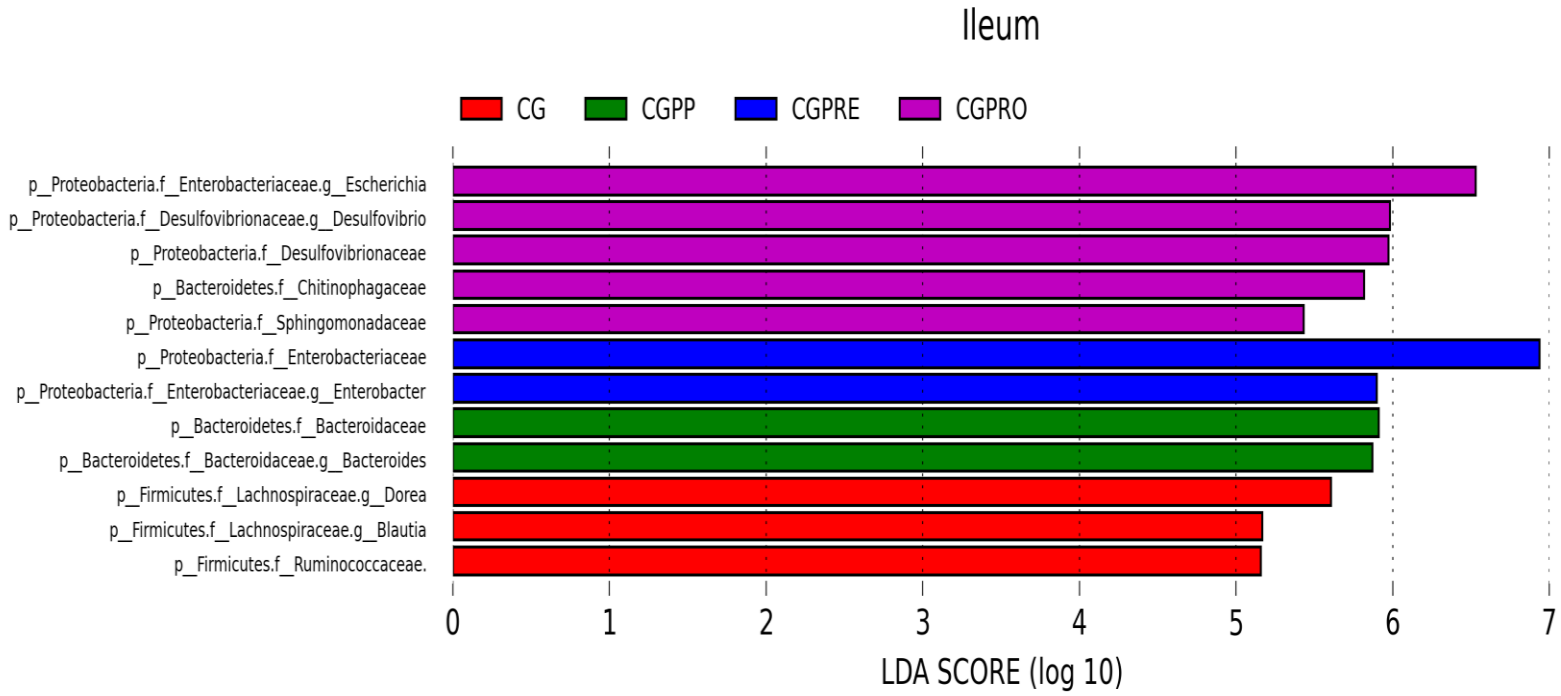


Figure 4.5. Phylogenetic comparisons of ileal mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the control group. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

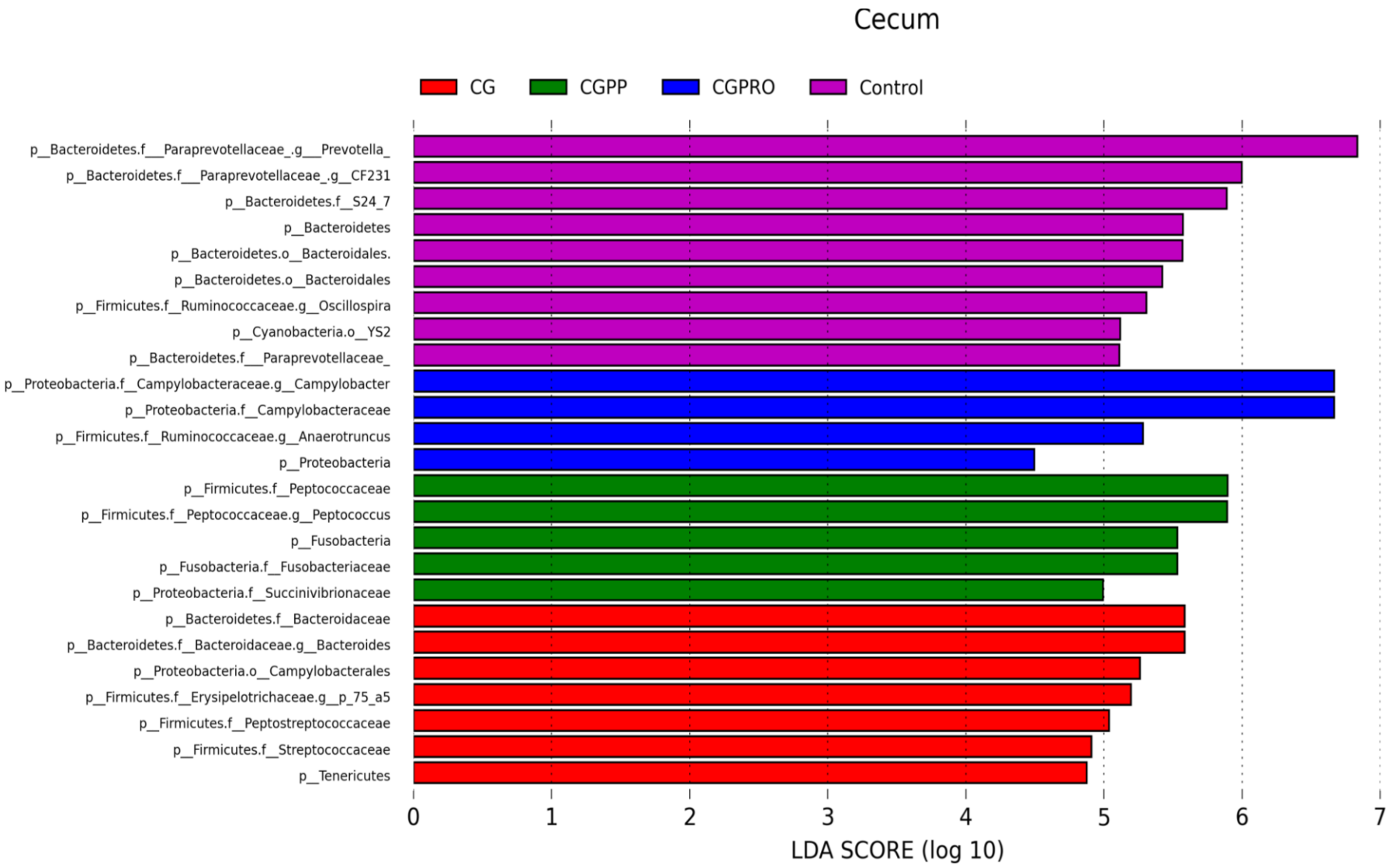

Cecum
CG
CGPP
CGPRO
Control
p__Bacteroidetes.f__Paraprevotellaceae_.g__Prevotella_
p__Bacteroidetes.f__Paraprevotellaceae_.g__CF231
p__Bacteroidetes.f__S24_7
p__Bacteroidetes
p__Bacteroidetes.o__Bacteroidales.
p__Bacteroidetes.o__Bacteroidales
p__Firmicutes.f__Ruminococcaceae.g__Oscillospira
p__Cyanobacteria.o__YS2
p__Bacteroidetes.f__Paraprevotellaceae_
p__Proteobacteria.f__Campylobacteraceae.g__Campylobacter
p__Proteobacteria.f__Campylobacteraceae
p__Firmicutes.f__Ruminococcaceae.g__Anaerotruncus
p__Proteobacteria
p__Firmicutes.f__Peptococcaceae
p__Firmicutes.f__Peptococcaceae.g__Peptococcus
p__Fusobacteria
p__Fusobacteria.f__Fusobacteriaceae
p__Proteobacteria.f__Succinivibrionaceae
p__Bacteroidetes.f__Bacteroidaceae
p__Bacteroidetes.f__Bacteroidaceae.g__Bacteroides
p__Proteobacteria.o__Campylobacterales
p__Firmicutes.f__Erysipelotrichaceae.g__p_75_a5
p__Firmicutes.f__Peptostreptococcaceae
p__Firmicutes.f__Streptococcaceae
p__Tenericutes
0
1
2
3
4
5
6
7
LDA SCORE (log 10)

Figure 4.6. Phylogenetic comparisons of cecal mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the CGPRE group. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

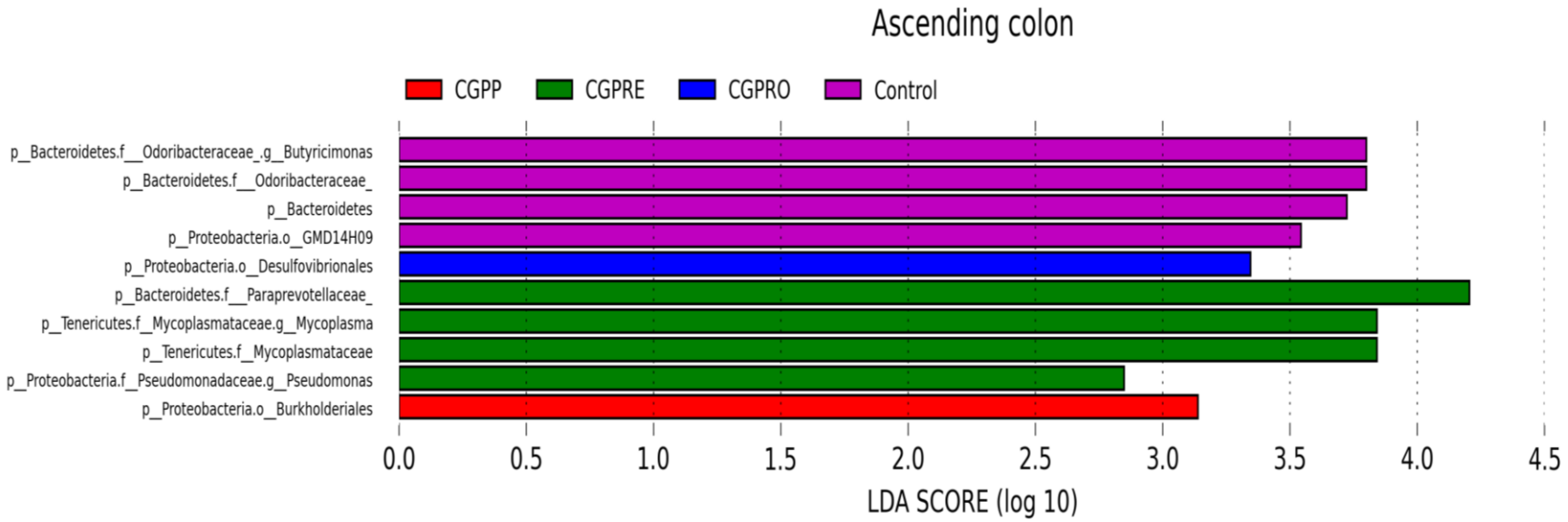


Figure 4.7. Phylogenetic comparisons of ascending colon mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the CG group. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

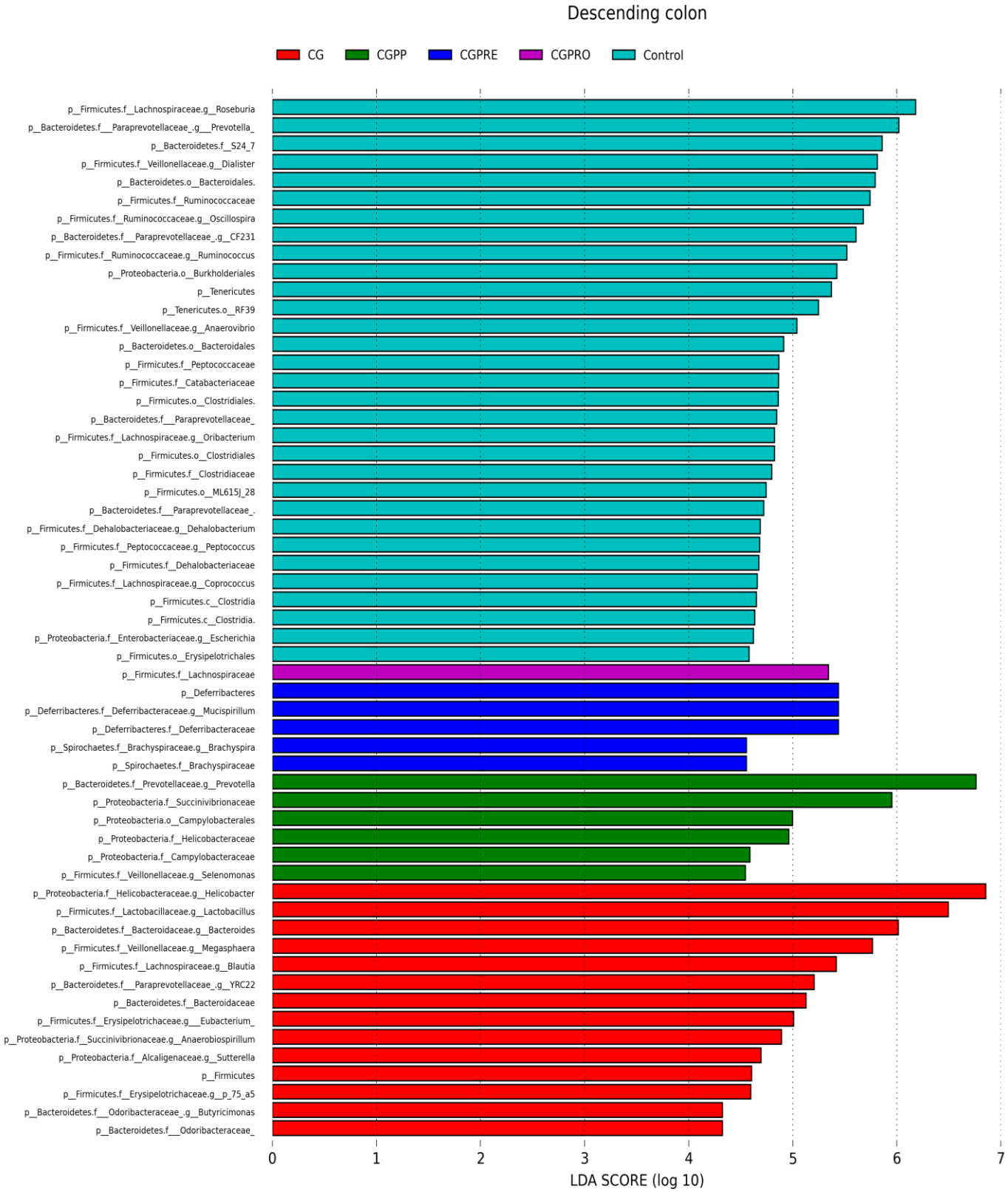

Descending colon
CG
CGPP
CGPRE
CGPRO
Control
p__Firmicutes.f__Lachnospiraceae.g__Roseburia
p__Bacteroidetes.f__Paraprevotellaceae_.g__Prevotella_
p__Bacteroidetes.f__S24_7
p__Firmicutes.f__Veillonellaceae.g__Dialister
p__Bacteroidetes.o__Bacteroidales.
p__Firmicutes.f__Ruminococcaceae
p__Firmicutes.f__Ruminococcaceae.g__Oscillospira
p__Bacteroidetes.f__Paraprevotellaceae_.g__CF231
p__Firmicutes.f__Ruminococcaceae.g__Ruminococcus
p__Proteobacteria.o__Burkholderiales
p__Tenericutes
p__Tenericutes.o__RF39
p__Firmicutes.f__Veillonellaceae.g__Anaerovibrio
p__Bacteroidetes.o__Bacteroidales
p__Firmicutes.f__Peptococcaceae
p__Firmicutes.f__Catabacteriaceae
p__Firmicutes.o__Clostridiales.
p__Bacteroidetes.f__Paraprevotellaceae_
p__Firmicutes.f__Lachnospiraceae.g__Oribacterium
p__Firmicutes.o__Clostridiales
p__Firmicutes.f__Clostridiaceae
p__Firmicutes.o__ML615J_28
p__Bacteroidetes.f__Paraprevotellaceae_.
p__Firmicutes.f__Dehalobacteriaceae.g__Dehalobacterium
p__Firmicutes.f__Peptococcaceae.g__Peptococcus
p__Firmicutes.f__Dehalobacteriaceae
p__Firmicutes.f__Lachnospiraceae.g__Coprococcus
p__Firmicutes.c__Clostridia
p__Firmicutes.c__Clostridia.
p__Proteobacteria.f__Enterobacteriaceae.g__Escherichia
p__Firmicutes.o__Erysipelotrichales
p__Firmicutes.f__Lachnospiraceae
p__Deferribacteres
p__Deferribacteres.f__Deferribacteraceae.g__Mucispirillum
p__Deferribacteres.f__Deferribacteraceae
p__Spirochaetes.f__Brachyspiraceae.g__Brachyspira
p__Spirochaetes.f__Brachyspiraceae
p__Bacteroidetes.f__Prevotellaceae.g__Prevotella
p__Proteobacteria.f__Succinivibrionaceae
p__Proteobacteria.o__Campylobacterales
p__Proteobacteria.f__Helicobacteraceae
p__Proteobacteria.f__Campylobacteraceae
p__Firmicutes.f__Veillonellaceae.g__Selenomonas
p__Proteobacteria.f__Helicobacteraceae.g__Helicobacter
p__Firmicutes.f__Lactobacillaceae.g__Lactobacillus
p__Bacteroidetes.f__Bacteroidaceae.g__Bacteroides
p__Firmicutes.f__Veillonellaceae.g__Megasphaera
p__Firmicutes.f__Lachnospiraceae.g__Blautia
p__Bacteroidetes.f__Paraprevotellaceae_.g__YRC22
p__Bacteroidetes.f__Bacteroidaceae
p__Firmicutes.f__Erysipelotrichaceae.g__Eubacterium_
p__Proteobacteria.f__Succinivibrionaceae.g__Anaerobiospirillum
p__Proteobacteria.f__Alcaligenaceae.g__Sutterella
p__Firmicutes
p__Firmicutes.f__Erysipelotrichaceae.g__p_75_a5
p__Bacteroidetes.f__Odoribacteraceae_.g__Butyricimonas
p__Bacteroidetes.f__Odoribacteraceae_
0
1
2
3
4
5
6
7
LDA SCORE (log 10)

Figure 4.8. Phylogenetic comparisons of descending colon mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

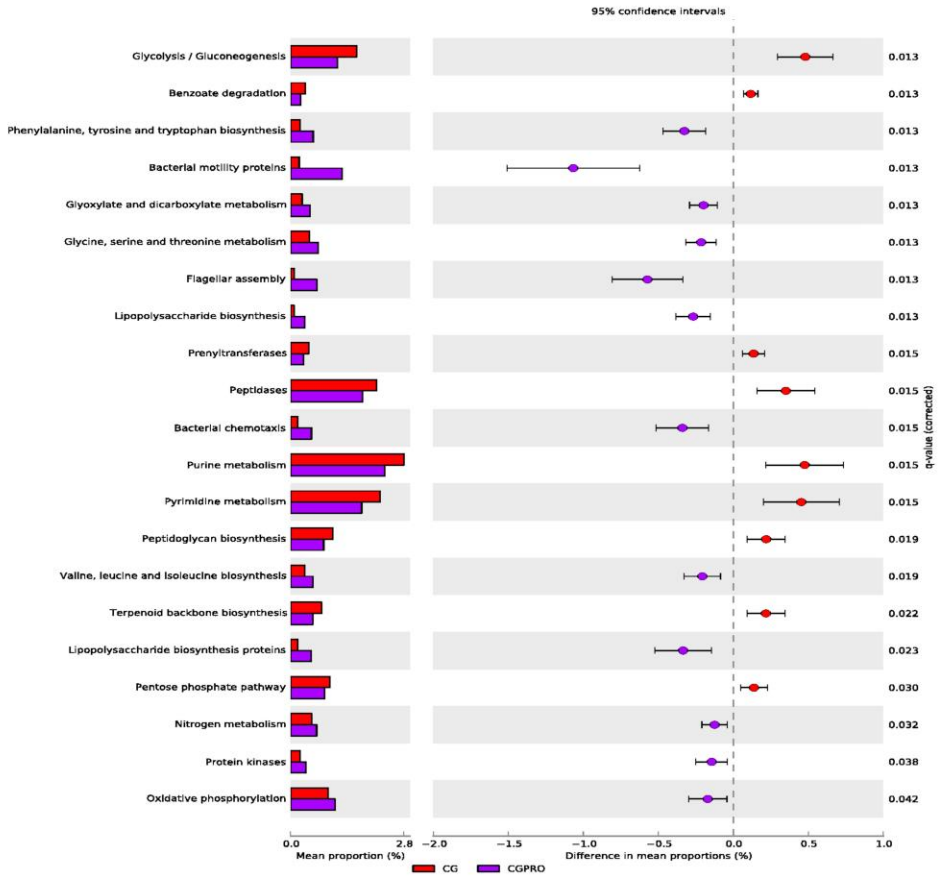
Table 4.7. Linear discriminant analysis with effect size (LEfSe) analysis summary of the significant clades/taxa in each intestinal segment of pigs treated with degraded carrageenan gum (CG), prebiotics (resistant starch), and probiotics (*Escherichia coli* UM2 & UM7)[1]

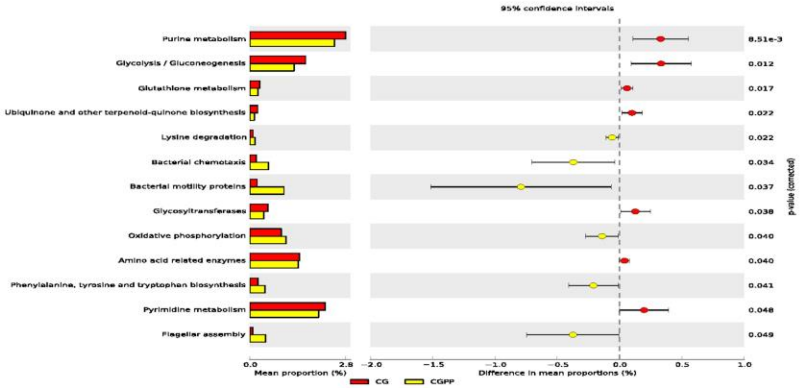
	Treatments[2]				
Tissue	**Control**	**CG**	**CGPRE**	**CGPRO**	**CGPP**
Ileum		*Dorea* *Blautia* Ruminococcaceae	Enterobacteraceae *Enterobacter*	*Escherichia* *Desulfovibrio* Desulfovibrionaceae Chitinosphagaceae Sphigomonadaceae	Bacteroidaceae *Bacteroides*
Cecum	*Prevotella, Oscillospira* *CF231* S24_7 Bacteroidetes, Bacteroidales YS2 Paraprevotellaceae	Bacteroidaceae Bacteroides Campylobacterales *P_75_a5* Peptostreptococaceae Streptococcaceae Tenericutes		*Campylobacter* Campylobacteraceae *Anaerotruncus* Proteobacteria	Peptococcaceae *Peptococcus* Fusobacteria Fusobacteriaceae Succinivibrionaceae
Ascending colon	GMD14H09 Bacteroidetes *Butyricimonas*		*Pseudomonas* Mycoplasmataceae *Mycoplasma* Paraprevotellaceae	Desulfovibrionales	Burkholderiales
Descending colon	*Prevotella*, *Dialister* *CF231*, Tenericutes Burkholderiales Ruminococcaceae S24_7, RF39 Bacteroidales, ML615j_28 *Oscillospira*, Peptococcaceae Catabacteriaceae *Anaerovibrio*, *Coprococcus* Dehalobacteriaceae Paraprevotellaceae	*Blautia* *Sutterella* *P_75_a5* *Eubacterium* Bacteroidaceae Bacteroides *Helicobacter* *Lactobacillus* *Megasphaera* *Butyricimonas* Odoribacteraceae	Deferribacteres *Mucispirillum* Deferibacteraceae *Brachyspira* Brachyspiraceae	Lachnospiraceae	*Prevotella*, *Selenomonas* Succinivibrionaceae Campylobacteriales Helicobacteraceae Campylobacteraceae

	Ruminococcus, *Escherichia* Clostridia, Dehalobacterium Clostridiaceae, *Peptococcus* Clostridiales, Erysipelotrichales *Roseburia*, *Oribacterium*	Firmicutes *YRC22* *Anaerobiospirillum*			

[1]CG Pigs received CG from d 1 of the study until the end of the study, whilst administration of the prebiotics (potato starch), and probiotics (*E. coli* UM2 & UM7) was started on d 8 until the end of the study.

[2]CG= pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.


95% confidence intervals
Glycolysis / Gluconeogenesis 0.013
Benzoate degradation 0.013
Phenylalanine, tyrosine and tryptophan biosynthesis 0.013
Bacterial motility proteins 0.013
Glyoxylate and dicarboxylate metabolism 0.013
Glycine, serine and threonine metabolism 0.013
Flagellar assembly 0.013
Lipopolysaccharide biosynthesis 0.013
Prenyltransferases 0.015
Peptidases 0.015
Bacterial chemotaxis 0.015
Purine metabolism 0.015
Pyrimidine metabolism 0.015
Peptidoglycan biosynthesis 0.019
Valine, leucine and isoleucine biosynthesis 0.019
Terpenoid backbone biosynthesis 0.022
Lipopolysaccharide biosynthesis proteins 0.023
Pentose phosphate pathway 0.030
Nitrogen metabolism 0.032
Protein kinases 0.038
Oxidative phosphorylation 0.042
q-value (corrected)
0.0 2.8 −2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0
Mean proportion (%)
Difference in mean proportions (%)
CG CGPRO


95% confidence intervals
Purine metabolism 8.51e-3
Glycolysis / Gluconeogenesis 0.012
Glutathione metabolism 0.017
Ubiquinone and other terpenoid-quinone biosynthesis 0.022
Lysine degradation 0.022
Bacterial chemotaxis 0.034
Bacterial motility proteins 0.037
Glycosyltransferases 0.038
Oxidative phosphorylation 0.040
Amino acid related enzymes 0.040
Phenylalanine, tyrosine and tryptophan biosynthesis 0.041
Pyrimidine metabolism 0.048
Flagellar assembly 0.049
p-value (corrected)
0.0 2.8 −2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0
Mean proportion (%)
Difference in mean proportions (%)
CG CGPP

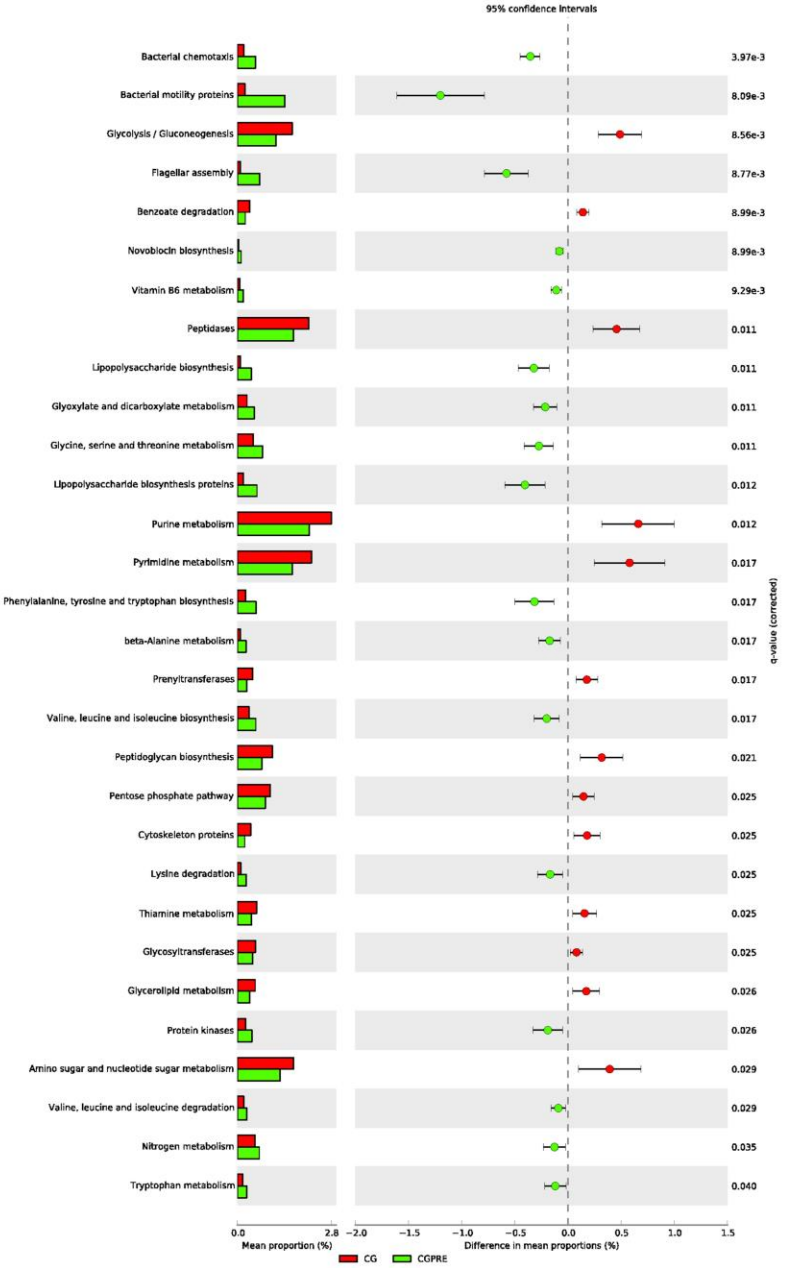
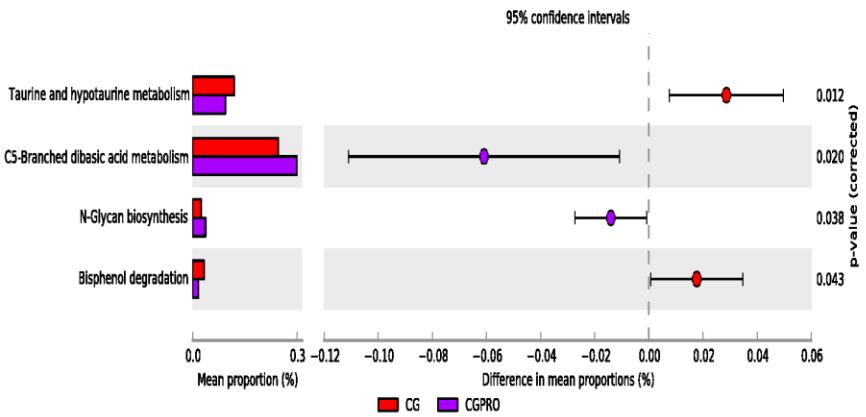
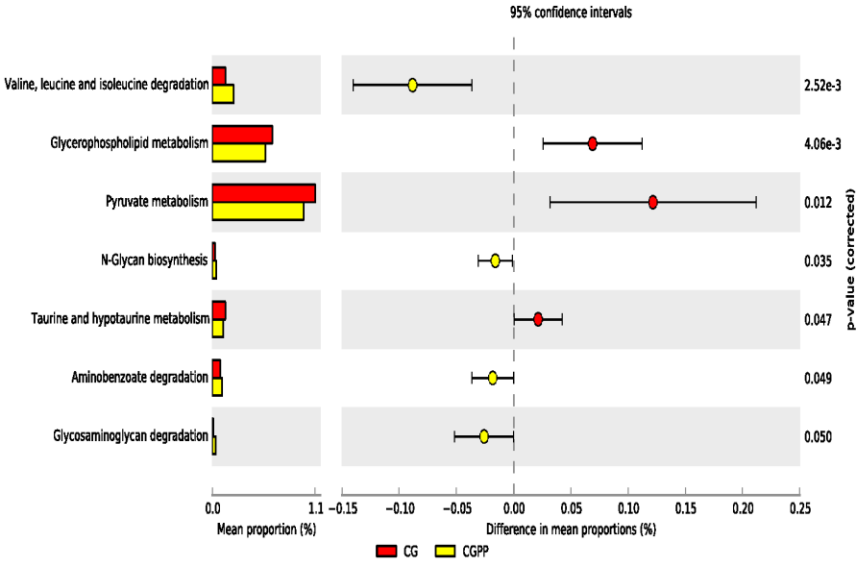

95% confidence intervals
Bacterial chemotaxis 3.97e-3
Bacterial motility proteins 8.09e-3
Glycolysis / Gluconeogenesis 8.56e-3
Flagellar assembly 8.77e-3
Benzoate degradation 8.99e-3
Novobiocin biosynthesis 8.99e-3
Vitamin B6 metabolism 9.29e-3
Peptidases 0.011
Lipopolysaccharide biosynthesis 0.011
Glyoxylate and dicarboxylate metabolism 0.011
Glycine, serine and threonine metabolism 0.011
Lipopolysaccharide biosynthesis proteins 0.012
Purine metabolism 0.012
Pyrimidine metabolism 0.017
Phenylalanine, tyrosine and tryptophan biosynthesis 0.017
beta-Alanine metabolism 0.017
Prenyltransferases 0.017
Valine, leucine and isoleucine biosynthesis 0.017
Peptidoglycan biosynthesis 0.021
Pentose phosphate pathway 0.025
Cytoskeleton proteins 0.025
Lysine degradation 0.025
Thiamine metabolism 0.025
Glycosyltransferases 0.025
Glycerolipid metabolism 0.026
Protein kinases 0.026
Amino sugar and nucleotide sugar metabolism 0.029
Valine, leucine and isoleucine degradation 0.029
Nitrogen metabolism 0.035
Tryptophan metabolism 0.040
q-value (corrected)
0.0 2.8 −2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0 1.5
Mean proportion (%)
Difference in mean proportions (%)
CG CGPRE

Figure 4.9. Subsystems and pathways enriched or decreased in: CG (red) vs. CGPRO (purple) or CGPRE (light green) or CGPP (yellow) of the **ileal** mucosa samples from pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO. Corrected P-values were calculated using the Storey FDR correction.


95% confidence intervals
Taurine and hypotaurine metabolism
C5-Branched dibasic acid metabolism
N-Glycan biosynthesis
Bisphenol degradation
0.012
0.020
0.038
0.043
p-value (corrected)
0.0
0.3
Mean proportion (%)
−0.12
−0.10
−0.08
−0.06
−0.04
−0.02
0.00
0.02
0.04
0.06
Difference in mean proportions (%)
CG
CGPRO


95% confidence intervals
Valine, leucine and isoleucine degradation
Glycerophospholipid metabolism
Pyruvate metabolism
N-Glycan biosynthesis
Taurine and hypotaurine metabolism
Aminobenzoate degradation
Glycosaminoglycan degradation
2.52e-3
4.06e-3
0.012
0.035
0.047
0.049
0.050
p-value (corrected)
0.0
1.1
Mean proportion (%)
−0.15
−0.10
−0.05
0.00
0.05
0.10
0.15
0.20
0.25
Difference in mean proportions (%)
CG
CGPP

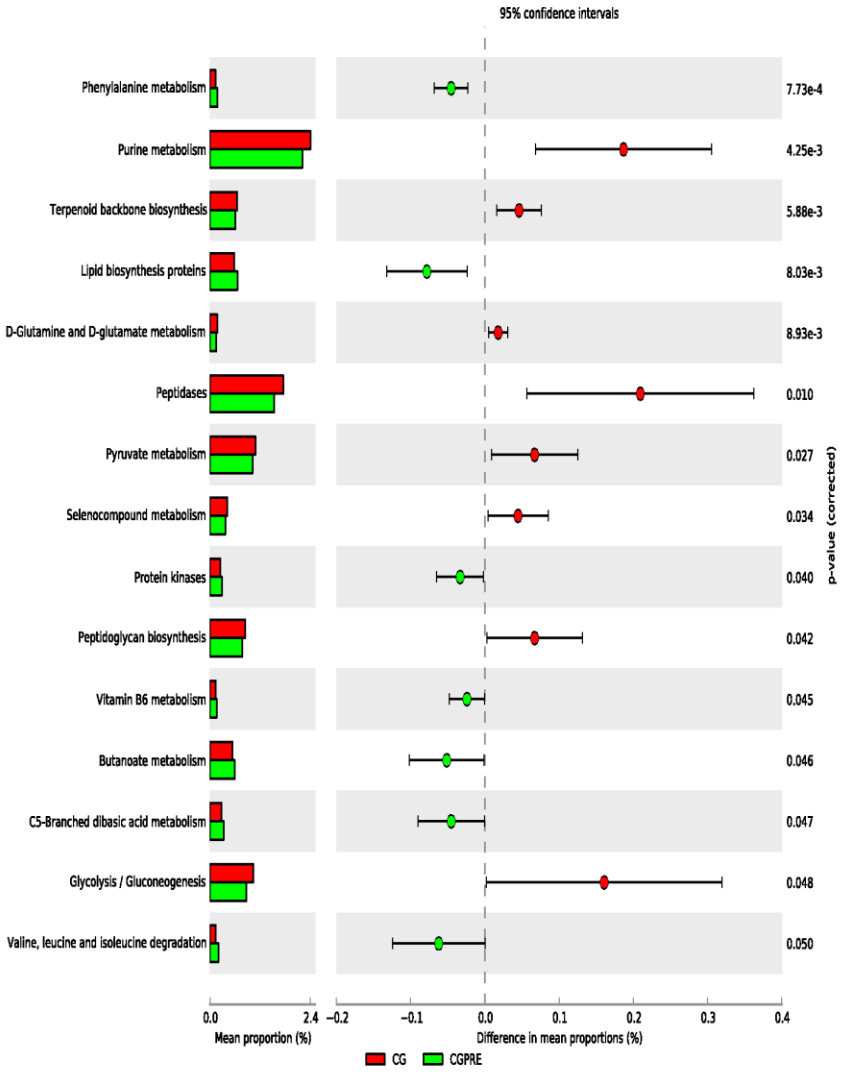

95% confidence intervals
Phenylalanine metabolism
Purine metabolism
Terpenoid backbone biosynthesis
Lipid biosynthesis proteins
D-Glutamine and D-glutamate metabolism
Peptidases
Pyruvate metabolism
Selenocompound metabolism
Protein kinases
Peptidoglycan biosynthesis
Vitamin B6 metabolism
Butanoate metabolism
C5-Branched dibasic acid metabolism
Glycolysis / Gluconeogenesis
Valine, leucine and isoleucine degradation
7.73e-4
4.25e-3
5.88e-3
8.03e-3
8.93e-3
0.010
0.027
0.034
0.040
0.042
0.045
0.046
0.047
0.048
0.050
p-value (corrected)
0.0
2.4
Mean proportion (%)
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
Difference in mean proportions (%)
CG
CGPRE

Figure 4.10. Subsystems and pathways enriched or decreased in: CG (red) vs. CGPRO (purple) or CGPRE (light green) or CGPP (yellow) of the **cecal** mucosa samples from pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

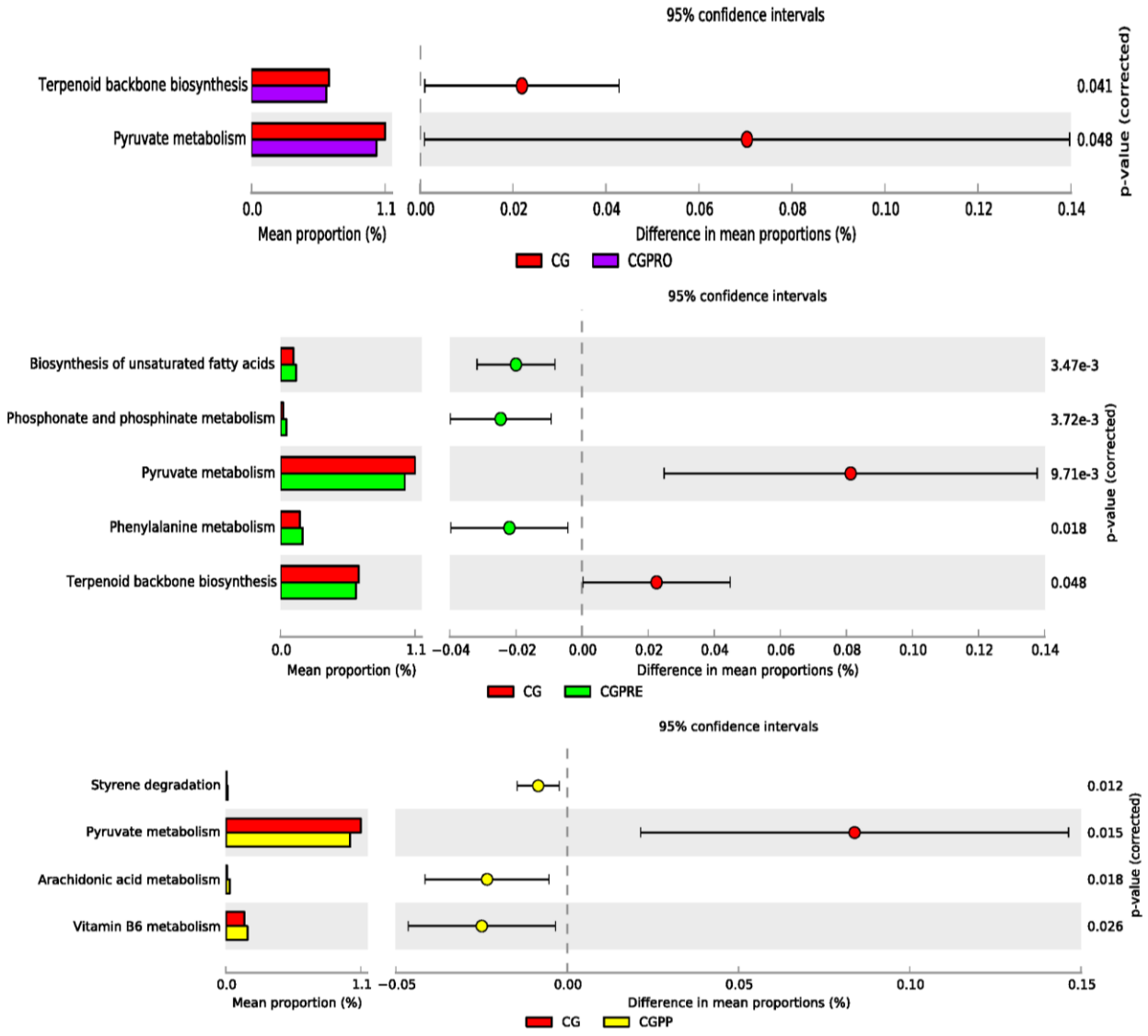
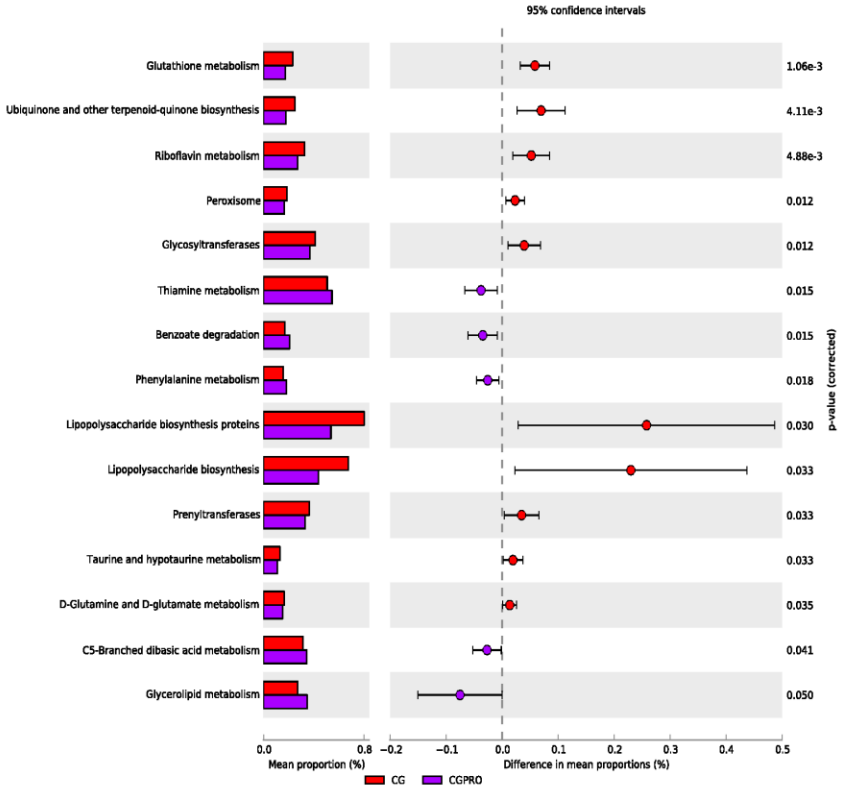
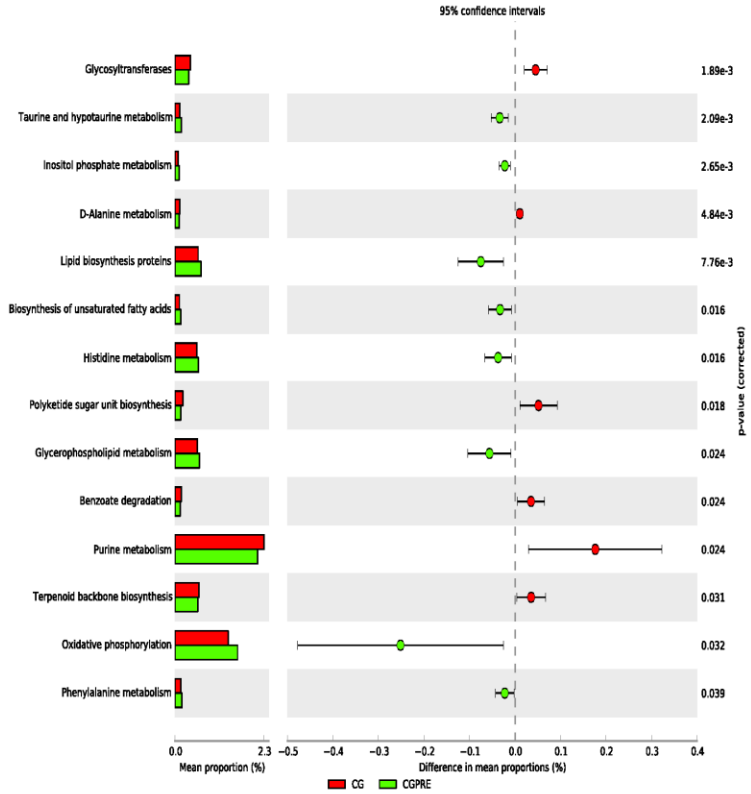

95% confidence intervals
Terpenoid backbone biosynthesis
Pyruvate metabolism
0.041
0.048
p-value (corrected)
0.0
1.1
Mean proportion (%)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Difference in mean proportions (%)
CG
CGPRO
95% confidence intervals
Biosynthesis of unsaturated fatty acids
Phosphonate and phosphinate metabolism
Pyruvate metabolism
Phenylalanine metabolism
Terpenoid backbone biosynthesis
3.47e-3
3.72e-3
9.71e-3
0.018
0.048
p-value (corrected)
0.0
1.1
Mean proportion (%)
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Difference in mean proportions (%)
CG
CGPRE
95% confidence intervals
Styrene degradation
Pyruvate metabolism
Arachidonic acid metabolism
Vitamin B6 metabolism
0.012
0.015
0.018
0.026
p-value (corrected)
0.0
1.1
Mean proportion (%)
−0.05
0.00
0.05
0.10
0.15
Difference in mean proportions (%)
CG
CGPP

Figure 4.11. Subsystems and pathways enriched or decreased in: CG (red) vs. CGPRO (purple) or CGPRE (light green) or CGPP (yellow) of the **ascending colon** mucosa samples from pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.


95% confidence intervals
Glutathione metabolism
Ubiquinone and other terpenoid-quinone biosynthesis
Riboflavin metabolism
Peroxisome
Glycosyltransferases
Thiamine metabolism
Benzoate degradation
Phenylalanine metabolism
Lipopolysaccharide biosynthesis proteins
Lipopolysaccharide biosynthesis
Prenyltransferases
Taurine and hypotaurine metabolism
D-Glutamine and D-glutamate metabolism
C5-Branched dibasic acid metabolism
Glycerolipid metabolism
1.06e-3
4.11e-3
4.88e-3
0.012
0.012
0.015
0.015
0.018
0.030
0.033
0.033
0.033
0.035
0.041
0.050
p-value (corrected)
0.0
0.8
Mean proportion (%)
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
0.5
Difference in mean proportions (%)
CG
CGPRO


95% confidence intervals
Glycosyltransferases
Taurine and hypotaurine metabolism
Inositol phosphate metabolism
D-Alanine metabolism
Lipid biosynthesis proteins
Biosynthesis of unsaturated fatty acids
Histidine metabolism
Polyketide sugar unit biosynthesis
Glycerophospholipid metabolism
Benzoate degradation
Purine metabolism
Terpenoid backbone biosynthesis
Oxidative phosphorylation
Phenylalanine metabolism
1.89e-3
2.09e-3
2.65e-3
4.84e-3
7.76e-3
0.016
0.016
0.018
0.024
0.024
0.024
0.031
0.032
0.039
p-value (corrected)
0.0
2.3
Mean proportion (%)
−0.5
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
Difference in mean proportions (%)
CG
CGPRE

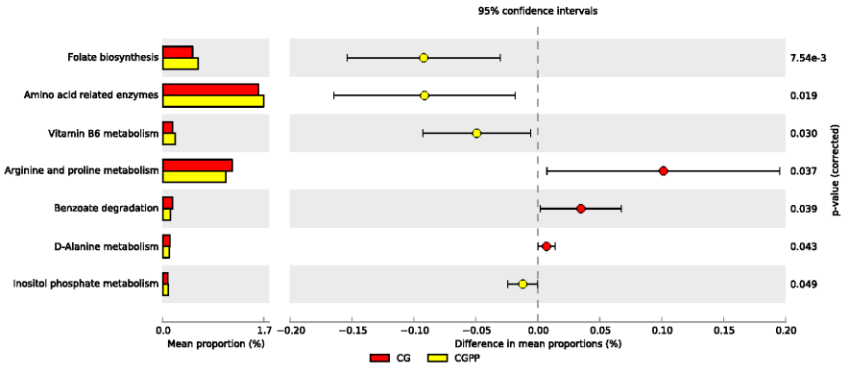

95% confidence intervals
Folate biosynthesis
Amino acid related enzymes
Vitamin B6 metabolism
Arginine and proline metabolism
Benzoate degradation
D-Alanine metabolism
Inositol phosphate metabolism
7.54e-3
0.019
0.030
0.037
0.039
0.043
0.049
p-value (corrected)
0.0
1.7
Mean proportion (%)
−0.20
−0.15
−0.10
−0.05
0.00
0.05
0.10
0.15
0.20
Difference in mean proportions (%)
CG
CGPP

Figure 4.12. Subsystems and pathways enriched or decreased in: CG (red) vs. CGPRO (purple) or CGPRE (light green) or CGPP (yellow) of the **descending colon** mucosa samples from pigs treated with degraded carrageenan gum (CG) and fed diets supplemented with prebiotics and probiotics. CG: pigs received 1% CG only in drinking water daily from day 1 until end of the study; CGPRE: pigs received 1 % CG daily from day 1 of the study, and basal diet supplemented with 5% resistant starch from day 8 until the end of the study; CGPRO: pigs received 1 % CG daily from day 1 of the study, and an overnight culture of probiotics *E. coli* strains UM2 and UM7 (50 mL per pig; 10^8 cfu/mL) from d 8 until end of of the study; the probiotic culture was mixed with small amount of feed every morning and the pigs were allowed to consume the freshly mixed feed first. CGPP: pigs were treated as in CGPRE and CGPRO.

4.5. DISCUSSION

Starch is a substantial component of the human and swine diets and it is generally accepted that most of the starch is digested in the small intestine. However, a fraction of starch [resistant starch (RS)] resists small intestinal digestion and enters the large bowel, where it is fermented, and acts as a substrate for commensal bacteria (Higgins and Brown, 2013; Giuberti et al., 2015). In this study, we investigated the impact of RS prebiotic and *E. coli* probiotics UM2 & UM7 on composition and inferred functional activities of intestinal mucosa-associated bacteria in a pig model of CG-induced colitis. The results demonstrate that CG-induced colitis caused bacterial dysbiosis but therapeutic effects of RS and probiotics used in this study although promising, were not very strong in modulating the dysbiosis.

Decrease in bacterial diversity and changes in the bacterial composition are conditions commonly reported in IBD patients (Ott et al., 2004; Lupp et al., 2007; Sepehri et al., 2007; Sokol et al., 2008; Sokol et al., 2009; Friswell et al., 2010; Qiu et al., 2013; Cao et al., 2014; Walters et al., 2014; Sheehan et al., 2015; Wright et al., 2015). In this study, α-diversity was generally lowest in the CG treatment and highest in the control, whereas the intervention treatments were intermediate especially in the cecum and ascending colon. Also, as shown in beta diversity analysis, the UniFrac distance between some treatment groups in different intestinal segments was quite significant, suggesting differences in the bacterial community composition. In addition, although the phyla results were not consistent among different treatment groups, phylum Proteobacteria and Firmicutes were relatively higher and lower respectively in other groups compared to the control, and hence, the effect of the resistant starch and the probiotics were transient or not evident in the colitic pigs at phylum level. Nevertheless, a marked increase in the phylum Proteobacteria, or a decrease in the phylum Firmicutes has been

reported in IBD patients (Ott et al., 2004; Lupp et al., 2007; Sepehri et al., 2007; Sokol et al., 2008; Sokol et al., 2009; Friswell et al., 2010; Qiu et al., 2013; Cao et al., 2014; Walters et al., 2014; Wright et al., 2015), which supports the efficacy of CG in mimicking colitis, but again suggests lack of influence by the therapeutic measures employed in our study. The lack of clear or greater effects of the therapeutic measures on bacterial diversity and composition could be due to the short duration of their administration as the intestinal microbiota may require relatively longer time to adapt to different additives.

Enterobacteriaceae family especially *Escherichia* have long been associated with IBD conditions (Darfeuille-Michaud et al., 1998; Sellon et al., 1998; Schuppler et al., 2004; Sepehri et al., 2007; Sepehri et al., 2011; Wright et al., 2015). However, not all members in this group accounts for pathogenicity as *Escherichia coli* consists of many biotypes some of which are commensal colonizers and have probiotic effects. In this regard, besides the probiotic strains used in this study, other members of *Escherichia coli* have been identified to have probiotic effects including *E. coli* Nissle 1917 and *E. coli* strain HS (Maltby et al., 2013; Sassone-Corsi and Raffatellu, 2015). This therefore supports the presence of both Enterobacteriaceae and *Escherichia* in the CGPRE, CGPRO and control treatments in our study. Also, members of Ruminococcaceae and Lachnospiraceae are particularly abundant in the large intestines and are reduced in CD (Packey and Sartor, 2009; Frank et al., 2011). They also include the major butyrate-producing species as well as species that convert lactate to butyrate or propionate (Barcenilla et al., 2000; Duncan et al., 2004; Louis and Flint, 2009; Louis et al., 2010), and together with other Firmicutes and Bacteroidetes (e.g *Bacteroides* and *Prevotella),* they also play key roles in polysaccharide degradation.

Other bacteria that were enriched in the control, CGPRE, CGPRO, and CGPP treatment groups such as *Prevotella*, *CF231*, GMD14H09, *Butyricimonas*, *Oribacterium*, *Anaerotruncus* and *Bacteroides*, positively associated with short chain fatty acids (SCFA), and therefore, may suggest enhanced production of SCFA, the end products of fermentation of dietary fibres, which have been shown to exert multiple beneficial effects on mammalian energy metabolism. Moreover, SCFA are known for their immunoregulatory properties and contribution to gut health (Sartor, 2008; Canani et al., 2011; Smith et al., 2013). In this study, colonic acetate and butyrate were significantly higher in both the control and CGPP treatments compared to other treatments, suggesting enhanced growth of volatile fatty acids-producing bacteria in the colon of colitic pigs that received a combination of prebiotics and probiotics (CGPP), which supports positive synergistic effects of both the prebiotics and the probiotics. Together, these results may partly explain the mechanism through which prebiotics and probiotics relatively ameliorated the effects of CG. Another possible mechanism could be through modulation of local inflammatory responses as CGPRE treatment down-regulated the level of inflammatory cytokines: IL-1β, IL-6 and TNF-α in the descending colon, cecum and ileum respectively. Inflammatory response is characterized by massive recruitment of polymorphonuclear and mononuclear cells, and consequently increased levels of proinflammatory cytokines such as IL-1β, IL-6, TNF-α among others, which have also been implicated in IBD (Crabtree et al., 1991; Neurath, 2014; Moldoveanu et al., 2015). Therefore the down-regulation of these cytokines may suggest some anti-inflammatory properties of RS. Moreover, some taxa (eg. unclassified members of Proteobacteria and Enterobacteraceae and genera *Enterobacter*, that were enriched in CGPRE and CGPRO negatively correlated with pro-inflammatory cytokines TNF-α and IL-8 further suggesting anti-inflammatory effects of the RS and probiotics. However, *Bacteroides*,

Brachyspira, *Campylobacter*, *Oscillospira*, and *Escherichia*, which were associated with the control or the therapeutic treatments may also partially promote inflammation as indicated by their positive correlation with pro-inflammatory (IL-8, IL-6, TNF-α) and or negative correlation with anti-inflammatory (IL-10) cytokines, although *Campylobacter* was also positively associated with IL-10, suggesting redundant effects. This could be partially explained by the fact that each taxon represents a group of organisms belonging to different species or sub-species that may play different roles. Genera *Lactobacillus* is generally known for it's beneficial effects; however, *Lactobacillus* was enriched in the colon of the colitic pigs. The species of *Lactobacillus* are phylogenetically diverse (Goh and Klaenhammer, 2009), and therefore, characterization of *Lactobacillus* genus at lower levels may help to elucidate on specific members that could be associated with colitis. Similar results have been reported in colonic mucosal biopsy samples of patients with active IBD (Wang et al., 2014), and therefore, there is need to determine which species of *Lactobacillus* could be associated with IBD conditions during active phase and their possible roles.

Since the total quantity of bacterial microbiota outnumbers host cells, it should be expected that the host/microbial interactions within the gut exert direct and indirect effects on host physiology, biochemistry, and immunology (Barbara et al., 2005; Pimentel et al., 2012). As such, several bacterial metabolic pathways and cellular functions were influenced by the CG treatment as well as the therapeutic measures employed, which may have indirect impacts on the host. In this regard, folate biosynthesis, metabolism of vitamin B6 and metabolism/ biosynthesis of various amino acids associated with the prebiotic and probiotic based treatments may be beneficial to the host as part of these vitamins/amino acids can be utilized by the host. Although the CG treatment was also associated with metabolism of few amino acids, it was also

characterized by glycolysis/gluconeogenesis and peptidases, which account for breakdown of energy and protein reserves, and may therefore suggest increased energy and protein utilization in microbiota of colitic pigs.

In conclusion, CG-induced colitis caused bacterial dysbiosis whereas inclusion of RS and or the probiotics slightly modulated bacterial dysbiosis, and several taxa that could be beneficial to the host were enriched in the intestines of the pigs receiving probiotics and RS. Therefore, bearing in mind that the probiotics and prebiotics were introduced when the pigs had already been exposed to colitis and for a short period of time, and that, longer time may be required for the intestinal microbiota to adapt to a load of fermentable substrate (Gorvers et al., 1999; Martinez-Puig. et al., 2003), the few beneficial effects that were observed in these treatments could suggest a potential role for RS and probiotics *E. coli* UM2 and UM7 in ameliorating the effects of colitis. However, more research is required to further investigate on the precise action of RS and the probiotics used in this study, as their impact was relatively mild.

BRIDGE TO CHAPTER 5

The experiment described in chapter 4 showed that the effect of the probiotics and prebiotics used was minimal, which could be due to the duration of administration that might have been short for the probiotics and prebiotics to exert a noticeable beneficial effect. Also, it might be possible that the timing of application was not optimal. The next step would therefore include further investigations on these prebiotics and probiotics. However, the experiment described in chapter 5 was designed to further investigate the role of the prebiotics (resistant starch) only, in which case both the protective and therapeutic role of resistant starch were tested in a pig model of experimental colitis over a relatively longer period of time (40 days).

Rationale: Previous studies have shown that, a relatively longer period of time may be required for the intestinal microbiota to adapt to a load of fermentable substrates (Gorvers et al., 1999; Martinez-Puig. et al., 2003). Therefore, it is apparent that inclusion of resistant starch over a relatively longer period than used in our previous study would be more beneficial in pig model of colitis. Also, studies utilizing mice models of colitis have demonstrated potential role of RS in IBD by partially preventing or ameliorating clinical disease or disease severity and prevention of inflammatory lesion development (Bassaganya-Riera et al., 2011; Le Leu et al., 2013), and hence, investigating protective and therapeutic role of RS in a pig model of colitis might shed more light in this topic.

CHAPTER 5

MANUSCRIPT III

Protective and therapeutic use of resistant starch (MSPrebiotic) modulates gut microbiota profile, histological structure and local gene expression in a pig model of experimental colitis

A version of the material presented in chapter five of this thesis is being prepared for peer review journal publication and will be submitted in PLOS One journal. The authors are: Peris M Munyaka, Jean-Eric Ghia and Ehsan Khafipour.

5.1. ABSTRACT

There are numerous emerging therapeutic strategies that could be used in the alleviation of chronic intestinal inflammation and microbial dysbiosis including dietary supply of non-digestible saccharides such as resistant starch (RS), but their efficacy has not been fully substantiated. In a 40-day study, we investigated protective and therapeutic effects of RS (MSPrebiotic) in a pig model of experimental colitis. We demonstrate that, inclusion of RS as therapy or prevention measure improved fecal consistency, modulated local inflammation, and improved histological structure. Also, inclusion of RS either as prevention or therapeutic measure influenced bacterial structure especially in the large intestines. Linear discriminant analysis (LDA) effect size (LEfSe) revealed significant differences in bacterial groups between colitic (CG) and non-colitic pigs as well as both in protective and therapeutic groups in various intestinal segments. *Desulfovibrio*, *Mucispirillum*, *Fusobacterum*, Enterobacteriaceae, Deferribacteraceae, Fusobacteria and Deferribacteres phyla were significantly enriched in CG group. In contrast, *Bifidobacterium*, *Peptococcus*, *Succinivibrio*, *Ruminococcus*, *Campylobacter*, Xanthomonadales, *Mollicutes*, Aeromonadales and other taxa were significantly enriched in the intestinal mucosa of control and the pigs receiving RS alone or as a therapeutic measure, but no such significant taxa were observed in the protective goup. The observed changes in bacterial composition were accompanied by significant differences in various functional gene contents as predicted using PICRUSt. In conclusion, RS showed both preventive and therapeutic effects in a pig model of experimental colitis, however, the therapeutic effects were more prominent relative to the protective effects.

Key words: resistant starch, pig, dysbiosis, protective, therapy

5.2. INTRODUCTION

Ulcerative colitis and Crohn's disease are defined by a common term of inflammatory bowel disease, and they result in significant morbidity and mortality. While there are no cures for these diseases, major advancements in understanding of the immunological, pathological and physiological features of chronic intestinal inflammation have been achieved, which can be attributed to a steadily increasing number of different experimental animal models. In this context, animal models have been established to provide a uniquely accurate and tractable model for studying the gut microbiota, including the molecular and cellular mechanisms driving chronic intestinal inflammation (Panwala et al., 1998; Shah et al., 1998; Pizarro et al., 2003; Sartor, 2006; Wirtz et al., 2007). The models of inflammatory bowel disease facilitate a mechanistic evaluation of the contribution of the gut microbiota to the initiation and perpetuation of chronic intestinal inflammation, as occurs in human IBD (Sartor, 2006).

There are numerous emerging therapeutic strategies, which may be useful in the alleviation of chronic intestinal inflammation, including dietary supply of non-digestible saccharides such as resistant starch (RS), inulins, fructo-oligosaccharides among others, which are carbohydrate food ingredients designated as prebiotics (Cummings and Englyst, 1987; Cummings et al., 1997; Higgins and Brown, 2013). Resistant starch represents a diverse range of indigestible starch-based dietary carbohydrates that is not digested and absorbed in the upper digestive tract and, so, passes into the large bowel, where it undergoes microbial fermentation (Asp, 1987; Topping and Clifton, 2001; Higgins and Brown, 2013). It has been investigated in the past for its effects on bowel health (pH, epithelial thickness, and apoptosis of colorectal cancer cells), reduction in postprandial glycemia; increased insulin sensitivity; and effects on the gut microbiome (Higgins and Brown, 2013). The microbial complex in the colon, comprising

apparently 10^{14} microbes of several hundred species, represents a large ecosystem, which in the right composition has a beneficial effect on the host. In this regard, the intestinal microbiota, with their immunological potency, may play an essential role in intestinal barrier resistance to ulcerative colitis (UC) (Butzner et al., 1996), and could be important in promoting large bowel health and preventing IBD among other gut ailments (Topping and Clifton, 2001). Studies utilizing mice models of colitis have demonstrated potential role of RS in IBD by partially preventing or ameliorating clinical disease or disease severity and prevention of inflammatory lesions (Bassaganya-Riera et al., 2011; Le Leu et al., 2013). This study therefore seeks to investigate resistant starch (MSPrebiotic) as a potential preventive and or therapeutic tool for ulcerative colitis in a pig model of experimental colitis.

5.3. MATERIALS AND METHODS

5.3.1. Animals and housing.

In a Forty-day study, a total of 35 pigs [Duroc x (Yorkshire x Landrace)] weaned at 20 ±1 d (average 6 kg) were obtained from Glenlea research station, University of Manitoba, MB, Canada. Upon arrival, the piglets were housed in group pens for 3 days after which they were randomly assigned to experimental treatments (based on body weight) with 7 pigs per treatment, moved to individual pens, and allowed 3 more days for acclimatization before the start of experimental procedures. The Pigs were housed in temperature-controlled rooms within T. K. Cheung Center for Animal Science Research, University of Manitoba, Winnipeg, MB, Canada. Room temperatures were maintained at 30°C during wk 1 and the temperatures were reduced by 1°C every week, with a 16 h lighting system. All pigs had *ad libitum* access to water and basal diet in mash form formulated to meet or exceed the national research council (NRC, 2012) recommendations for a 7 to 11 kg and 11 to 25 kg pigs.

5.3.2. Ethical considerations.

The procedures were approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee (F12-040/1/2), and the pigs were cared for according to the guidelines of the Canadian Council of Animal Care.(CCAC, 1993)

5.3.3. Degraded carrageenan gum (CG).

Carrageenan gum powder (CarboMer, Inc. San Diego, CA, USA) was prepared and administered as described in manuscript I and II.

5.3.4. Resistant starch (RS): MSPrebiotic.

Potato resistant starch (RS; MSPrebiotic) in powder form was provided by the McPharma Neutraceuticals, Carberry, Manitoba, Canada, in pouches of 454 g each. Each pouch contained a 10 g scoop. The RS was mixed with little amount of feed every morning and pigs were allowed to finish the mixed feed first.

5.3.5. Diet and Experimental Treatments

A basal corn-soybean-based diet formulated to meet or exceed NRC (2012) nutrient recommendations for a 7 to 11 kg pig, and for an 11 to 25 kg pig was used. The 40-day study was divided into two experiments. Pigs were weighed and randomly assigned to the treatment groups with one pig per pen and 7 replicate pens per treatment.

Experiment 1: The aim of this experiment was to investigate microbiota profile and local immune responses in pigs exposed to CG-induced colitis, and in pigs supplemented with RS with no colitis. The treatments were: 1; control (basal diet only), 2; CG (as in 1 and 1% CG starting from wk 1 of the study), and 3; RS (as in 1 and MSPrebiotic (RS) starting from wk 1 of the study). Pigs in treatment one received basal diet only through out the experimental period, pigs in treatment two received basal diet and 1 % CG solution through out the experimental period,

whereas pigs in treatment three received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period.

Experiment 2: The aim of this experiment was to investigate role of resistant starch (MSPrebiotic) as a potential preventive or therapeutic tool for ulcerative colitis in a pig model of experimental colitis. The treatments were: 1; CG (as used in experiment one), 2; RST (as in CG and MSPrebiotic starting from wk 3 of the study), and 3; RSP (as in RS and 1% CG starting from wk 3 of the study). In treatment RST, pigs received CG alone during the first 14 d of the experiment and MSPrebiotic (RS) was introduced (15 g) from 15th day of the study up to the end of the experimental period. In this case MSprebiotic (RS) was used as a therapy for CG-induced colitis and the purpose was to investigate whether RS could induce remission or reduce the severity of colitis in patients with active colitis. In Treatment RSP, pigs received 10 g daily RS alone for the first 14 d of the study, and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d to compensate for the changes in growth of the pigs. Here the RS was used for prevention or protection against CG-induced colitis and the aim was to investigate whether consumption of RS can protect healthy individuals from developing colitis.

5.3.6. Fecal scoring

Severity of diarrhea was characterized using the fecal consistency scoring system described by Marquardt et al. (Marquardt et al., 1999); (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea). The scoring was done daily, and each week's average score was used for statistical analysis.

5.3.7. Digesta and Tissue sampling

At the end of the study (d 40), blood was collected from the jugular vein and all pigs were sedated by intramuscular injection of Ketamine: Xylazine (20:2 mg/kg BW) and euthanized by an intracardiac injection of 110 mg/kg BW sodium pentobarbital (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). The abdominal cavity was opened from sternum to pubis to expose the gastrointestinal tract without damaging the wall of the digestive tract. The small intestine was stripped free of its mesentery and feces, ileal, cecal and colon digesta samples were obtained, and transferred to sterile sample containers. The fecal and digesta samples were used for determination of pH, and immediately 0.1N HCl was added (1:1 v/w), and the samples were transferred to -20 °C for later analysis of volatile fatty acids (VFA).

Tissue samples (three segments) were collected from the ileum, cecum, ascending, and descending colon, flushed with sterile saline to remove excess lumen contents, and used for histological analysis, microbial analyses, and for local gene expression. Samples for microbial analysis and gene expression were immediately frozen in liquid nitrogen and transferred to - 80° C until use.

5.3.8. Analysis of pH and volatile fatty acids (VFA).

pH was measured immediately after digesta collection using an Accumet Basic 15 pH meter (Fisher Scientific, Fairlawn, NJ) equipped with a Sensorex 450C Flat Surface Combination pH/Reference Electrode (Sensorex, Stanton, CA), which was standardized with certified pH 4 and 7 buffer solutions, whereas volatile fatty acids were determined using gas chromatography (Bhandari et al., 2007).

5.3.9. Histological examination

Immediately following sacrifice, sections of the intestinal tissue samples were removed and cleaned in saline solution to remove luminal contents. The tissue samples were then placed

into 10% buffered formalin and kept in formalin for 2 – 3 days. Tissues were embedded in paraffin then sections stained with hemotoxylin and eosin (H&E) prior to microscopic observation. Colonic damage was scored based on a published scoring system that considers architectural derangements, goblet cell depletion, edema/ulceration and degree of inflammatory cell infiltration (Cooper et al., 1993)

5.3.10. RNA isolation and analysis of local gene expression by reverse transcriptase quantitative real time PCR (RT-qPCR)

Real time RT-qPCR analysis was used to measure gene expression of various biomarkers in the ileum, cecum, ascending and descending colon tissue samples. Total RNA was extracted from the tissue samples and purified using MagMax Total RNA Isolation Kit (Life Technologies, CA, USA), according to the manufacturer's instructions. Reverse transcriptase cDNA synthesis was done using superscript II RT and random primers (Invitrogen), following the manufacturer's protocol. Real-time PCR was performed using SsoFast EvaGreen Supermix (Bio Rad Laboratories, Inc). The real-time PCR amplification was run on a LightCycler (Bio-Rad Laboratories, Inc) using the following conditions: enzyme activation for 3 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C. Porcine primers were designed using Primer 3 v.0.4.0 as used in a previous study (Lee et al., 2009b) and are shown in **Table 5.1**.The threshold cycle (C_T) was determined by CFX manager software, and the relative mRNA expression of the genes was calculated using the ΔΔCt method, with porcine β-actin as the housekeeping gene.

5.3.11. DNA extraction and quality control.

DNA was extracted from tissue samples using ZR DNA extraction kits as described previously (Munyaka et al 2016 a, b).

Table 5.1. Porcine primers used for RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Accession no.
β-actin	GGATGCAGAAGGAGATCACG	ATCTGCTGGAAGGTGGACAG	130	U07786
IL-8	TGGCAGTTTTCCTGCTTTCT	CAGTGGGGTCCACTCTCAAT	154	M86923
IFN-γ	CCATTCAAAGGAGCATGGAT	GAGTTCACTGATGGCTTTGC	146	AY188090
IL-1β	CAAAGGCCGCCAAGATATAA	GAAATTCAGGCAGCAACAT	147	NM_214055
IL-17	TCATGATCCCACAAAGTCCA	AGTCCATGGTGAGGTGAAGC	146	NM_001005729
IL-10	TGATGGGGAGGATATCAAGG	TGGAGCTTGCTAAAGGCACT	150	NM_2114041
TGF-β	CGAGCCCTGGATACCAACTA	AGGCTCCAGATGTAGGGACA	164	Y00111
IL-12p40	TTTCAGACCCGACGAACTCT	CATTGGGGTACCAGTCCAAC	160	NM_214013
Foxp3	CTGACAAGGGTTCCTGCTGT	GAAATCTGGGAACGTGCTGT	149	NM_001128438
MUC1	ACCAAGTCCCCTAACCCATC	TTGGAATTTTCCAGGCAGTC	101	XM_001926883
MUC2	ACCCGCACTATGTCACCTTC	GGGATCGCAGTGGTAGTTGT	131	NM_002457

5.3.12. Library construction and Illumina sequencing.

Library construction and Illumina sequencing was done as described in manuscript I (Munyaka et al 2016 a,b).

5.3.13. Bioinformatic analyses.

All bioinformatics analyses were performed as described in manuscript I (Munyaka et al 2016 a, b)

5.3.14. Alpha- and beta-diversity analyses.

Alpha-diversity was calculated using Chao 1(Chao, 1984). Beta-diversity was measured by calculating the unweighted and weighted UniFrac distances using QIIME (Lozupone and Knight, 2005) and the *P* values were calculated using PERMANOVA analyses of Bray-Curtis distances (Anderson, 2005).

5.3.15. Real time PCR determination of 16S rRNA gene copy number: absolute quantification.

To determine the 16S rRNA copy numbers in tissue DNA samples, the universal primers F338 primer for forward primer and R806 were used (Caporaso et al., 2012). The real-time PCR amplification was run on a LightCycler (Bio Rad Laboratories, Inc), and the threshold cycle (C_T) was determined by CFX manager software. The PCR was performed in triplicate, and each reaction mixture was prepared using the SsoFast Eva Green Supermix (Bio Rad Laboratories, Inc) in a total volume of 20 µl: 6.4 µl PCR-grade water, 0.8 µl of each primer (final concentration 0.5 µM), 10 µl Eva green master mix, and 2.0 µl template DNA. The thermal cycling protocol was as follows: enzyme activation for 3 min at 95°C followed by 40 cycles of 10 s at 95°C, and 30 s at 58°C, and the fluorescence signal was measured at the end of each extension step.

5.3.16. Linear discriminant analysis with effect size (Lefse) analysis

LEfSe (Segata et al., 2011) was used to identify overrepresented taxa between pigs with CG-induced colitis and non-colitic pigs (control and RS), and between pigs with CG-induced colitis and those with RS as a protective (RSP) or therapeutic (RST) measure.

5.3.17. 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), as described in manuscript I (Munyaka et al., 2016 a, b).

5.3.18. Statistical analysis.

For the volatile fatty acids, pH, alpha-diversity and phylum data, the effect of treatment was evaluated in a completely randomized design and the data was subjected to ANOVA using the PROC MIXED procedure of SAS (SAS 9.3). Differences between means were determined using Pdiff and the UNIVARIATE procedure of SAS was used to test the normality of residuals. The differences between treatments were considered significant at $P < 0.05$. One-way ANOVA followed by Tukey's multiple-comparison test was used to compare the histological scores and local inflammatory markers between treatment groups using Graphpad Prism 5.0c (Graphpad Prism, La jolla, CA, USA). The significance level was adjusted at 0.05.

5.4. RESULTS

5.4.1. Experiment 1: Effect of CG-induced colitis and RS on fecal consistency, local inflammation, histological structure, and microbiota composition and functional gene contents.

5.4.1.1. Fecal score

No diarrhea was observed in the control and RS treatments, however, exposure to CG-induced colitis increased fecal score through out the experimental period (**Figure 5.1**).

5.4.1.2. Local inflammation

Pro-inflammatory cytokines: IL-1β, IL-8, IL-12p40, IFN-γ, and IL-17 were measured in the ileum, cecum, ascending and descending colon (**Figure 5.2 a - e**). IL-1β was not significantly different among treatment groups across the intestinal segments. Compared to the control and RS treated pigs, CG-induced colitis significantly up-regulated IL-8 in all intestinal segments, as well as IFN-γ, and IL-17 in the ileum and ascending colon. IL-12p40 was only significantly upregulated in the ileum of colitic pigs but a down-regulation was observed in the cecum compared to the control and RS treated pigs. Expression of anti-inflammatory cytokines IL-10 and TGF-β as well as the regulatory cytokine Foxp3 were also tested, but no significant differences were observed across the intestinal segments (**Figure 5.2 f- h**). To further assess mucosal integrity, expression of MUC1 and MUC2 genes was determined (**Figure 5.2 i – j**). Both genes were up regulated in the cecum of RS group, and in the ascending colon of RS and CG treated pigs compared to the control. MUC2 was significantly lower in the descending colon of control pigs compared to CG.

5.4.1.3. Histological structure in the colon tissue of pigs with CG-induced colitis.

The H&E staining analysis indicated that administration of CG markedly increased the severity of colitis compared to the control and RS pigs, in both the ascending and descending colon tissues (**Figure 5.3 a–d**). The lesions of the colon in the CG-treated group manifested mucosal erosion, loss of epithelial and goblet cells, and shortening and collapse of crypts.

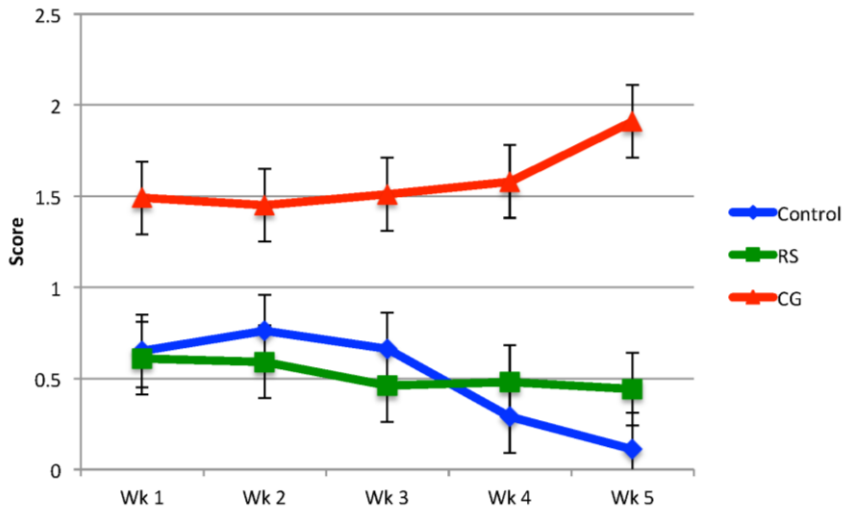


Figure 5.1 Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on fecal score as determined by stool consistency. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40. Severity of diarrhea was characterized using an established fecal consistency (FC) score system in pigs (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea.

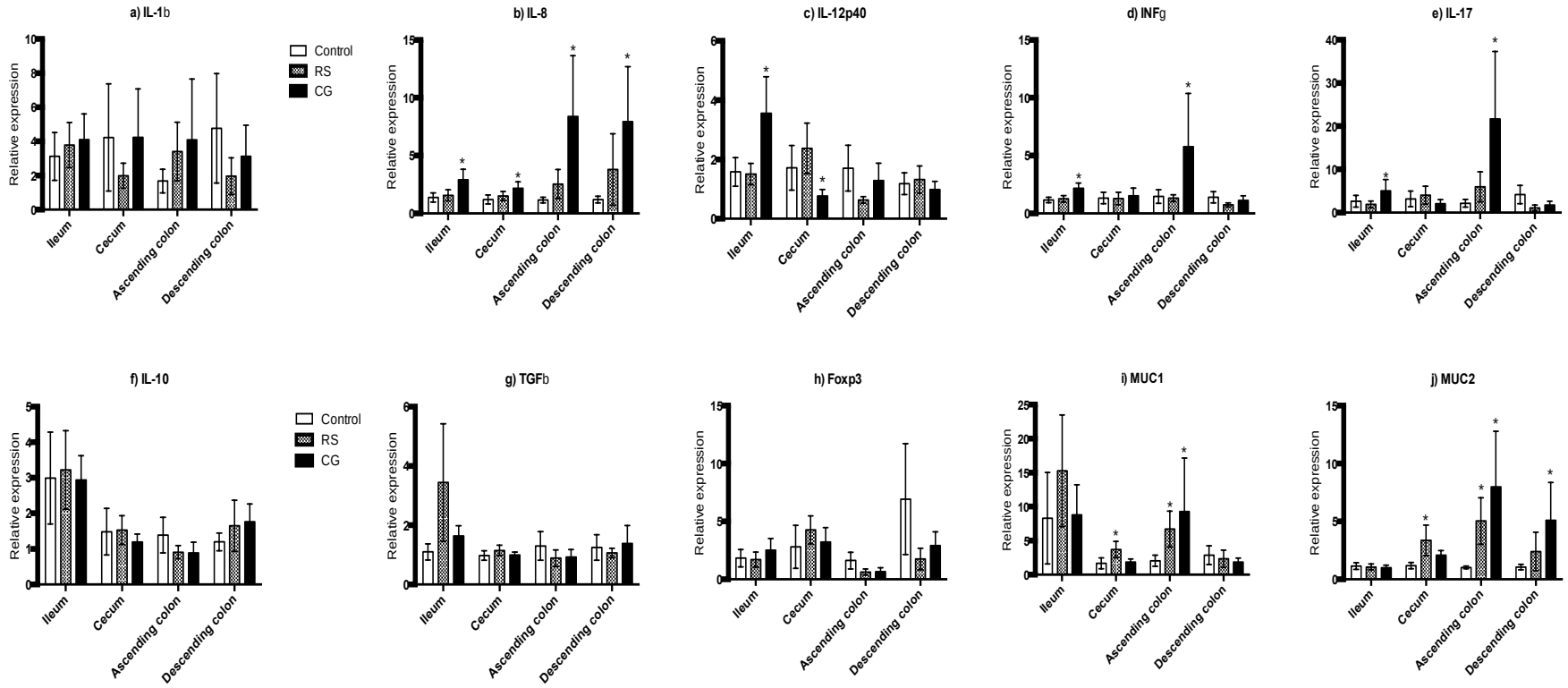


Figure 5.2. Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on local gene expression as determined by RT-PCR. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40. The relative expression fold changes were determined using the ΔΔCt method with porcine β-actin as the housekeeping gene. * Designates a significant difference; $P \leq 0.05$.

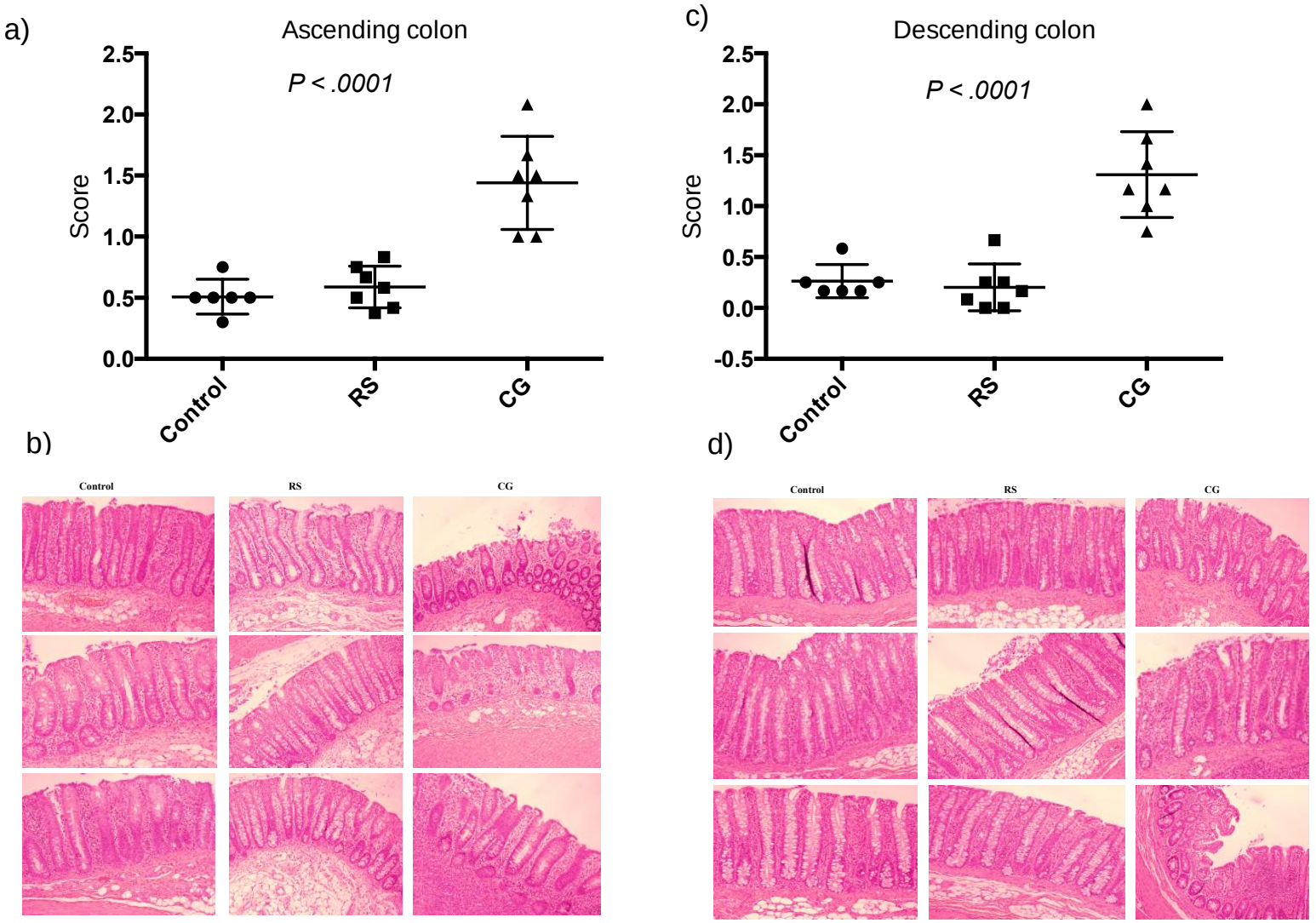

a)
Ascending colon
P < .0001
Score
2.5
2.0
1.5
1.0
0.5
0.0
Control
RS
CG
c)
Descending colon
P < .0001
Score
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Control
RS
CG
b)
Control
RS
CG
d)
Control
RS
CG

Figure 5.3. Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on histological structure. a) and b) shows histological score and the extend of histological damage, respectively in the ascending colon. c) and d) shows the histological score and the extend of histological damage, respectively in the descending colon. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

5.4.1.4. Alpha- and Beta-diversity

Alpha diversity as measured by Chao 1 was not different among treatment groups and across the intestinal segments evaluated (**Figure 5.4**). The results of bacterial structure are shown in **Figure 5.5 a-h**. Ileal bacterial structre was not different among treatment groups based on both weighted and unweighted Unifrac distances. Cecal unweighted Unifrac distance was not significantly different among treatment groups, however, based on the weighted Unifrac distance, the bacterial structure was significantly different between the control and CG treated pigs (P = 0.0229), and there was also a trend (P = 0.0984) between control and RS treated pigs, suggesting that the differences were due to abundant species. Similar differences were also observed in the ascending colon where the bacterial structure of CG (P = 0.0123) and RS (P = 0.033) treated pigs differed significantly from the control pigs in the weighted Unifrac distance, as well as in the unweighted Unifrac distance between CG and control (P = 0.03) pigs. This indicates that the differences were due to both rare and abundant species. Bacterial composition in the descending colon was only significantly different in the unweighted Unifrac distance implying that the differences were due to rare species. In this context, bacterial structure of CG treated pigs differed significantly from that of RS (P = 0.0047) and that of control (P = 0.0009) pigs.

5.4.1.5. Bacterial density

Bacterial density was not significantly different among treatment groups across all intestinal segments evaluated (ileum, cecum, ascending and descending colon) (**Figure 5.6**).

5.4.1.6. Bacterial composition at phylum level

Figure 5.7 a-d shows the results of bacterial composition at phylum level. Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes were the most abundant and dominant phyla

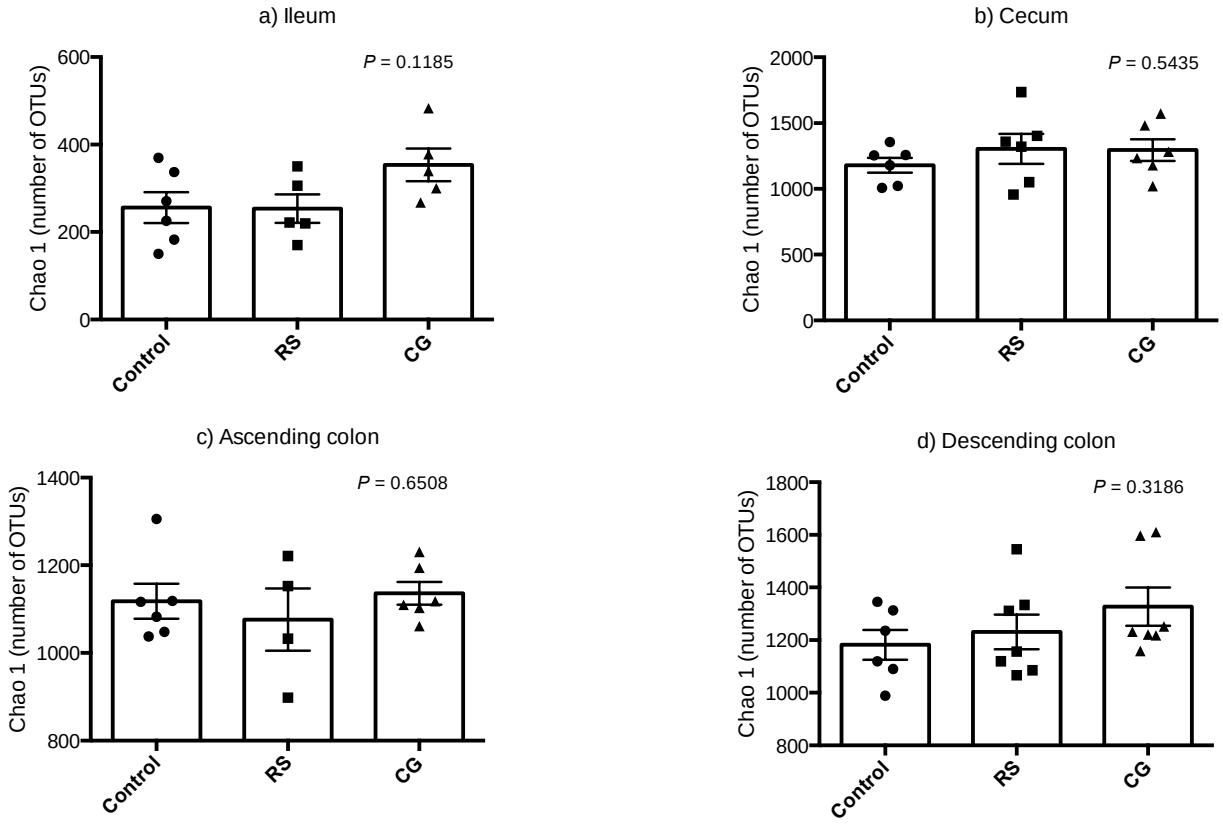


Figure 5.4 Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on bacterial richness/ Alpha-diversity in the ileum, cecum, ascending and descending colon. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40. No significant differences were observed.

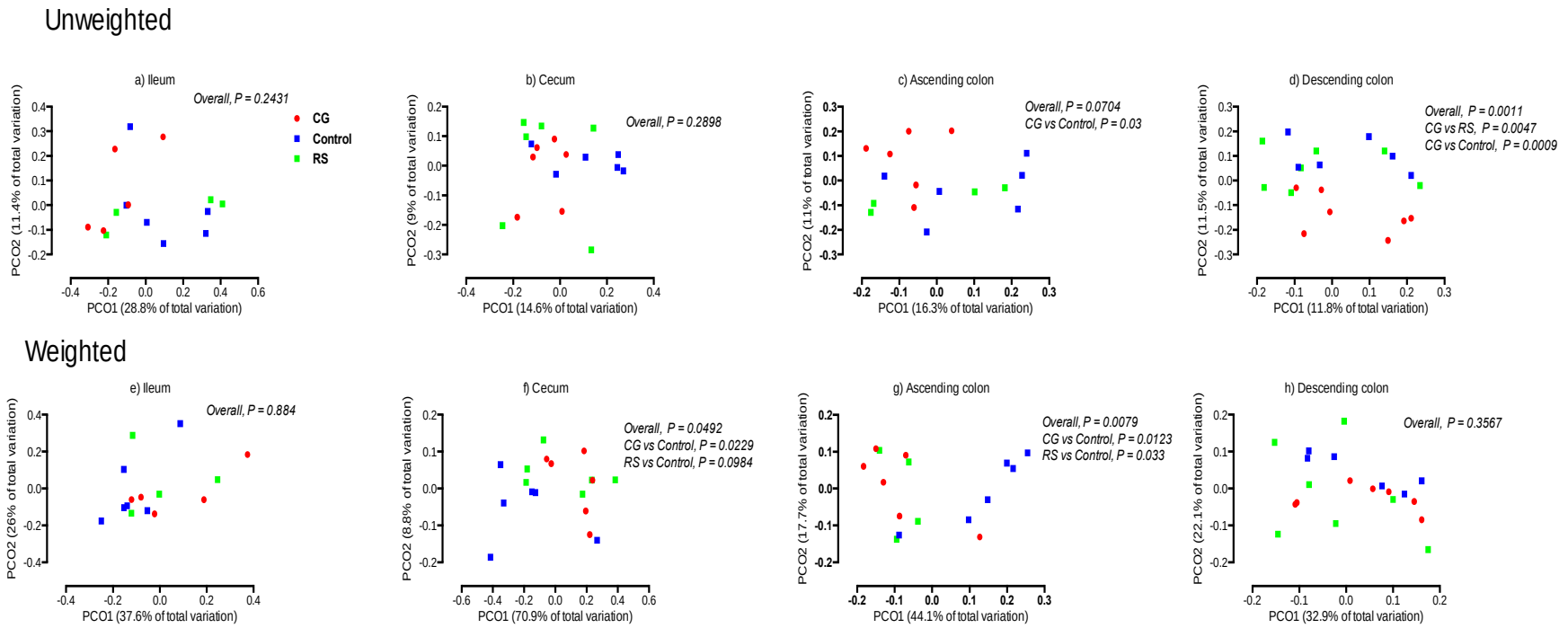


Figure 5.5. Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on bacterial structure/ Beta-diversity in the ileum, cecum, ascending and descending colon. The upper pannel shows the figures based on the unweighted UniFrac distance (accounts for abundance of observed organisms), whereas the lower pannel shows similar figures but based on weighted UniFrac distance (considers the presence or absence of the organisms only). CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40. The P values for both overall and pairwise analyses were calculated using PERMANOVA.

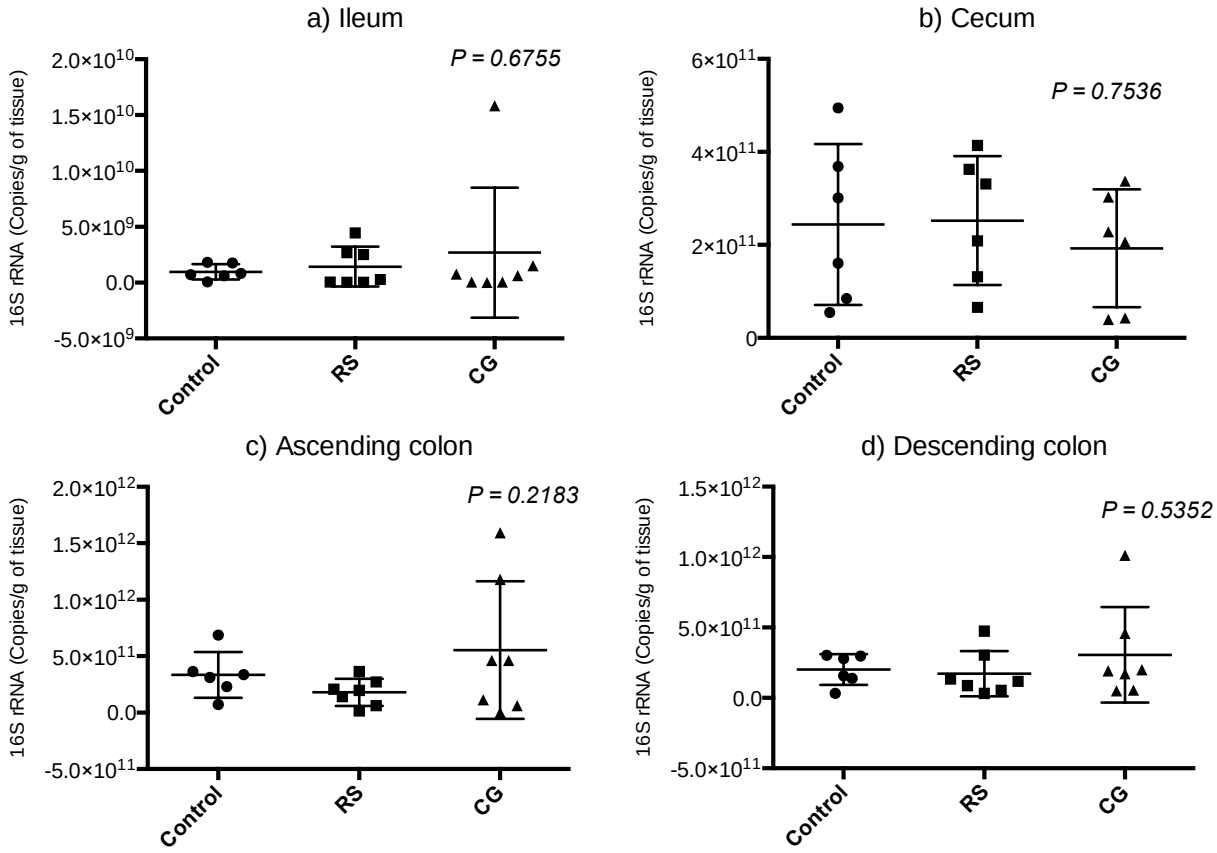


Figure 5.6. Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on bacterial density in the ileum, cecum, ascending and descending colon. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40. No significant differences were observed.

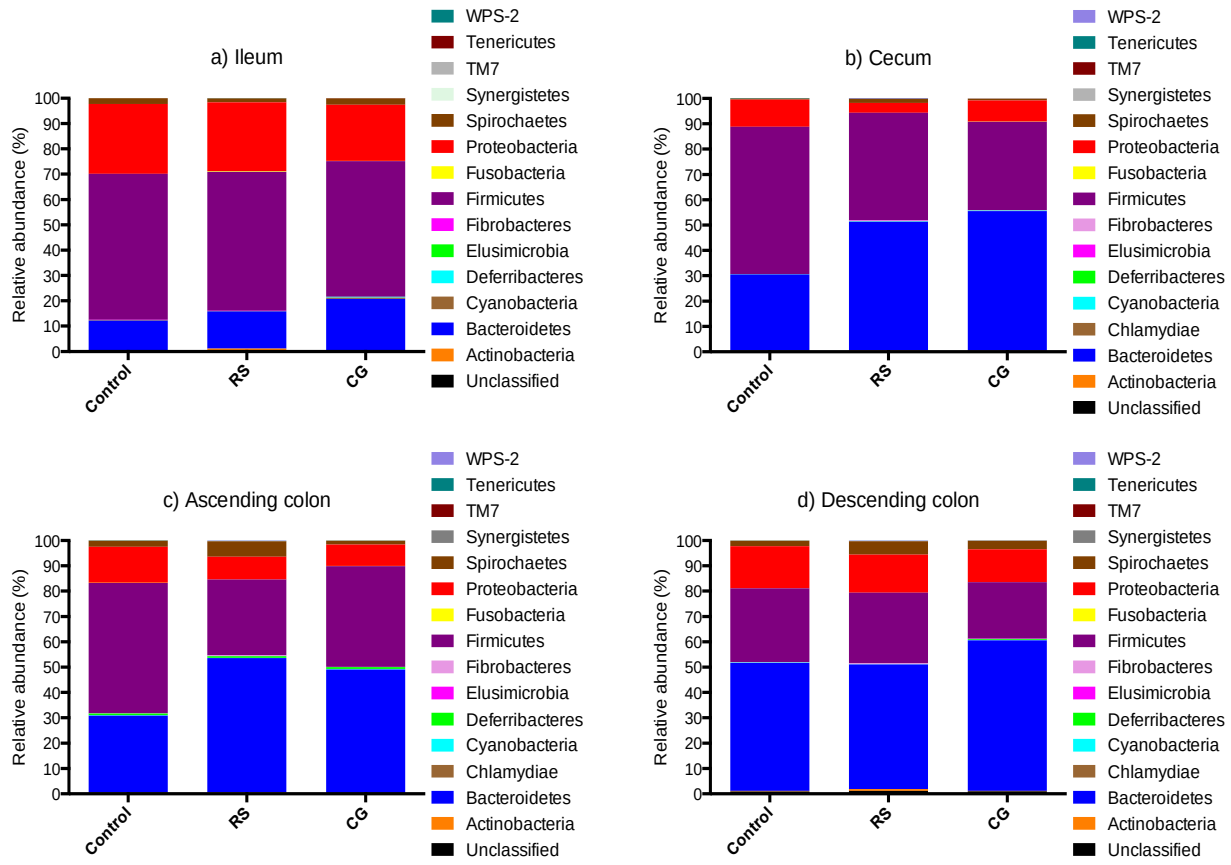


Figure 5.7. Effect of degraded carrageenan gum (CG) and resistant starch (RS; MSPrebiotic) on bacterial composition at phylum level in the ileum, cecum, ascending and descending colon. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

across the intestinal segments, whereas other phyla were less represented. Among the most abundant phyla, no significant differences were observed between treatment groups in the ileum. The population of Bacteroidetes in the cecum was significantly higher in the CG and RS treated pigs compared to the control pigs, whereas Firmicutes and Proteobacteria were lower in RS and CG treated pigs compared to the control. Bacteroidetes and Firmicutes were significantly lower and higher, respectively in the ascending colon of CG and RS treated pigs compared to the control pigs, whereas Spirochaetes was significantly higher in RS treated pigs compared to the CG and control pigs. In the descending colon, Actinobacteria were significantly higher in the RS treated pigs, Deferribacteres and Fusobacteria were higher in the CG treated pigs compared to other treatment groups, whereas Synergistetes and Tenericutes were lowest in the CG and control respectively.

5.4.1.7. Taxa characterizing treatment groups within tissue sites.

Ileum: The most differentially abundant bacterial taxon in colitic pigs belonged to phyla Proteobacteria including genera *Klebsiella,* whereas overrepresented genera in RS pigs include clades of Bifidobacteriaceae and genera *Bifidobacterium*. No taxa were significantly enriched in the ileum of control pigs (**Figure 5.8**).

Cecum: The most differentially abundant bacterial taxa in colitic pigs belonged to phylum Proteobacteria and Bacteroidetes, including genera *Prevotella*, *Helicobacter*, and other clades of Enterobacteriaceae. RS treated pigs were characterized by taxa within the Tenericutes phylum and included clades of Anaeroplasmatales, whereas the most differentially abundant bacterial taxa in control pigs belonged to phylum Proteobacteria including genera *Campylobacter*, and other clades of Campylobacteraceae (**Figure 5.9**).

Ascending colon: Colitic pigs were majorly characterized by clades within the phylum

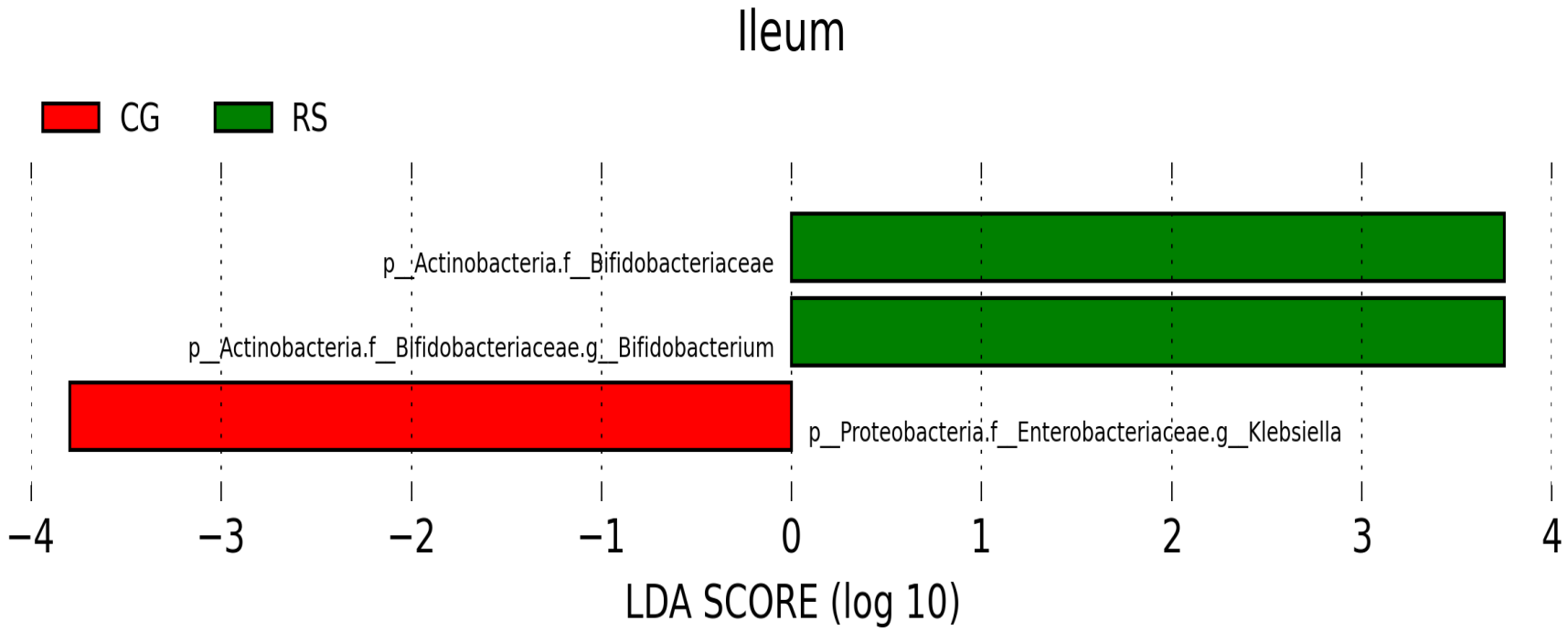


Figure 5.8. Phylogenetic comparisons of ileal mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the Control group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

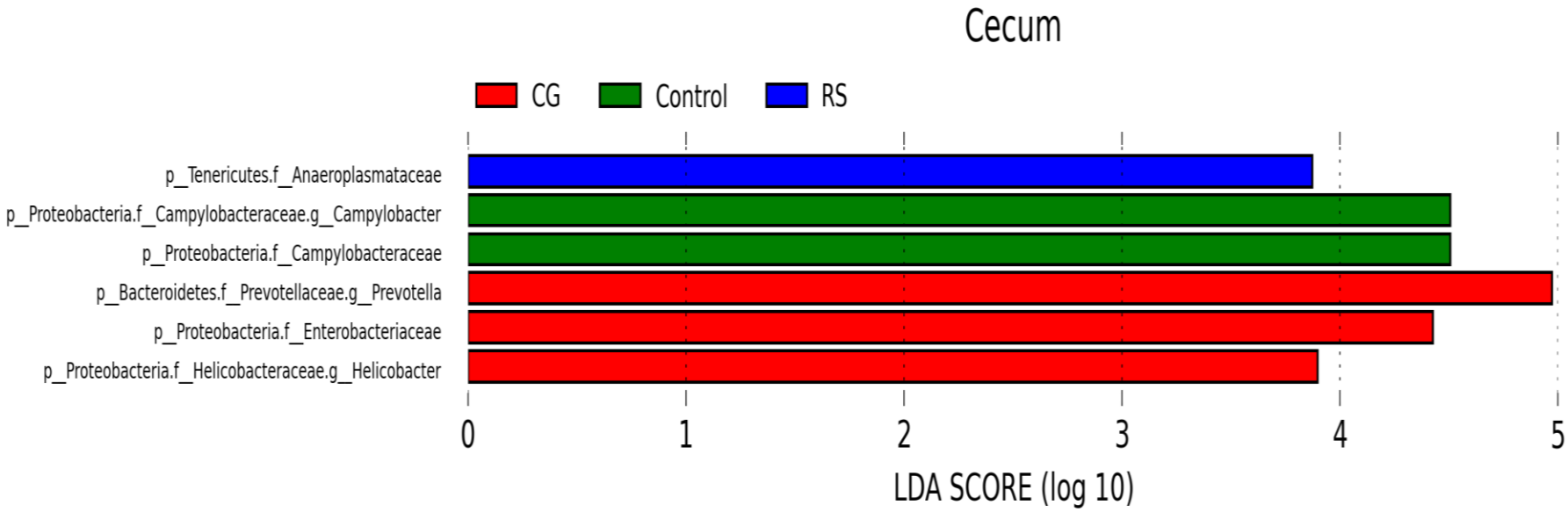


Figure 5.9. Phylogenetic comparisons of cecal mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

Fusobacteria including genera *Fusobacterium*, and clades of Fusobacteriaceae, whereas RS-treated pigs were enriched with taxa within Phylum Proteobacteria including clades of Xanthomodaceae, Succinivibrionaceae and GMD14H09. Majority of the most differentially abundant bacterial taxa in control pigs belonged to phyla Cyanobacteria, Synergistetes, Proteobacteria, and Firmicutes, including genera *Campylobacter* and *Pyramidobacter*, and other clades of Veillonellaceae, Campylobacteraceae, and Dethiosulfovibrionaceae (**Figure 5.10**).

Descending colon: The most differentially abundant bacterial taxa in colitic pigs belong to phyla Proteobacteria, Deferribacteres, Fusobacteria, Firmicutes, and Bacteroidetes, including genera *Butyricimonas*, *Fusobacterium*, *Faecalibacterium*, *Mucispirillum*, and *Desulfovibrio*, and clades of Deferribacteraceae, Enterobacteriaceae, Fusobacteraceae, Odoribacteraceae, and Lachnospiraceae. RS treated pigs were characterized by taxa within phyla Proteobacteria, Actinobacteria, Firmicutes, and Tenericutes, including genera *Bifidobacterium*, *Peptococcus*, and clades of GMD14H09, Succinivibrionaceae, Bifidobacteraceae, Coriobacteriaceae, and Anaeroplasmataceae. Control pigs were enriched with taxa within phyla Synegistetes and Firmicutes, including genera *Sharpea*, and *Pyramidobacter*, and clades of Dethiosulfovibrionaceae (**Figure 5.11**).

5.4.1.8. Predicted functional pathways/activities

Several pathways were enriched in the ileal mucosa-associated microbiota of the colitic pigs compared to the RS and control pigs. In this context, biosynthesis of ansamycins, ascorbate and aldarate metabolism, pentose and glucuronate interconversions, phenylpropanoid biosynthesis, arachidonic metabolism, and retinol metabolism were enriched in the CG-treated pigs. Primary and secondary bile biosynthesis was enriched in the ileal microbiota of control pigs and there were no differences between the control and RS treated pigs (**Figure 5.12**).

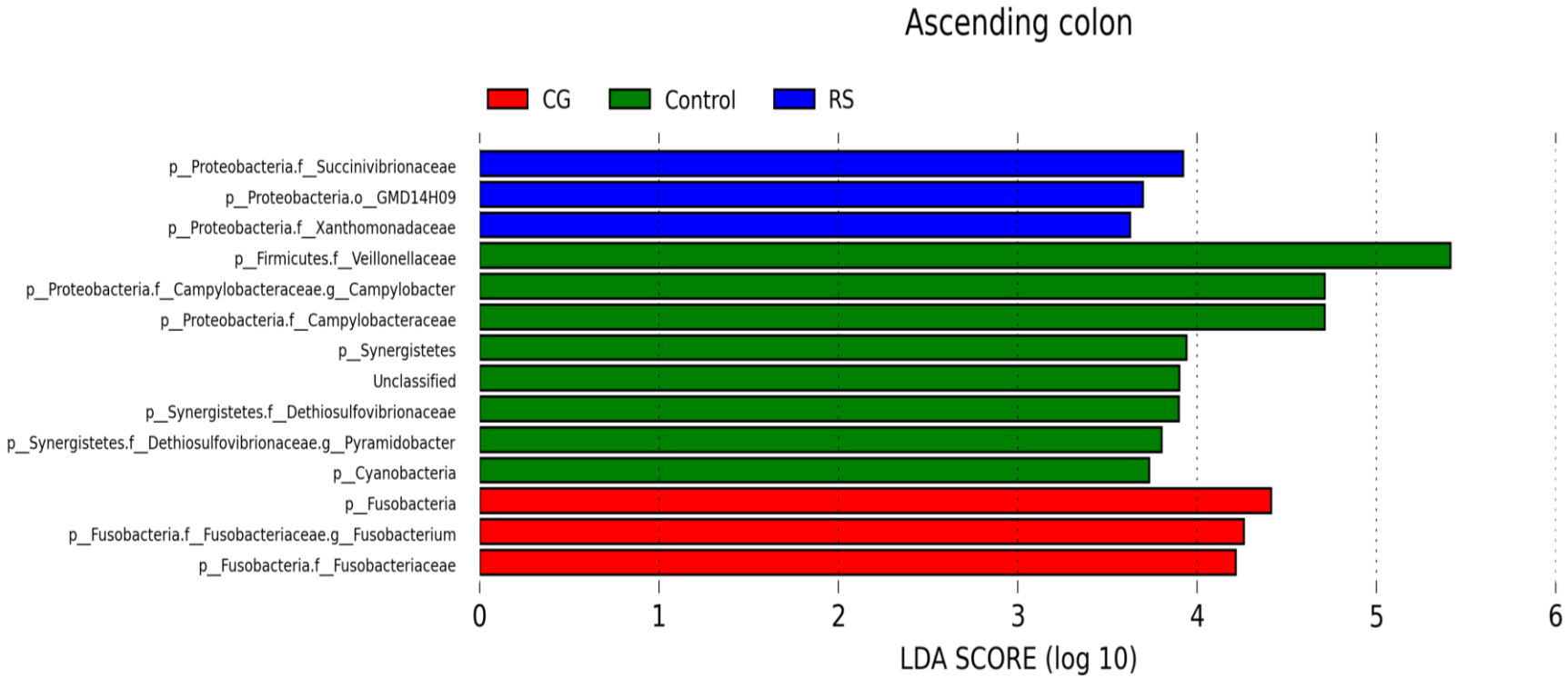


Figure 5.10. Phylogenetic comparisons of ascending colon mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

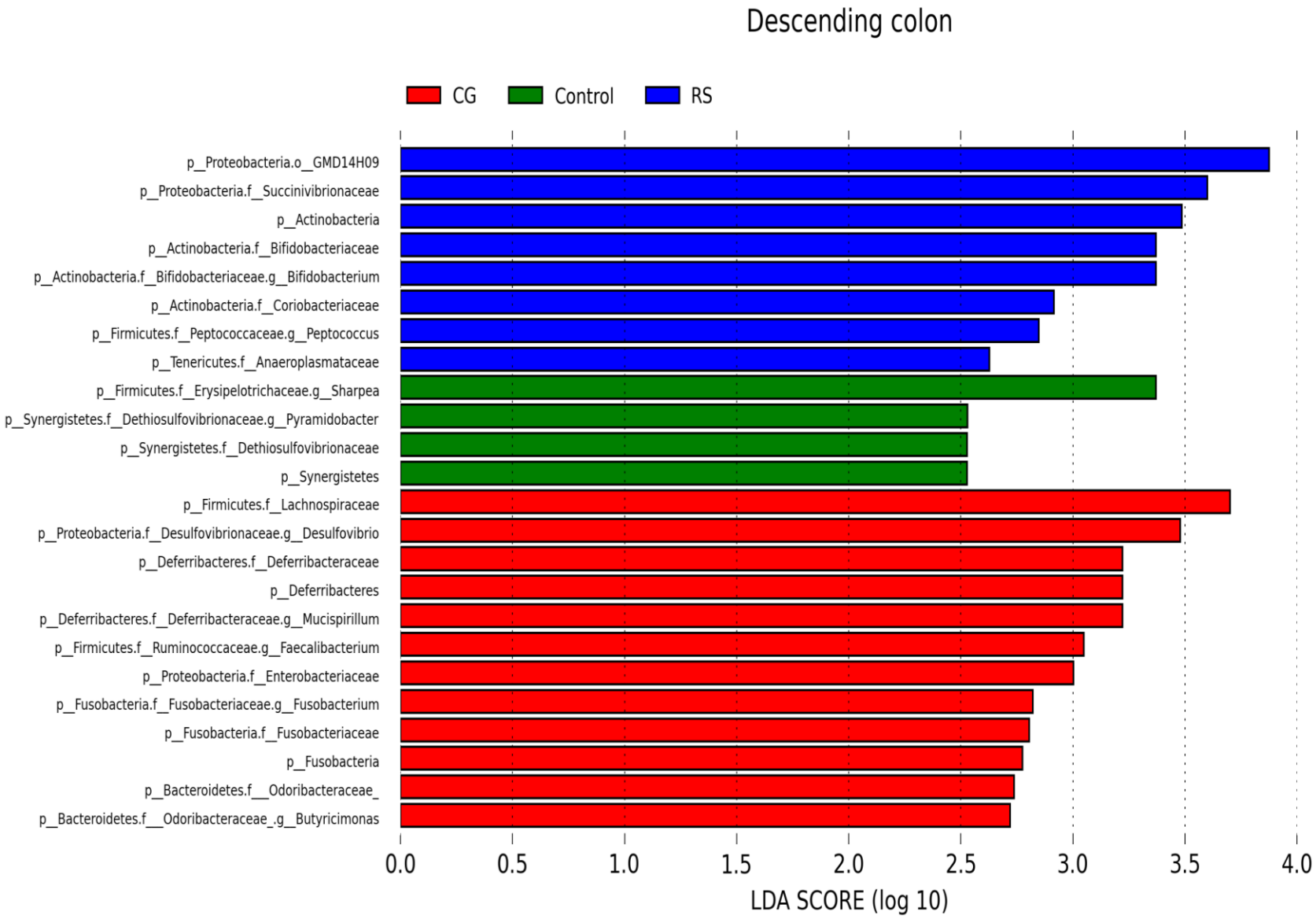

Descending colon
CG
Control
RS
p__Proteobacteria.o__GMD14H09
p__Proteobacteria.f__Succinivibrionaceae
p__Actinobacteria
p__Actinobacteria.f__Bifidobacteriaceae
p__Actinobacteria.f__Bifidobacteriaceae.g__Bifidobacterium
p__Actinobacteria.f__Coriobacteriaceae
p__Firmicutes.f__Peptococcaceae.g__Peptococcus
p__Tenericutes.f__Anaeroplasmataceae
p__Firmicutes.f__Erysipelotrichaceae.g__Sharpea
p__Synergistetes.f__Dethiosulfovibrionaceae.g__Pyramidobacter
p__Synergistetes.f__Dethiosulfovibrionaceae
p__Synergistetes
p__Firmicutes.f__Lachnospiraceae
p__Proteobacteria.f__Desulfovibrionaceae.g__Desulfovibrio
p__Deferribacteres.f__Deferribacteraceae
p__Deferribacteres
p__Deferribacteres.f__Deferribacteraceae.g__Mucispirillum
p__Firmicutes.f__Ruminococcaceae.g__Faecalibacterium
p__Proteobacteria.f__Enterobacteriaceae
p__Fusobacteria.f__Fusobacteriaceae.g__Fusobacterium
p__Fusobacteria.f__Fusobacteriaceae
p__Fusobacteria
p__Bacteroidetes.f__Odoribacteraceae_
p__Bacteroidetes.f__Odoribacteraceae_.g__Butyricimonas
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
LDA SCORE (log 10)

Figure 5.11. Phylogenetic comparisons of descending colon mucosa-associated microbiota in different treatment groups. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

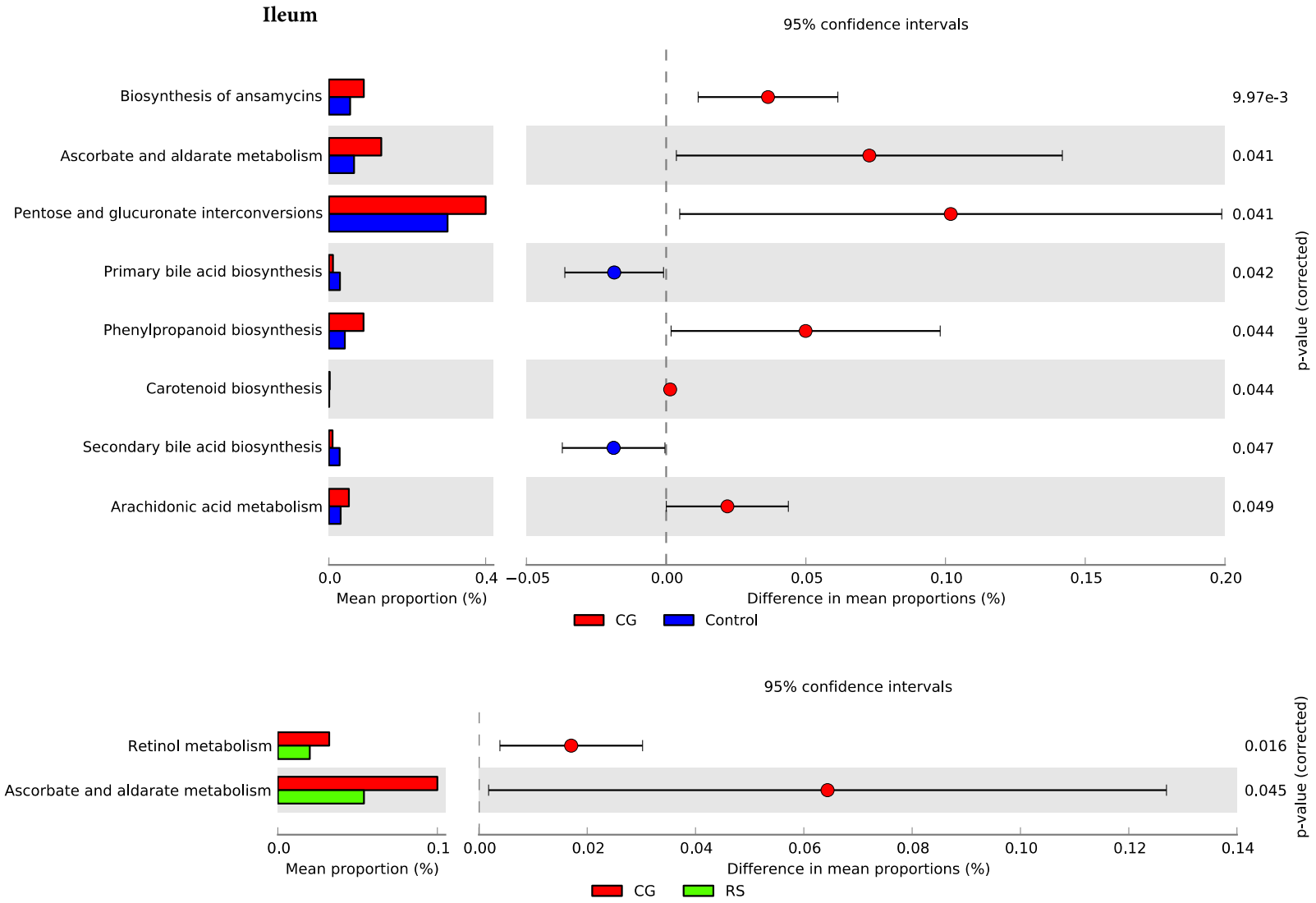
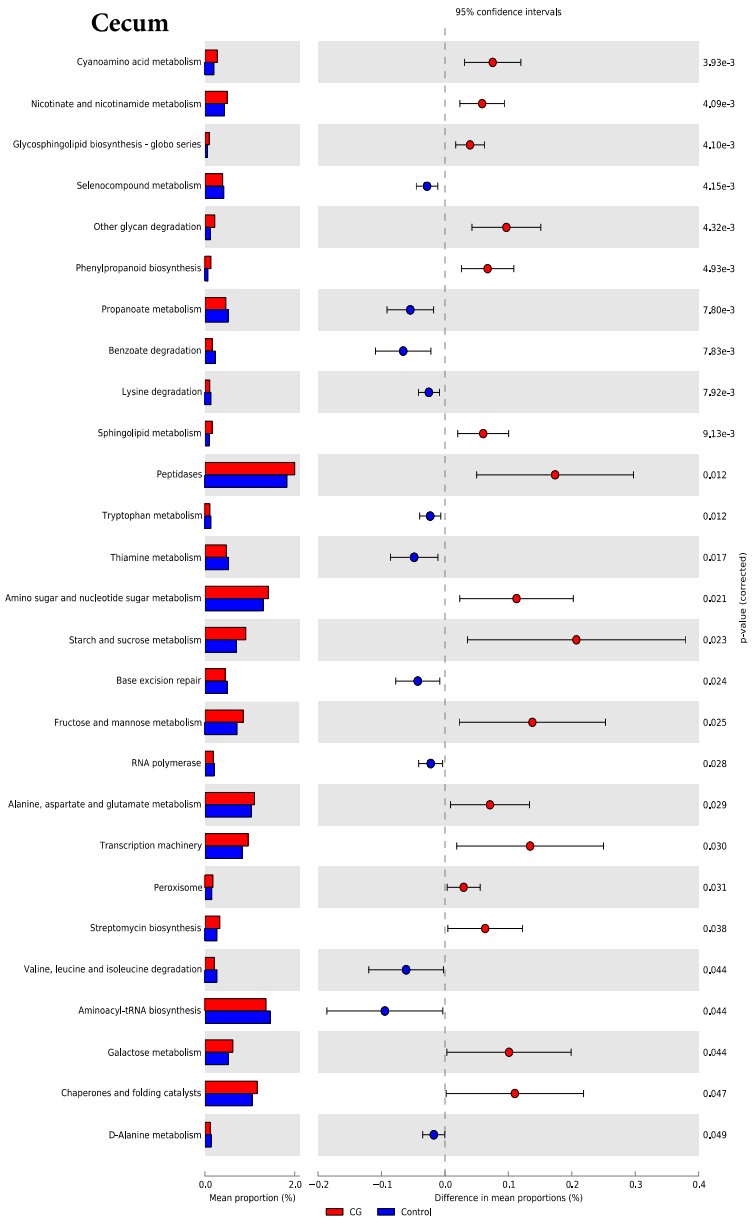

Ileum
95% confidence intervals
Biosynthesis of ansamycins
Ascorbate and aldarate metabolism
Pentose and glucuronate interconversions
Primary bile acid biosynthesis
Phenylpropanoid biosynthesis
Carotenoid biosynthesis
Secondary bile acid biosynthesis
Arachidonic acid metabolism
9.97e-3
0.041
0.041
0.042
0.044
0.044
0.047
0.049
p-value (corrected)
0.0
0.4
Mean proportion (%)
−0.05
0.00
0.05
0.10
0.15
0.20
Difference in mean proportions (%)
CG
Control
95% confidence intervals
Retinol metabolism
Ascorbate and aldarate metabolism
0.016
0.045
p-value (corrected)
0.0
0.1
Mean proportion (%)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Difference in mean proportions (%)
CG
RS

Figure 5.12. Sub-systems or pathways enriched in the ileal mucosa-associated microbiota in CG treatment compared to RS and control groups. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period. All pigs were euthanized on day 40.

Compared to the control, cecal mucosa-associated microbiota of the pigs exposed to CG colitis was enriched with several functional pathways including galactose metabolism, starch and sucrose metabolism, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, alanine, aspartate and glutamate metabolism, peptidases, among other functional pathways. Very few pathways were significantly different between colitic and RS treated pigs in the cecum, whereas compared to the control, RS treated pigs were enriched with several pathways including peptidoglycan biosynthesis, amino sugar and nucleotide sugar metabolism, peptidases, one carbon pool by carbonate, cyanoamino acid metabolism, among other pathways (**Figures 5.13**).

Most of the functional pathways in the ascending colon were significantly enriched in the colitic pigs, and in RS treated pigs when compared to the control pigs. However, there was no significantly different functional pathways between colitic and RS treated pigs. Methane metabolism, peptidoglycan biosynthesis, fructose and mannose metabolism, peptidases, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, naphthalene degradation among other pathways were enriched in the CG treated pigs, whereas alanine, aspartate and glutamate metabolism, taurine and hypotaurine metabolism, streptomycin biosynthesis, peptidases, cyanoamino acid metabolism, sphigolipid metabolism, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, among other functional pathways were enriched in the microbiota of RS treated pigs compared to the control. (**Figure 5.14**).

Only few bacterial functional pathways were significantly enriched in the descending colon of colitic pigs compared to RS or the control pigs. In this case, cyanoamino acid metabolism, phenylpropanoid biosynthesis, and butirosin and neomycin biosynthesis were significantly enriched in the colitic pigs compared to the control, whereas only riboflavin


Cecum
95% confidence intervals
Cyanoamino acid metabolism
Nicotinate and nicotinamide metabolism
Glycosphingolipid biosynthesis - globo series
Selenocompound metabolism
Other glycan degradation
Phenylpropanoid biosynthesis
Propanoate metabolism
Benzoate degradation
Lysine degradation
Sphingolipid metabolism
Peptidases
Tryptophan metabolism
Thiamine metabolism
Amino sugar and nucleotide sugar metabolism
Starch and sucrose metabolism
Base excision repair
Fructose and mannose metabolism
RNA polymerase
Alanine, aspartate and glutamate metabolism
Transcription machinery
Peroxisome
Streptomycin biosynthesis
Valine, leucine and isoleucine degradation
Aminoacyl-tRNA biosynthesis
Galactose metabolism
Chaperones and folding catalysts
D-Alanine metabolism
3.93e-3
4.09e-3
4.10e-3
4.15e-3
4.32e-3
4.93e-3
7.80e-3
7.83e-3
7.92e-3
9.13e-3
0.012
0.012
0.017
0.021
0.023
0.024
0.025
0.028
0.029
0.030
0.031
0.038
0.044
0.044
0.044
0.047
0.049
p-value (corrected)
0.0
2.0
Mean proportion (%)
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
Difference in mean proportions (%)
CG
Control

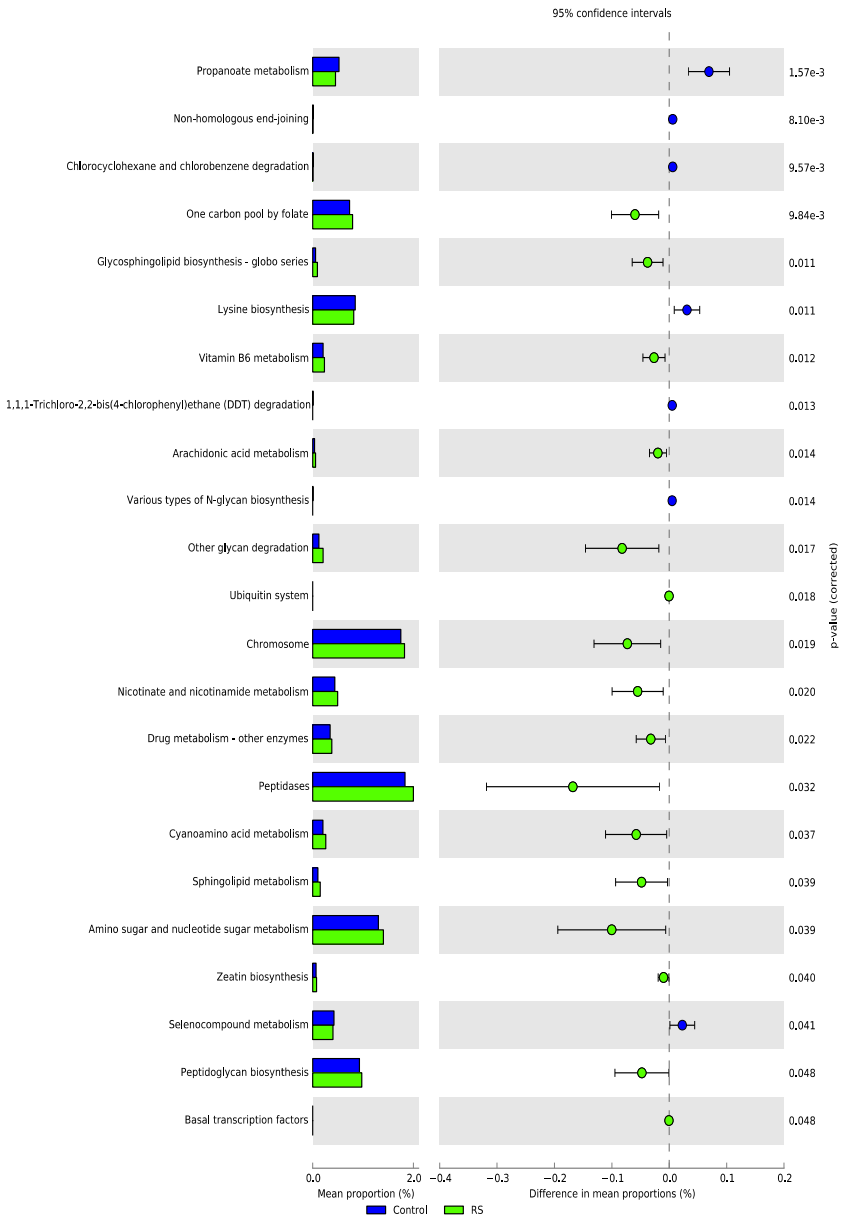

95% confidence intervals
Propanoate metabolism
Non-homologous end-joining
Chlorocyclohexane and chlorobenzene degradation
One carbon pool by folate
Glycosphingolipid biosynthesis - globo series
Lysine biosynthesis
Vitamin B6 metabolism
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation
Arachidonic acid metabolism
Various types of N-glycan biosynthesis
Other glycan degradation
Ubiquitin system
Chromosome
Nicotinate and nicotinamide metabolism
Drug metabolism - other enzymes
Peptidases
Cyanoamino acid metabolism
Sphingolipid metabolism
Amino sugar and nucleotide sugar metabolism
Zeatin biosynthesis
Selenocompound metabolism
Peptidoglycan biosynthesis
Basal transcription factors
1.57e-3
8.10e-3
9.57e-3
9.84e-3
0.011
0.011
0.012
0.013
0.014
0.014
0.017
0.018
0.019
0.020
0.022
0.032
0.037
0.039
0.039
0.040
0.041
0.048
0.048
p-value (corrected)
0.0
2.0
Mean proportion (%)
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
Difference in mean proportions (%)
Control
RS

Figure 5.13. Sub-systems or pathways enriched in the cecal mucosa-associated microbiota in CG treatment compared to RS and control groups. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period.

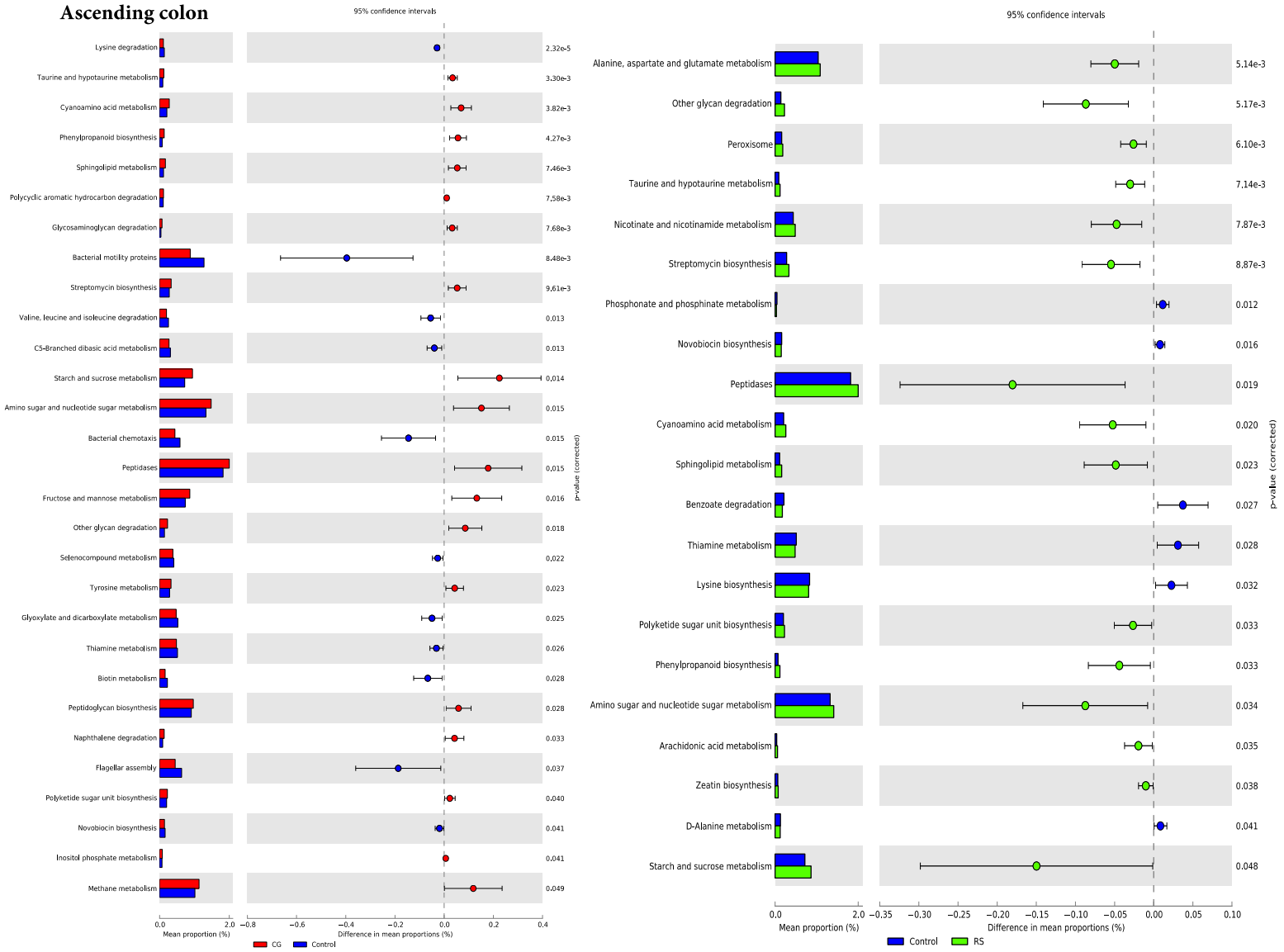

Ascending colon
95% confidence intervals
Lysine degradation
Taurine and hypotaurine metabolism
Cyanoamino acid metabolism
Phenylpropanoid biosynthesis
Sphingolipid metabolism
Polycyclic aromatic hydrocarbon degradation
Glycosaminoglycan degradation
Bacterial motility proteins
Streptomycin biosynthesis
Valine, leucine and isoleucine degradation
C5-Branched dibasic acid metabolism
Starch and sucrose metabolism
Amino sugar and nucleotide sugar metabolism
Bacterial chemotaxis
Peptidases
Fructose and mannose metabolism
Other glycan degradation
Selenocompound metabolism
Tyrosine metabolism
Glyoxylate and dicarboxylate metabolism
Thiamine metabolism
Biotin metabolism
Peptidoglycan biosynthesis
Naphthalene degradation
Flagellar assembly
Polyketide sugar unit biosynthesis
Novobiocin biosynthesis
Inositol phosphate metabolism
Methane metabolism
2.32e-5
3.30e-3
3.82e-3
4.27e-3
7.46e-3
7.58e-3
7.68e-3
8.48e-3
9.61e-3
0.013
0.013
0.014
0.015
0.015
0.015
0.016
0.018
0.022
0.023
0.025
0.026
0.028
0.028
0.033
0.037
0.040
0.041
0.041
0.049
p-value (corrected)
0.0
2.0
Mean proportion (%)
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
Difference in mean proportions (%)
CG
Control
95% confidence intervals
Alanine, aspartate and glutamate metabolism
Other glycan degradation
Peroxisome
Taurine and hypotaurine metabolism
Nicotinate and nicotinamide metabolism
Streptomycin biosynthesis
Phosphonate and phosphinate metabolism
Novobiocin biosynthesis
Peptidases
Cyanoamino acid metabolism
Sphingolipid metabolism
Benzoate degradation
Thiamine metabolism
Lysine biosynthesis
Polyketide sugar unit biosynthesis
Phenylpropanoid biosynthesis
Amino sugar and nucleotide sugar metabolism
Arachidonic acid metabolism
Zeatin biosynthesis
D-Alanine metabolism
Starch and sucrose metabolism
5.14e-3
5.17e-3
6.10e-3
7.14e-3
7.87e-3
8.87e-3
0.012
0.016
0.019
0.020
0.023
0.027
0.028
0.032
0.033
0.033
0.034
0.035
0.038
0.041
0.048
p-value (corrected)
0.0
2.0
Mean proportion (%)
−0.35
−0.30
−0.25
−0.20
−0.15
−0.10
−0.05
0.00
0.05
0.10
Difference in mean proportions (%)
Control
RS

Figure 5.14. Sub-systems or pathways enriched in the ascending colon mucosa-associated microbiota in CG treatment compared to RS and control groups. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period.

metabolism was significantly enriched in the colitic pigs compared to RS treated pigs. On the other hand, valine, leucine and isoleucine degradation, fatty acid metabolism, limonene and pinene degradation, tryptophan metabolism, benzoate degradation, and phosphonate and phosphinate metabolism were enriched in the RS treated pigs compared to the colitic pigs (**Figure 5.15**).

5.4.2. Experiment 2: Inhibitory and therapeutic effects of resistant starch (RS; MSprebiotic) on fecal consistency, local inflammation, histological damage, and microbiota composition and functional gene contents in pigs with CG-induced colitis.

5.4.2.1. Fecal score

The protective and therapeutic effects of RS on fecal consistency are shown in **Figure 5.16**. Therapeutic use of resistant starch after two weeks of colitis (RST) drastically reduced fecal score, however, after the introduction of colitis in the prevention group (RSP), there was a slow and gradual increase in the fecal score, although the score was still below that of CG group.

5.4.2.2. Local pro-and anti-inflammatory responses, and gut barrier function.

As shown in **Figure 5.17 a-e**, inclusion of RS down regulated various pro-inflammatory cytokines including IL-1β in the cecum of RST group, IL-8 in the ascending colon of both RST and RSP, and in the descending colon of RSP group, and IFN-γ in the cecum and ascending colon of RSP and RST groups. However, IL-12p40 was up regulated in both the ileum and descending colon of the RST group, whereas, IL-17 was up regulated in the cecum of both RSP and RST treatment groups. Expression of anti-inflammatory cytokines IL-10, transforming growth factor (TGF)-β and regulatory forkhead box p3 (Foxp3) gene were also tested (**Figure 5.17 f-h**). IL-10 was up regulated in the ileum of RST group, both RST and RSP up-regulated IL-10 in the cecum, but a down regulation was observed in the ileum of RSP group, and in the


Descending colon
95% confidence intervals
Cyanoamino acid metabolism
Phenylpropanoid biosynthesis
Lysine degradation
Tryptophan metabolism
Butirosin and neomycin biosynthesis
Benzoate degradation
0.011
0.015
0.022
0.024
0.031
0.042
p-value (corrected)
0.0
0.3
Mean proportion (%)
−0.06
−0.04
−0.02
0.00
0.02
0.04
0.06
Difference in mean proportions (%)
CG
Control
95% confidence intervals
Valine, leucine and isoleucine degradation
Fatty acid metabolism
Limonene and pinene degradation
Tryptophan metabolism
Benzoate degradation
Phosphonate and phosphinate metabolism
Caprolactam degradation
Fluorobenzoate degradation
Riboflavin metabolism
0.013
0.019
0.028
0.030
0.036
0.036
0.048
0.048
0.049
p-value (corrected)
0.0
0.3
Mean proportion (%)
−0.08
−0.06
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
Difference in mean proportions (%)
CG
RS
95% confidence intervals
Insulin signaling pathway
D-Alanine metabolism
Primary bile acid biosynthesis
Riboflavin metabolism
5.95e-3
0.011
0.038
0.048
p-value (corrected)
0.0
0.3
Mean proportion (%)
−0.08
−0.06
−0.04
−0.02
0.00
0.02
Difference in mean proportions (%)
RS
Control

Figure 5.15 Sub-systems or pathways enriched in the descending colon mucosa-associated microbiota in CG treatment compared to RS and control groups. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RS= pigs received basal diet and one scoop (10 g) of MSPrebiotic during the first 14 days of the experiment after which the amount of MSPrebiotic was adjusted to one scoop and half (15 g) until the end of the experimental period.

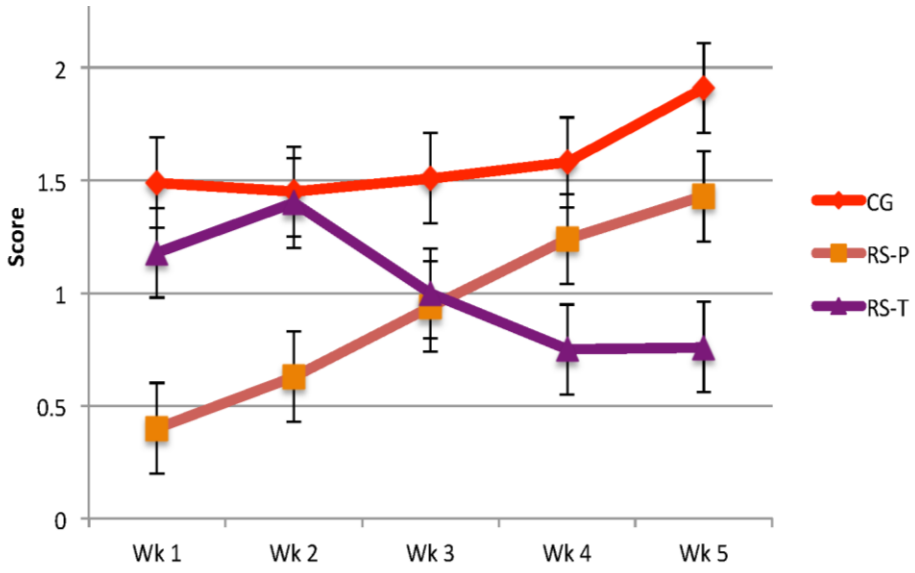


Figure 5.16. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on fecal score as determined by stool consistency in pigs exposed to degraded carrageenan gum (CG)-induced colitis. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period. Severity of diarrhea was characterized using an established fecal consistency (FC) score system in pigs (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea.

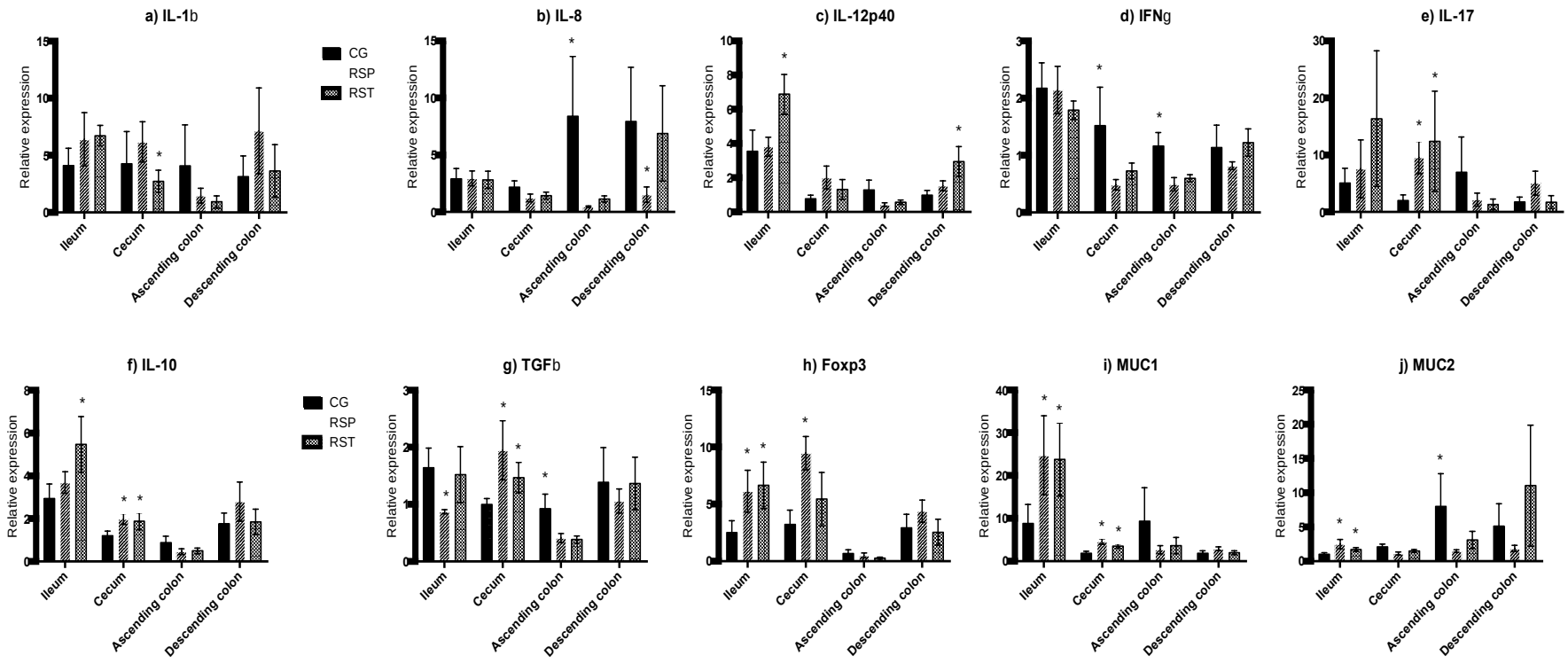


Figure 5.17. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on local gene expression as determined by RT-PCR in pigs exposed to degradeed carrageenan gum (CG)-induced colitis. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

ascending colon of both RSP and RST groups. Ileal Foxp3 was up regulated in both RST and RSP groups, but only RSP significantly up-regulated Foxp3 in the cecum. To further assess epithelial integrity, relative expression of the mucin genes MUC1 and MUC2 was investigated (**Figure 5.17 i-j**). Compared to CG, RSP and RST up-regulated both genes in the ileum; however, only the expression of MUC1 was up-regulated in the cecum of RSP and RST treated pigs, but there was a down-regulation of MUC2 in the ascending colon of RSP and RST treated pigs.

5.4.2.3. Protective and therapeutic effect of MSPrebiotic on histological damage in the colon tissue of pigs with CG-induced colitis.

The inhibitory and therapeutic effects of RS on CG-induced colitis damage on the ascending and descending colon are shown in **Figures 5.18 a-d**. Protective and therapeutic administration of resistant starch reduced colon lesions in CG-induced colitis; however, the therapeutic effects of RS on ascending colon were less prominent compared to the protective effects.

5.4.2.4. Alpha- and Beta-diversity

Alpha diversity as measured by Chao 1 was not different among treatment groups across the intestinal segments, except a trend (P = 0.0864) of decreased diversity in the ileum of RSP group (**Figure 5.19 a-d**). **Figure 5.20 a-d** shows differences in bacterial composition between treatment groups across the intestinal segments. Based on the unweighted Unifrac distance, ileal RSP bacterial structure tended to be different from RST (P = 0.0951), whereas the bacterial structure of RSP tended to be different from both CG (P = 0.0546) and RST (P = 0.0821) based on weighted Unifrac distance. Bacterial structure was not significantly different among treatment groups in the cecum, whereas in the ascending colon, CG and RST pigs were significantly

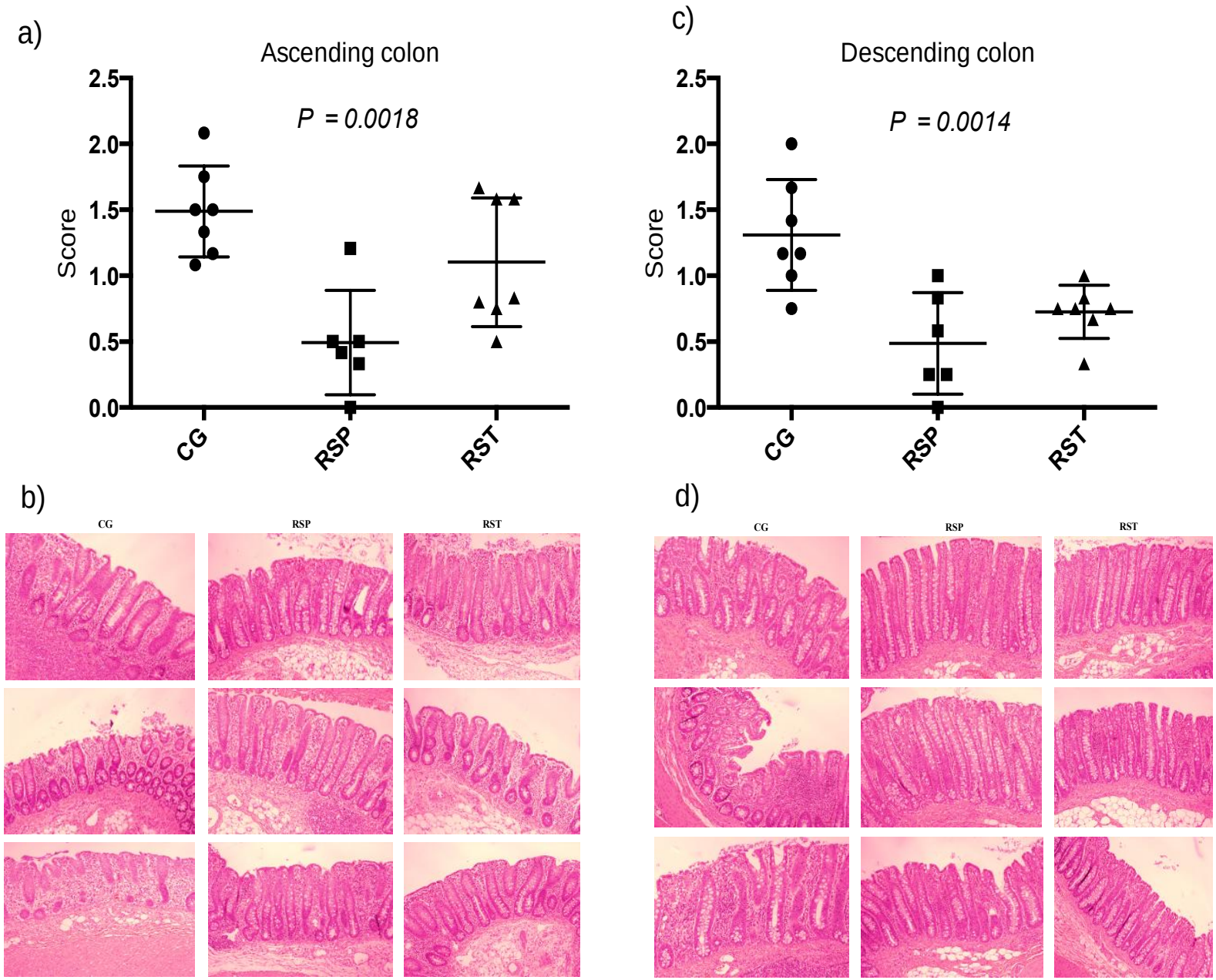

a)
Ascending colon
P = 0.0018
Score
2.5
2.0
1.5
1.0
0.5
0.0
CG
RSP
RST
c)
Descending colon
P = 0.0014
Score
2.5
2.0
1.5
1.0
0.5
0.0
CG
RSP
RST
b)
CG
RSP
RST
d)
CG
RSP
RST

Figure 5.18. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on histological structure in pigs exposed to degraded carrageenan gum (CG)-induced colitis. a) and b) shows histological score and the extent of histological damage, respectively in the ascending colon. c) and d) shows the histological score and the extent of histological damage, respectively in the descending colon. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15^{th} day of the study up to the end of the experimental period.

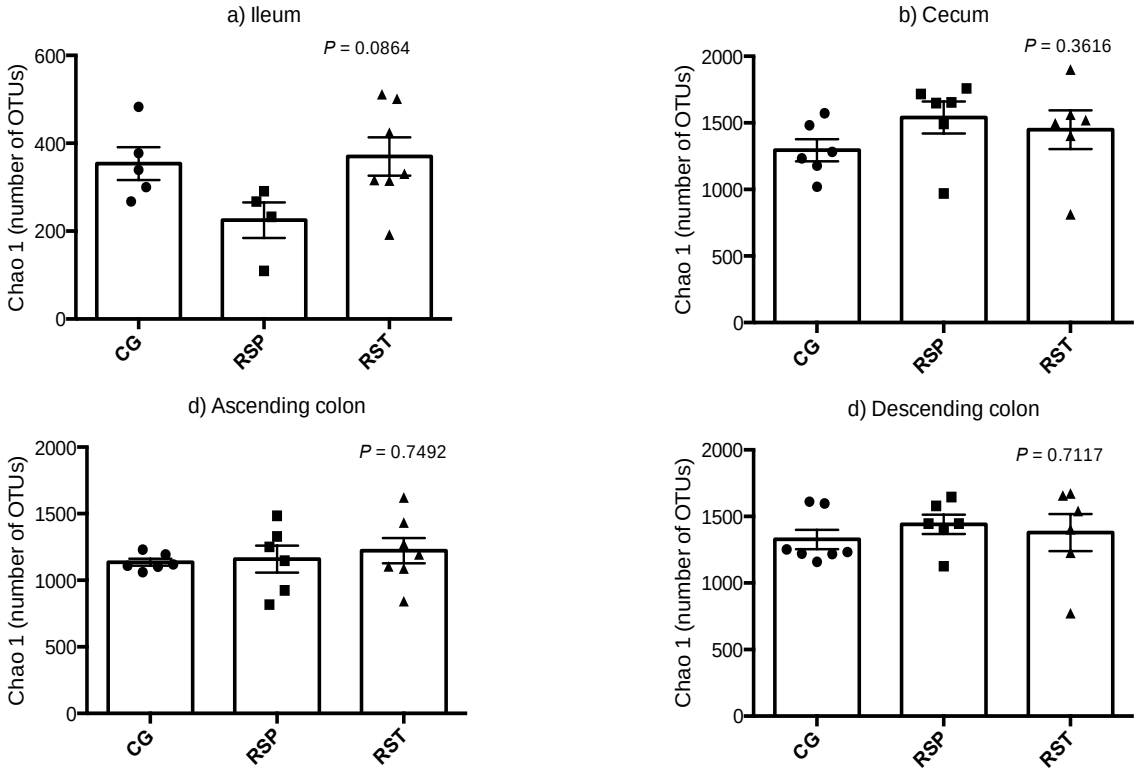


Figure 5.19. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on bacterial richness/alpha-diversity in pigs exposed to degraded carrageenan gum (CG)-induced colitis. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

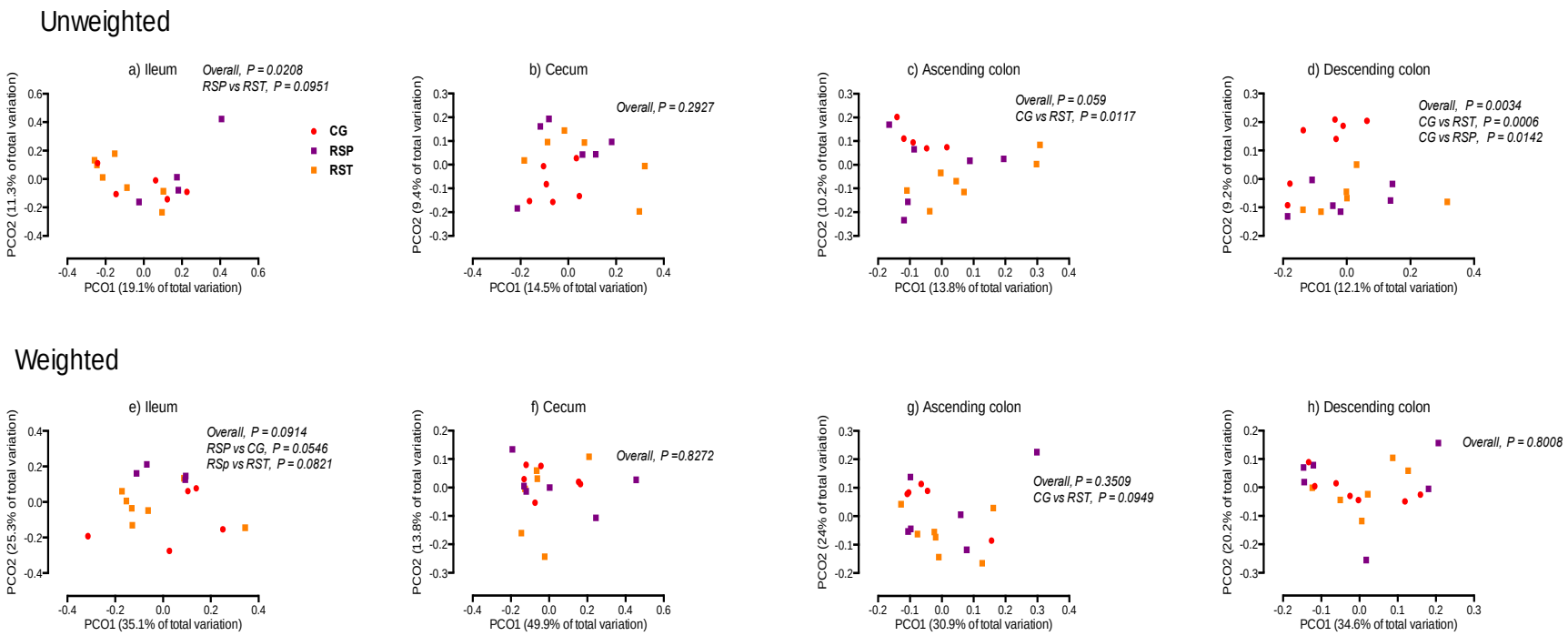


Figure 5.20. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on bacterial structure/beta-diversity in pigs exposed to degraded carrageenan gum (CG)-induced colitis. The upper pannel shows the figures based on the unweighted UniFrac distance (accounts for abundance of observed organisms), whereas the lower pannel shows similar figures but based on weighted UniFrac distance (considers the presence or absence of the organisms only). CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

different based on the unweighted UniFrac distance (P = 0.0117), and a trend was observed based on the weighted UniFrac distance (P = 0.0949). Bacterial structure in the descending colon was only significantly different based on the unweighted UniFrac distance whereby; CG differed from both the RST (P = 0.0006) and RSP (P = 0.0142) groups.

5.4.2.5. Bacterial density

Bacterial density was not significantly different among treatment groups in the ileum, ascending and descending colon, however, cecal bacterial density was significantly lower in the RSP and RST treatment groups compared to CG (**Figure 5.21 a-d**).

5.4.2.6. Bacterial composition at phylum level

Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes were the most abundant and dominant phyla across the intestinal segments, whereas other phyla were less represented. No significant differences were observed between treatment groups across the intestinal segments (data not shown).

5.4.2.7. Taxa characterizing treatment groups within tissue sites.

Ileum: The most differentially abundant bacterial taxa in CG pigs belong to phylum Proteobacteria, including genera *Actinobacillus*, whereas RST treated pigs were characterized by taxa belonging to Proteobacteria and Firmicutes phyla, including genera *Bulleidia* and *Ruminococcus*, and clades of Succinivibrionaceae. No significant taxa were enriched in RSP group (**Figure 5.22**).

Cecum: The most differentially abundant bacterial taxa in colitic pigs belong to phylum Deferribacteres, including genera *Mucispirillum*, and clades of Deferribacteraceae, whereas RST pigs were only enriched with clades of Succinivibrionaceae, and no significant taxa were associated with RSP compared to other groups (**Figure 5.23**).

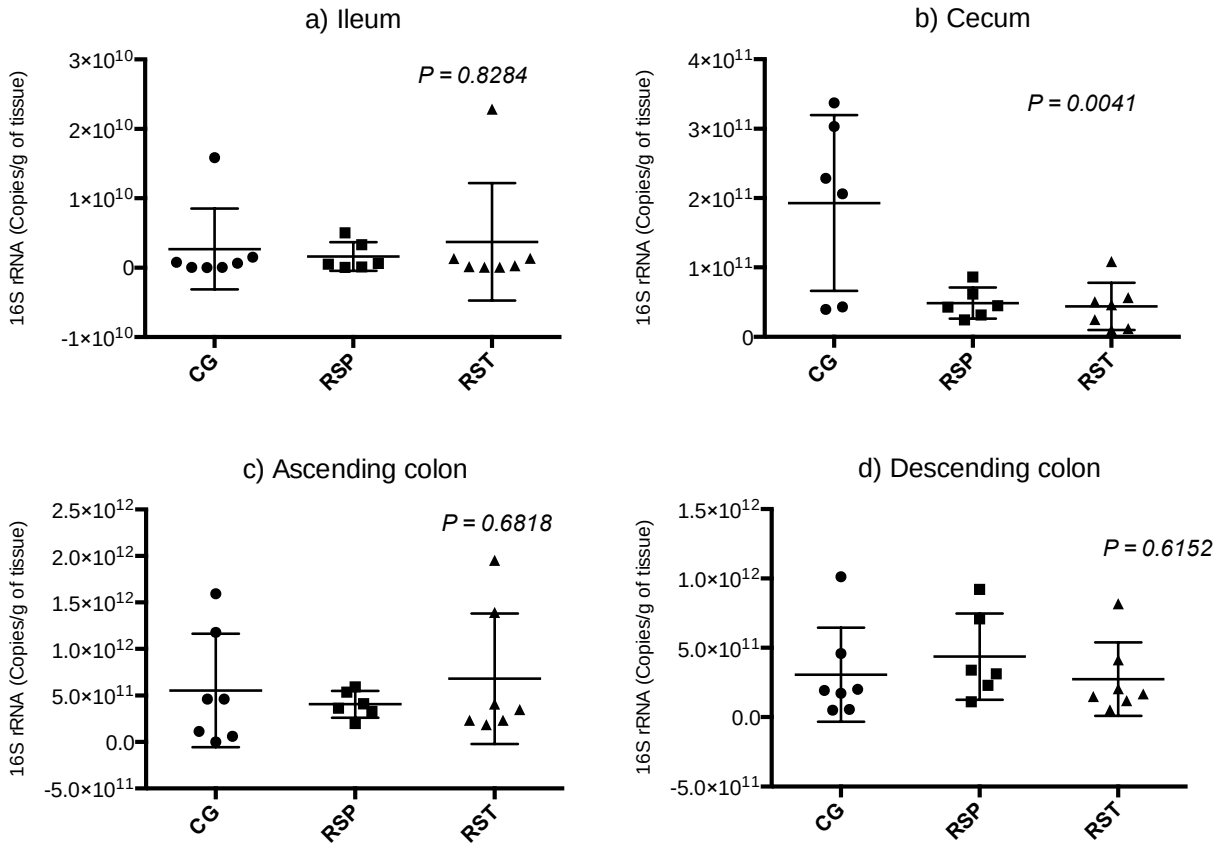


Figure 5.21. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on bacterial density in pigs exposed to degraded carrageenan gum (CG)-induced colitis. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

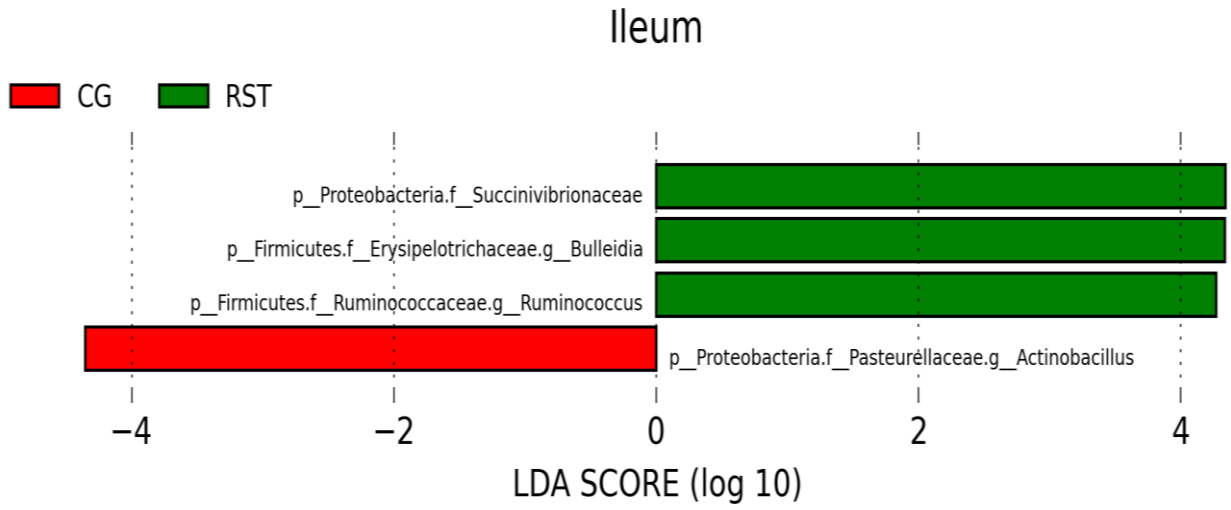


Figure 5.22. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on phylogenetic comparisons of ileal mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the RSP group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

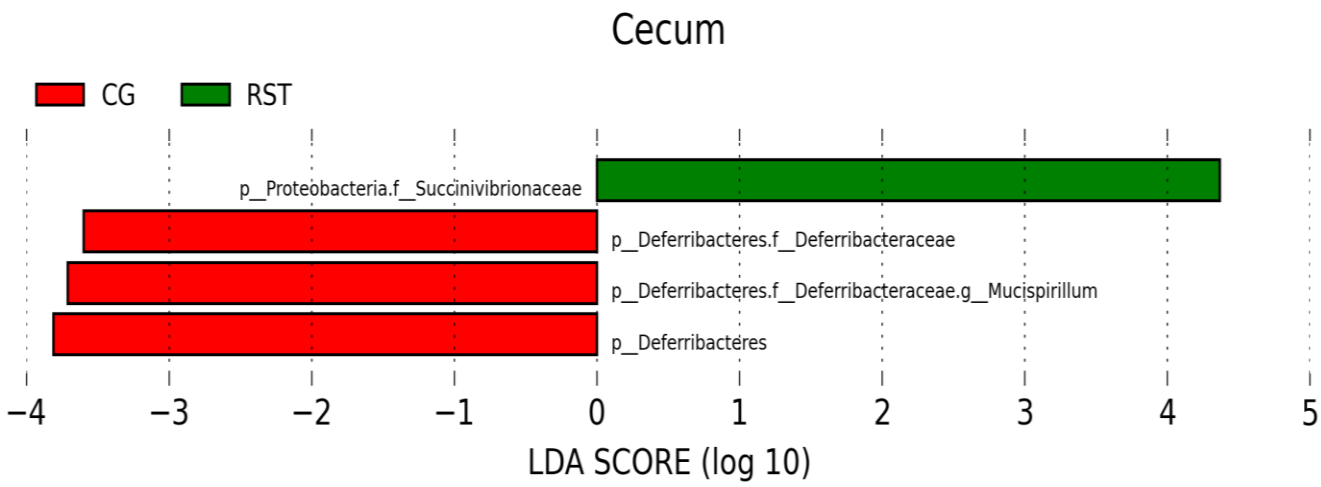


Figure 5.23 Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on phylogenetic comparisons of cecal mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the RSP group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

Ascending colon: CG pigs were majorly characterized by clades within the phylum Deferribacteres, including genera *Mucispirillum*, and clades of Deferribacteraceae, whereas RST group was enriched with members of phyla Proteobacteria, Bacteroidetes, Firmicutes, Tenericutes and Chlamydiae, including genera *rc4_4*, *Chlamydia*, *Sutterella* and *Anaeroplasa*, and clades of Succinivibrionaceae, RF16, Alcaligenaceae and Chlamydiaceae. No significant taxa were associated with RSP compared to other groups (**Figure 5.24**).

Descending colon: The most differentially abundant bacterial taxa in CG pigs belong to phyla Deferribacteres and Bacteroidetes including genera *Butyricimonas* and *Mucispirillum* and other unclassified members of Deferribacteraceae and Odoribacteraceae. RSP pigs were only enriched with genera *Desulfovibrio*, whereas RST was enriched with members of Proteobacteria, Bacteroidetes and WPS_2 including clades of Succinivibrionaceae, S24_7 and RF16 (**Figure 5.25**).

5.4.2.8. Predicted functional pathways/activities

Few functional pathways including retinol metabolism, inositol phosphate metabolism, and taurine and hypotaurine metabolism were significantly enriched in the ileal microbiota of CG group compared to the RSP group, and there were no significant differences between CG and RST (**Figure 5.26**). Likewise, no much significant functional pathways were observed among treatment groups in the cecum and ascending colon. On the other hand, lipid acid metabolism was significantly enriched in the descending colon of RSP pigs, whereas secondary bile acid biosynthesis, biosynthesis of ansamycins, and sulfur metabolism were significantly enriched in the descending colon of CG treated pigs. In addition, several microbial functional pathways were significantly enriched in the descending colon of RST group compared to the CG group including penicillin and cephalosporin biosynthesis, geraniol degradation, caprolacta

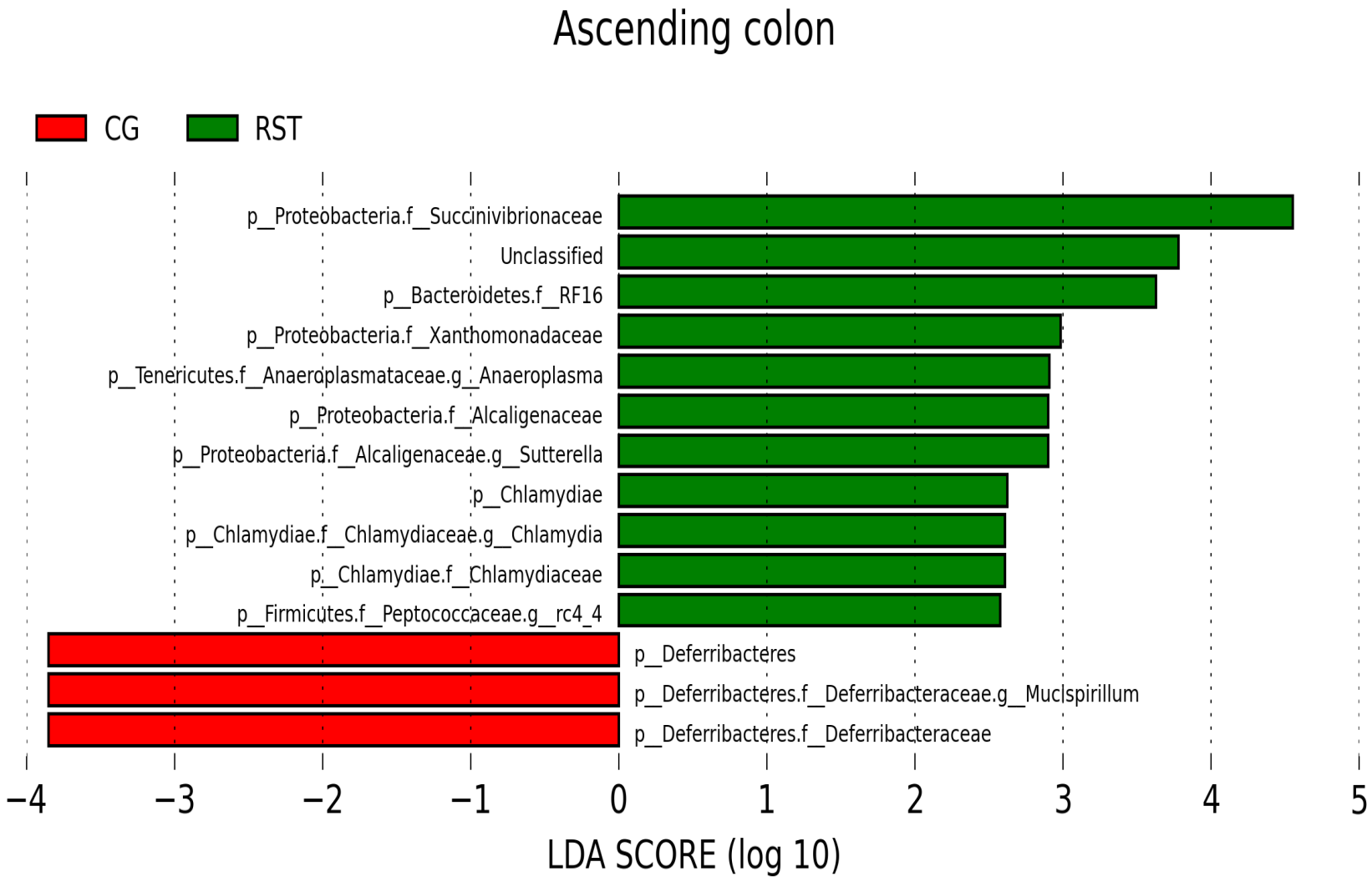

Ascending colon
CG
RST
p__Proteobacteria.f__Succinivibrionaceae
Unclassified
p__Bacteroidetes.f__RF16
p__Proteobacteria.f__Xanthomonadaceae
p__Tenericutes.f__Anaeroplasmataceae.g__Anaeroplasma
p__Proteobacteria.f__Alcaligenaceae
p__Proteobacteria.f__Alcaligenaceae.g__Sutterella
p__Chlamydiae
p__Chlamydiae.f__Chlamydiaceae.g__Chlamydia
p__Chlamydiae.f__Chlamydiaceae
p__Firmicutes.f__Peptococcaceae.g__rc4_4
p__Deferribacteres
p__Deferribacteres.f__Deferribacteraceae.g__Mucispirillum
p__Deferribacteres.f__Deferribacteraceae
−4
−3
−2
−1
0
1
2
3
4
5
LDA SCORE (log 10)

Figure 5.24. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on phylogenetic comparisons of ascending colon mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the RSP group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15^{th} day of the study up to the end of the experimental period.

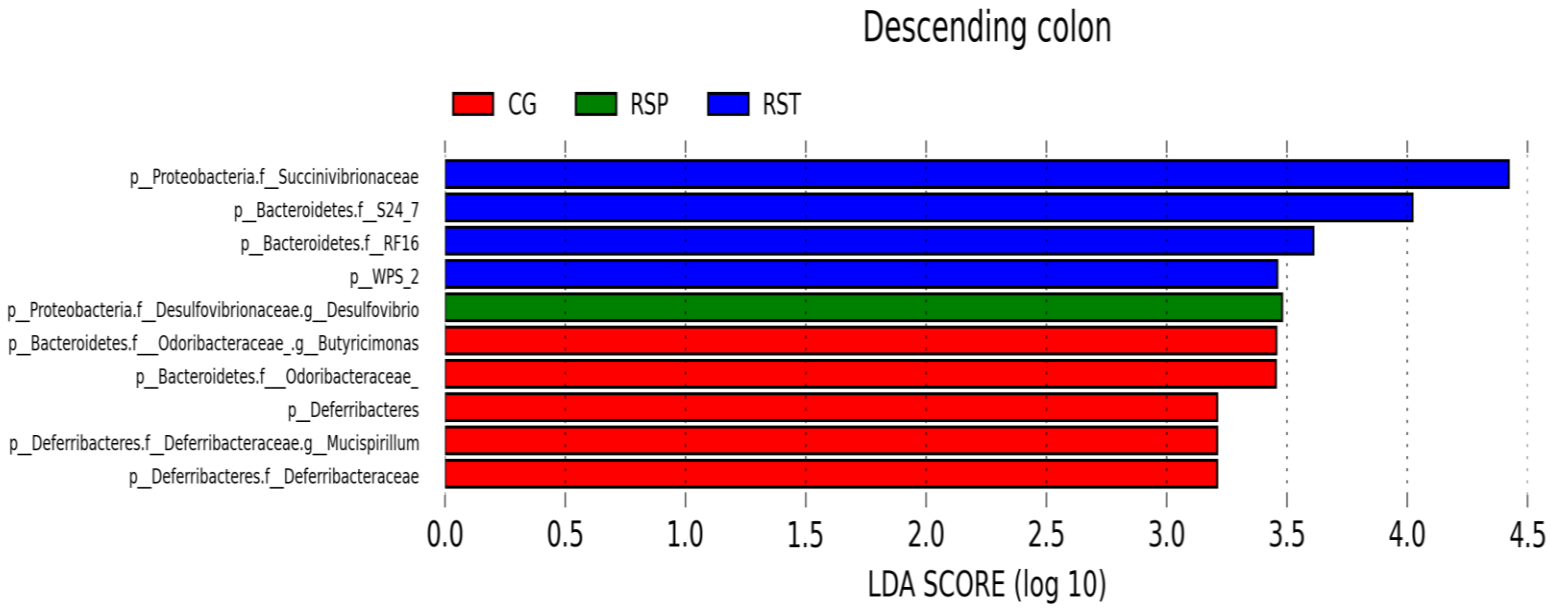


Figure 5.25. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on phylogenetic comparisons of descending colon mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. Lefse was used to determine differentially abundant taxa in each treatment group. Color code represents specific treatment group and only variables that were significantly enriched in each treatment group are shown. There were no significant variables associated with the RSP group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

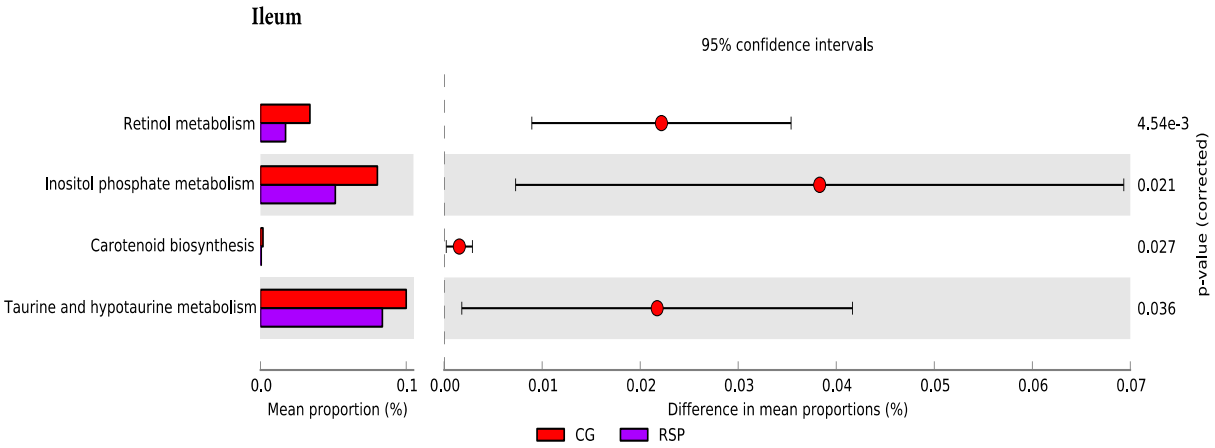


Figure 5.26. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on sub-systems or pathways enriched in the ileal mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15th day of the study up to the end of the experimental period.

degradation, tryptophan metabolism, fatty acid metabolism, novobiocin biosynthesis, phenylalanine metabolism, lysine degradation, lipid biosynthesis proteins, among other pathways (**Figure 5.27**).

5.5. DISCUSSION

Our previous findings had indicated that CG could lead to a considerable dysbiosis especially in the large intestines of pigs (Munyaka et al., 2016b), and that resistant starch (RS) and *E. coli* probiotics UM2&7 may attenuate CG-induced bacterial dysbiosis, however, the effects of RS and the probiotics were very minimal (manuscript II). In this study, we investigated protective and therapeutic role of RS (MSprebiotic) in a pig model of experimental colitis over a relatively longer period of time. Our results have demonstrated both therapeutic and protective effects of RS in CG-induced colitis through attenuation of diarrhea, intestinal damage, modulation of local gene expression and intestinal dysbiosis. In this context, administering RS in pigs pre-exposed to colitis showed merit in decreasing fecal score and although the use of RS as a preventive measure also ameliorated diarrhea compared to CG pigs, the preventive effects were less prominent compared to the therapeutic effects. This could be explained by the fact that, healthy animals adjust quickly to non-toxic products and therefore establish a relatively stable physiological environment; however, exposure to a toxic substance may immediately disturb the established stable environment, which might require some time to counteract the toxin. Nevertheless, the fact that the fecal score was below that of CG still explains merit on the use of RS in protecting against diarrhea in experimental colitis. A lower fecal score in weaned pigs supplemented with potato resistant starch either in powder form or in capsules has been reported previously (Bhandari et al., 2009; Heo et al., 2014), and this further supports efficacy of RS in ameliorating post-weaning diarrhea in pigs, as well as in experimental models of colitis.

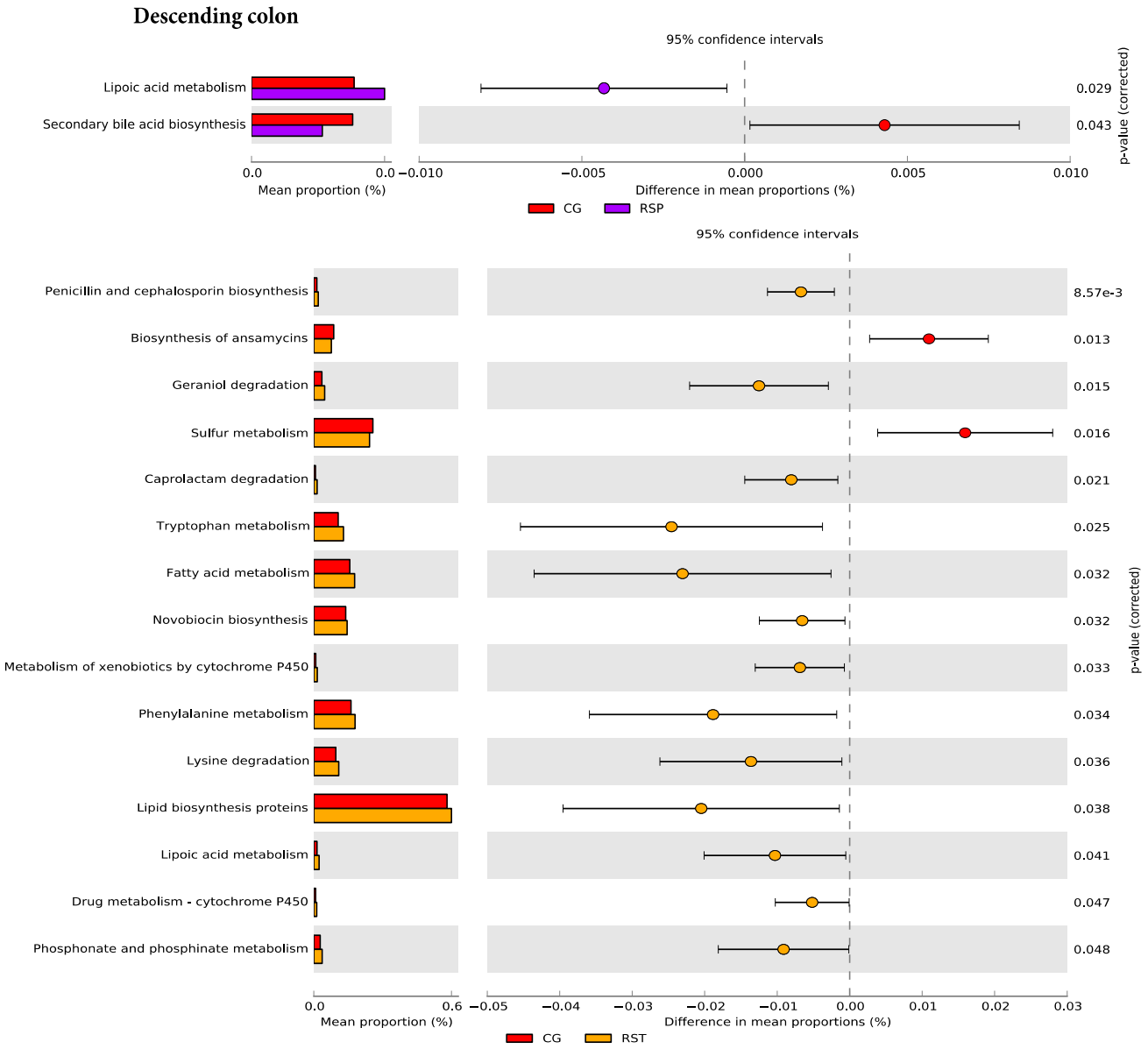

Descending colon
95% confidence intervals
Lipoic acid metabolism
Secondary bile acid biosynthesis
0.029
0.043
p-value (corrected)
0.0
0.0
−0.010
−0.005
0.000
0.005
0.010
Mean proportion (%)
Difference in mean proportions (%)
CG
RSP
95% confidence intervals
Penicillin and cephalosporin biosynthesis
Biosynthesis of ansamycins
Geraniol degradation
Sulfur metabolism
Caprolactam degradation
Tryptophan metabolism
Fatty acid metabolism
Novobiocin biosynthesis
Metabolism of xenobiotics by cytochrome P450
Phenylalanine metabolism
Lysine degradation
Lipid biosynthesis proteins
Lipoic acid metabolism
Drug metabolism - cytochrome P450
Phosphonate and phosphinate metabolism
8.57e-3
0.013
0.015
0.016
0.021
0.025
0.032
0.032
0.033
0.034
0.036
0.038
0.041
0.047
0.048
p-value (corrected)
0.0
0.6
−0.05
−0.04
−0.03
−0.02
−0.01
0.00
0.01
0.02
0.03
Mean proportion (%)
Difference in mean proportions (%)
CG
RST

Figure 5.27. Therapeutic or/and protective effect of resistant starch (RS; MSPrebiotic) on sub-systems or pathways enriched in the descending colon mucosa-associated microbiota in pigs exposed to degraded carrageenan gum (CG)-induced colitis. The color code shows sub-systems or pathways significantly enriched in each treatment group. CG was administered from d 1 of the experiment. Control = pigs received basal diet only, CG= pigs received 1% CG only in drinking water on daily basis, RSP= pigs received 10 g daily RS alone for the first 14 d of the study and CG was introduced at the start of d 15 until the end of the experimental period. Also starting from d 15 of the experiment, the amount of RS was adjusted to 15 g/d, RST=pigs received CG alone during the first 14 d of the experiment and RS was introduced (15 g) from 15^{th} day of the study up to the end of the experimental period.

Therapeutic restoration of mucosal barrier function could improve pathophysiology and clinical outcomes in IBD. One of the main histological pathophysiology of models of colitis is profound colonic inflammation characterized by crypt shortening and destruction, mucosal ulceration, erosions, and infiltration of immune cells into the mucosal tissue, which is accompanied by an increase in pro-inflammatory cytokines (Kwon et al., 2005; Ghia et al., 2007a; Ghia et al., 2007b; Ghia et al., 2009; Lee et al., 2009b; Laroui et al., 2012; Rabbi et al., 2014; Munyaka et al., 2015; Munyaka et al., 2016a). However, whereas pigs exposed to CG-induced colitis showed erosions, crypt shortening and destruction, mucosal ulcerations and up regulation of pro inflammatory cytokines such as IL-1β, IL-8, IFN-γ, therapeutic and protective effects of RS were evident in diminishing the histological damage, and in modulating local inflammatory responses, indicating merit in the use of RS in attenuating and reversing CG-induced destructive responses. RS-containing diet was also recently shown to reduce inflammation, and restore the architecture of colonic lamina propria in a rat model of colitis-associated colorectal cancer (Hu et al., 2016). Cytokines play an important role in disease progression, and increased levels of pro-inflammatory cytokines such as IL-8, IFN-γ, IL-1β, IL-12p40 and other cytokines, are most commonly involved in innate immune pathogenesis of IBD, and have been reported in patients with IBD, and in mice and pigs with DSS-induced colitis (Daig et al., 1996; Egger et al., 2000; Lee et al., 2009b). However, RS significantly reduced local expression of IFN-γ, a known driver of inflammatory responses, and IL-8, a chemokine that contributes to inflammatory-mediated pathology through recruitment of neutrophils (Nielsen et al., 1997), and also increased the expression of TGF-β and Foxp3, which play major roles in maintaining immune homeostasis. With regard to inflammatory responses, IL-17 has a controversial role as an effector or protector (Fuss, 2011). Some studies have shown its

pathogenic role (Fujino et al., 2003), while on the contrary it has been reported to have a protective role in different models of IBD (Yang et al., 2008; O'Connor et al., 2009; Eken et al., 2014). Whereas inclusion of RS down regulated IL-17 in some intestinal segments compared to CG, there was an up regulation in the cecum of RSP and RST pigs, which could be explained by the perceived dual role of IL-17. Also, IL-10 is a known anti-inflammatory cytokine but it is also known to be pleiotropic and therefore, it has important effects on the intestinal immune system depending on cell type, time and location of release, strength of signal and interaction with other cytokines (Paul et al., 2012).

In the intestinal mucosa, the mucus layer acts as a physical barrier to protect and maintain mucosal epithelial integrity (Tai et al., 2007). Mice lacking the capacity to produce mucin are known to spontaneously develop colitis-like phenotype, and are also more susceptible to chemical-induced colitis (Van der Sluis et al., 2006; Johansson et al., 2008). In addition, decreased expression of mucin genes has been reported during DSS-induced colitis in both mice and porcine models (Tai et al., 2007; Lee et al., 2009b). In the current study, relative gene expression of MUC1 and MUC2 were found to be decreased as a result of CG-induced colitis in the ileum and cecum, however, this was inhibited and restored by protective and therapeutic use of RS in the ileum, but only MUC1 was positively influenced by protective and therapeutic use of RS in the cecum, whereas the protective and therapeutic use of RS had negative effects on the expression of MUC2 in the ascending colon. In this case, the positive effects of RS were more pronounced in the membrane-bound mucin MUC1 which plays a role in cell signaling (Bergstrom and Xia, 2013) and Injury repair (Hoebler et al., 2006; Jiang et al., 2011), compared to the secreted form; MUC2. Generally, an increase in the expression of mucus genes can be considered a beneficial response contributing to an enhanced mucus barrier (Boltin et al., 2013;

Dorofeyev et al., 2013; Pastorelli et al., 2013). Also, although different protective or therapeutic measures may fail to directly stimulate the rejuvenation of mucosal barrier system, they may indirectly help in promoting remission in IBD conditions through manipulation of the luminal environment (McGuckin et al., 2009).

We found that feeding RS diet was linked to a beneficial manipulation of the gut microbiota, reflected by substantial shifts in the composition of mucosa-associated microbiota. Principal coordinate analysis (PCoA) plots revealed significant differences between CG, the control, and RS groups, especially in the large intestines. The results suggest differences in the overall microbiota structure between healthy pigs and pigs with CG-induced colitis, which is in agreement with our previous findings (Munyaka et al., 2016b), and that, inclusion of RS in healthy animals could also modulate microbial composition. Of interest was the fact that RS exhibited both protective and therapeutic effects on the pigs with CG-induced colitis through modulation of bacterial composition, as RSP and RST bacterial composition differed from that of CG in the large intestines. These results are also in agreement with our previous findings where inclusion of RS shifted bacterial composition compared to CG (manuscript II), and other studies have also reported significant shifts in microbial composition following RS supplementation in a rat model of colitis-associated colorectal cancer (Hu et al., 2016).

The changes in bacterial structure were confirmed by linear discriminant analysis (LDA) effect size (LEfSe), where we observed significant enrichment of various bacterial taxa in specific groups. The consistent association of some members of Proteobacteria especially members of Gammaproteobacteria with CG-induced colitis agrees with our recent findings (Munyaka et al., 2016b), as well as previous reports by others in IBD patients and in animals with IBD or experimental IBD (Sellon et al., 1998; Darfeuille-Michaud et al., 2004; Schuppler et

al., 2004; Mylonaki et al., 2005; Kotlowski et al., 2007; Lupp et al., 2007; Xenoulis et al., 2008; Sepehri et al., 2011; Wright et al., 2015). In addition, members of Deferribacteres and Fusobacteria including *Mucispirillum* and *Fusobacterum* were also constantly associated with CG-induced colitis especially in the large intestines. Interestingly, the Deferribacteres and Fusobacteria were completely absent in the control, RS, and in the RST and RSP groups, suggesting their strong association with colitic conditions, and merit of preventive or therapeutic use of MSPrebiotic. Other studies in pigs also support beneficial effects of RS feeding with an increase in the growth of microbial populations producing SCFA and inhibition of a range of potentially pathogenic microbial species (Haenen et al., 2013). Also, Succinivibrionaceae was enriched in all intestinal segments only in the RS based treatments, suggesting it's strong association with the beneficial effects of RS. Generally, inclusion of RS increased the abundance of bacteria associated with RS fermentation including members of Bifidobacteriaceae, *Ruminococcus* and *Bifidobacterium*. However, we also observed large increases in bacteria not directly involved in RS fermentation, which may help in modulating the gastrointestinal environment through attenuation of inflammation or mucosa regeneration. Nevertheless, it is worth noting that, compared to CG and RST, there were no significant taxa associated with the RSP group across the intestinal segments suggesting less efficacy of the use of RS as a protective measure in modulating gut microbiota at lower taxonomical levels in our model of colitis. This was also supported by observations made at microbiota functional level as very few microbial functional pathways or activities were enriched in this group compared to other groups, again indicating less effects of the use of RS as a protective measure. The reasons why the protective measure of RS was not evident based on some parameters measured are not clear, however, it is possible that the microbiota of the healthy pigs were able to adjust to the RS supplementation

within a short period of time, and therefore established a stable and 'normal' ecosystem before induction of colitis, which was disrupted by the introduction of CG. Therefore, protective use of RS might not be very effective in influencing microbiota dysbiosis at lower taxonomical levels and, hence, the microbiota functioal activities in experimental colitis.

In conclusion, the results have shown that MSPrebiotic have both preventive and therapeutic potential in experimental colitis. In this context, inclusion of RS significantly inhibited histological damage, attenuated expression of pro inflammatory cytokines, as well as modulation of intestinal bacterial dysbiosis and gene functional content following CG challenge. However, based on some parameters, the protective effects were less effective compared to the therapeutic effects, an observation that may warrant further investigations.

BRIDGE TO CHAPTER 6

So far, in chapters 3, 4, and 5, we have demonstrated that CG-induced colitis does cause bacterial dysbiosis, and that resistant starch exhibited both therapeutic and protective effects in a pig model of experimental colitis. This summarizes the first two phases of the research described in this thesis in which a pig model of colitis was used. The next phase therefore describes studies in which a mice model of colitis was used. The main aim of the mice model was to investigate the role of antepartum use of antibiotics on the nature of offspring initial gut microbiota colonization, and whether this would predispose them to DSS-induced colitis later in life. However, the first step was to first investigate whether DSS alone causes bacterial dysbiosis. Therefore, Chapter 6 describes an experiment in which the role of DSS-induced colitis in bacterial dysbiosis was investigated in a mouse model of experimental colitis.

Rationale: DSS treatment has been associated with changes in the composition of gut microbiota, whose dynamics shift towards an unhealthy state (Nagalingam et al., 2011; Berry et al., 2012; Samanta et al., 2012; De Fazio et al., 2014; Mar et al., 2014; Schwab et al., 2014; Hakansson et al., 2015). However, most of the previous studies used 16S rRNA gene fingerprinting methods, such as terminal-restriction fragment-length polymorphism (T-RFLP), fluorescent in-situ hybridization (FISH) (Heimesaat et al., 2007; Nagalingam et al., 2011; Samanta et al., 2012; Smith et al., 2012; Brinkman et al., 2013; De Fazio et al., 2014; Hakansson et al., 2015), or sequencing of few clones per animal (Lupp et al., 2007; Nagalingam et al., 2011), which have either low accuracy and precision or are limited in data mining. Therefore, further studies on the structural and functional alterations of the intestinal microbiota in the DSS model are required especially with the use of high-throughput sequencing technologies.

CHAPTER 6

MANUSCRIPT IV

Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice.

A version of the material presented in chapter six of this thesis has been published in Journal of Basic Microbiology. The authors are: Peris Mumbi Munyaka, Mohammad Fazle Rabbi, Ehsan Khafipour, & Jean-Eric Ghia. J Basic Microbiol. 2016. 56:1-13.

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6.1. ABSTRACT

The most widely used and characterized experimental model of ulcerative colitis (UC) is the epithelial erosion, dextran sulfate sodium (DSS)-induced colitis, which is developed by administration of DSS in drinking water. We investigated fecal and colonic mucosa microbial composition and functional changes in mice treated with DSS. C57Bl/6 mice received 5% DSS in drinking water for 5 days. Inflammation was evaluated clinically and by analysis of colonic tissue cytokine levels and C-reactive protein (CRP) in the serum. Colonic mucosa and fecal samples were used for DNA extraction and the V4 region of bacterial 16S rRNA gene was subjected to MiSeq Illumina sequencing. Alpha- and beta-diversities, and compositional differences at phylum and genus levels were determined, and bacterial functional pathways were predicted. DSS increased disease severity, serum CRP and cytokines IL-1β and IL-6, but decreased bacterial species richness, and shifted bacterial community composition. *Bacteroides*, *Turicibacter*, *Escherichia*, *Clostridium*, Enterobacteriaceae, Clostridiaceae, Bacteroidaceae, Bacteroidales, among other taxa were associated with DSS treatment in fecal and colonic samples. Also, DSS altered microbial functional pathways in both colonic mucosa and fecal samples. The development of colitis in DSS model was accompanied with reduced microbial diversity and dysbiosis of gut microbiota at lower taxonomical levels.

Keywords. dextran sulfate sodium; colitis; mice; microbiota; dysbiosis

6.2. INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) represent the two major forms of inflammatory bowel disease (IBD) that are characterized by alternating phases of clinical relapse and remission (Strober et al., 2007; Cosnes et al., 2011; Manichanh et al., 2012; Haenen et al., 2013). The etiology of both CD and UC is unclear; however, accumulating data suggest that a

genetic predisposition or a combination of genetic susceptibility factors (e.g. CARD15/NOD2, JAK-2/STAT3) (Basso et al., 2014), defective mucosal barrier (Jager et al., 2013; Klag et al., 2013), altered innate and adaptive immune responses and their interactions with commensal gut microbiota in the enteric environment (Hooper et al., 2012; Basso et al., 2014; Wallace et al., 2014), and different environmental factors contributes to the initiation and the recurrence of these diseases.

Much of the recent progress in the understanding of immunity has been achieved using experimental animal models of intestinal inflammation (Wirtz and Neurath, 2000; Hibi et al., 2002; Wirtz and Neurath, 2007). Although these models do not represent the complexity of human disease and do not replace studies with patient samples, they are valuable tools for studying many important disease aspects that are difficult to address in humans and they, therefore, provide a platform through which some of the complex mechanisms can be systematically investigated (Elson et al., 1998). Most of these models require exogenous manipulation based either on chemical induction, immune cell transfer or gene targeting (Wirtz and Neurath, 2000; Hibi et al., 2002; Pizarro et al., 2003; Wirtz and Neurath, 2007), and only a few models occur spontaneously without any exogenous manipulation (Sundberg et al., 1994; Elson et al., 1995a; Pizarro et al., 2003). Chemically induced models of intestinal inflammation are among the most commonly used animal models of IBD as the onset of inflammation is immediate and the procedure is relatively straightforward. Even though they have limitations like all other models, they present some important immunological and histopathological aspects of IBD in humans (Solomon et al., 2010). The most widely used and characterized experimental model of erosion and inflammation related to UC in mice is the dextran sulphate sodium (DSS)-induced colitis, which is developed by DSS administration either in the drinking water (Solomon

et al., 2010; Chassaing et al., 2014a) or via intragastric catheter (Lee et al., 2009a; Kim et al., 2010). The DSS induces a reproducible acute colitis (Solomon et al., 2010); however, there is some controversy regarding the type of inflammation that is induced. For some, the DSS model is recognized as a true model of colitis, while for others the model represents a model of epithelial erosion, but it is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier (Kitajima et al., 2000; Wirtz and Neurath, 2007; Solomon et al., 2010). However, the model induces an acute colitis that is characterized by ulceration and infiltration, and also reflects many of the clinical features of IBD (Kitajima et al., 2000; Wirtz and Neurath, 2007; Melgar et al., 2008; Yan et al., 2009). For example, among other features, changing the DSS concentration or administration cycles can easily induce acute, chronic, and relapsing colitis. Our lab has extensive experience with this model (Ghia et al., 2006; Ghia et al., 2007a; Ghia et al., 2007b; Ghia et al., 2009; Rabbi et al., 2014).

The focus of most DSS studies has been on the dynamic and profile of mucosal response in relation to DSS treatment and its similarity to that observed in UC patients (Kitajima et al., 2000; Wirtz and Neurath, 2007; Melgar et al., 2008; Yan et al., 2009). These studies provide compelling evidence for changes in the gut mucosal immune response in mice treated with DSS compared to controls. In addition, other studies have investigated the role of defensins; antimicrobial, and anti-inflammatory components that are produced by paneth cells in the mucosa (Shi, 2007). In this regard, mice with impaired expression of α-defensins due to destruction of the epithelium, and hence, the paneth cells have been shown to be more susceptible to DSS-induced colitis, characterized by increased production of pro-inflammatory cytokines (Shi, 2007). On the other hand, notable shifts in gut microbiota composition (dysbiosis) have been highlighted in IBD patients at different stages of the disease (Tamboli et

al., 2004; Erickson et al., 2012; Morgan et al., 2012; Wills et al., 2014). However, given the widespread use of DSS, not many studies have so far investigated the compositional shifts in gut microbiota and changes in their metabolic capacity in relation to DSS treatment in mice or rat models (Nagalingam et al., 2011; Berry et al., 2012; Samanta et al., 2012; Smith et al., 2012; Brinkman et al., 2013; De Fazio et al., 2014; Liang et al., 2014; Mar et al., 2014; Schwab et al., 2014; Hakansson et al., 2015). In this context, DSS treatment has been associated with changes in the composition of gut microbiota, whose dynamics shift towards an unhealthy state (Nagalingam et al., 2011; Berry et al., 2012; Samanta et al., 2012; De Fazio et al., 2014; Mar et al., 2014; Schwab et al., 2014; Hakansson et al., 2015). The nature of gut microbiota was also reported to influence sensitivity to acute DSS-induced colitis independently of host genotype (Brinkman et al., 2013). In addition, interdependence of the mucosa-associated bacteria and chronic inflammation has also been reported (Smith et al., 2012). Therefore, whether DSS-induced colitis causes dysbiosis or the nature of existing microbial colonization affects susceptibility to colitis remains a topic of discussion worth more explorations. It is however important to note that, most of the previous studies used 16S rRNA gene fingerprinting methods, such as terminal-restriction fragment-length polymorphism (T-RFLP), fluorescent in-situ hybridization (FISH) (Heimesaat et al., 2007; Nagalingam et al., 2011; Samanta et al., 2012; Smith et al., 2012; Brinkman et al., 2013; De Fazio et al., 2014; Hakansson et al., 2015), or sequencing of few clones per animal (Lupp et al., 2007; Nagalingam et al., 2011), which had either low accuracy and precision or were limited in data mining. Furthermore, few studies investigated shifts in microbiota's functional potential or activity using metagenomic or metranscriptomic approaches (Berry et al., 2012; Schwab et al., 2014). Therefore, a detailed and clear understanding of the structural and functional alterations of the intestinal microbiota in the

DSS model is still required especially with the use of the high-throughput sequencing technologies. The aim of the present study was to utilize Illumina sequencing of the 16S rRNA gene and inferred metagenomics by open source software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) to investigate differences in microbiome composition and function in the fecal and colonic mucosa of mice treated with 5% DSS for 5 days. We demonstrated that oral administration of DSS in C57Bl/6 mice significantly decreased bacterial species richness and shifted bacterial community composition. Specific taxa were associated with DSS treatment that may be important as intervention targets against the disease. In addition, DSS largely altered microbial activities and their functional pathways.

6.3. MATERIAL AND METHODS

6.3.1. Animals

Male C57BL/6 (7–9 weeks old) mice were purchased from Charles River (Senneville, QC, Canada) and maintained in the animal care facility at the Faculty of Health Sciences, University of Manitoba. All experiments were approved by the University of Manitoba Animal Ethics Committee (10-073) and conducted according to the Canadian guidelines for animal research (CCAC).

6.3.2. DSS colitis

DSS (molecular weight; MW 40 kDa: MP Biomedicals, Soho, OH, USA) was added to the drinking water at a final concentration of 5% (wt/vol) and administered for 5 days. Controls were time-matched and consisted of mice that received normal drinking water only. Five and four mice were included in the DSS and control groups, respectively.

6.3.3. Disease activity index

Disease activity index; DIA is the combined score for weight loss, stool consistency and

bleeding, and the scores have historically correlated well with the pathological findings in DSS-induced model of IBD (Cooper et al., 1993). The 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, diarrhoea; and bleeding: 0, no blood; 2, presence of blood; and 4, gross blood. Blood was assessed using the Hemoccult II test (Beckman coulter, Oakville, ON, Canada). The DAI scoring was performed from d 0 to d 5 over the period of DSS treatment.

6.3.4. Macroscopic scores

Mice were euthanized at the end of d 5 of DSS administration, the abdominal cavity was opened, and the colon was located, isolated and opened longitudinally. Macroscopic damage was next evaluated on the full colon section, and macroscopic scores were assessed according to the rectal bleeding, rectal prolapse, diarrhea and colonic bleeding using a previously established scoring system (Cooper et al., 1993).

6.3.5. Characterization of inflammation

Colon tissue samples were collected 5 days post-DSS activation, and blood was collected by intracardiac puncture under isoflurane (Abbot, Mississaugua, ON, Canada) anaesthesia. Serum C-reactive protein (CRP); a marker for systemic inflammation was determined using an enzyme-linked immunosorbent assay (ELISA) commercial kit (R&D Systems, Minneapolis, MN, USA). Colonic samples were homogenized in 700 microliter of Tris-HCl buffer containing protease inhibitors (Sigma, Mississauga, ON, Canada) and centrifuged for 12 min at 10, 000 x g, and the supernatant was frozen at 80°C until assay. Cytokine levels (IL-6, IL-1β) were determined using an ELISA commercial kit (R&D Systems, Minneapolis, MN, USA).

6.3.6. DNA extraction and quality control

Approximately 200 mg of each fecal sample was used for DNA extraction using ZR fecal

DNA extraction kit (Zymo Research Corp., Irvine, CA, USA). For colonic samples, the tissue was cut open and approximately 50 mg of mucosa scrapings were taken. DNA extraction from colonic mucosa samples was done using ZR Tissue and Insect DNA kit (Zymo Research Corp., Irvine, CA, USA). Both DNA extraction kits included a bead-beating step for the mechanical lysis of microbial cells. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA samples were normalized to 20 ng/µl, and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. (Khafipour et al., 2009). Amplicons were verified by agarose gel electrophoresis.

6.3.7. Library construction and Illumina sequencing

The V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al., 2012) as described previously (Derakhshani et al., 2015). In brief, a 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, 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; finalized by 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, quantified by Picogreen dsDNA

(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 Qubit 2.0 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´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3´) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, San Diego, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada. The sequencing data are uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and are accessible through accession number SRR2830596.

6.3.8. Bioinformatic analyses

The PANDAseq assembler (Masella et al., 2012) was used to merge overlapping paired-end Illumina fastq files. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010b). 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. Taxonomies were assigned to the representative sequence of each OTU using 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., 2010a). Phylogenetic tree was built with FastTree 2.1.3. (Price et al., 2010) for further comparisons between microbial communities.

6.3.9. Alpha- and beta-diversity analyses

Within community diversity (α-diversity) was calculated by different indices of species richness and evenness such as Observed number of species, Chao1, ACE (abundance-based coverage estimators), Shannon, Simpson, InvSimpson, and Fisher using the open source R software. An even depth of 21,000 and 14,500 sequences per sample for fecal and colon mucosa samples, respectively, was used for the calculation of diversity indices. Beta-diversity was measured by calculating the weighted UniFrac distances using QIIME default scripts (Lozupone and Knight, 2005). Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using the open source R software (v. 3.1.0) and the *P* values were calculated using PERMANOVA analyses of Bray-Curtis distances (Anderson, 2005). Differences in alpha-diversity between DSS and control were determined using SAS (SAS 9.3, 2012). One colon sample in the control group was lost during DNA extraction and therefore only samples from three mice were included for all colon microbial analysis.

6.3.10. Partial least square discriminant analysis

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.02, Umetrics, Umea, Sweden) was performed on genus data to identify the effects of DSS. As described previously (Li et al., 2012) the PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the categories of a categorical variable on X. In this case,

X variables were bacterial taxa and Y was observations of different treatments compared together. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus, and genera with VIP < 0.5 were removed from the final model. R^2 estimates then were used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. Data was presented in loading scatter plots. The PLS-DA regression coefficients were used to identify taxa that were positively or negatively correlated with each treatment group.

6.3.11. Prediction of functional metagenome

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 (Langille et al., 2013). 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 and Beiko, 2010).

6.3.12. Statistical analysis

The UNIVARIATE procedure of SAS (SAS 9.3, 2012) was used to test the normality of residuals for α-diversity data. Data were used to assess the effect of treatment using MIXED procedure of SAS with treatment as the fixed and animal as the random factor (SAS 9.3, 2012). Phylum percentage data was also used to evaluate statistical differences between the DSS and control treatments. For the disease index activity, macroscopic scores, weight loss score and

inflammatory markers, statistical analysis was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison post hoc analysis, using prism (Prism 5, GraphPad, La jolla, CA, USA). The differences between groups were considered significant at $P < 0.05$.

6.4. RESULTS

6.4.1. Macroscopic score, disease activity index, C-reactive protein, and cytokines IL-1β and IL-6.

As shown in **Figure 6.1**(**a-f**), DSS increased disease activity index, weight loss score and macroscopic score ($P < 0.0001$). In this context, weight loss, and the presence of blood in the feces were increased while stool consistency was decreased. Also, the level of CRP, a marker of systemic inflammation, and inflammatory cytokines IL-1β and IL-6 increased in the DSS group ($P < .0001$). These results confirmed the presence of colitis in our DSS model.

6.4.2. Alpha-diversity of the fecal and colonic mucosa-associated microbiota (MAM).

Based on the different diversity indices used, DSS reduced bacterial species richness and evenness in the fecal samples compared to the control samples (**Figure 6.2 a**). Bacterial α-diversity in the colonic MAM was not significantly different between the control mice and the DSS mice samples, suggesting that both sample groups have similar bacterial species richness and evenness. (**Figure 6.2 b**). The significance levels were determined using SAS and the *P* values are shown on top of each bar.

6.4.3. Beta-diversity in fecal and colon samples.

As shown by the PCoA of weighted UniFrac distances, fecal samples clustered separately according to treatment group, suggesting that DSS and control samples were composed of distinct bacterial communities ($P = 0.007$) (**Figure 6.3 a**). Although clustered separately for a large portion of communities, the colonic MAM still had an overlap of some bacterial

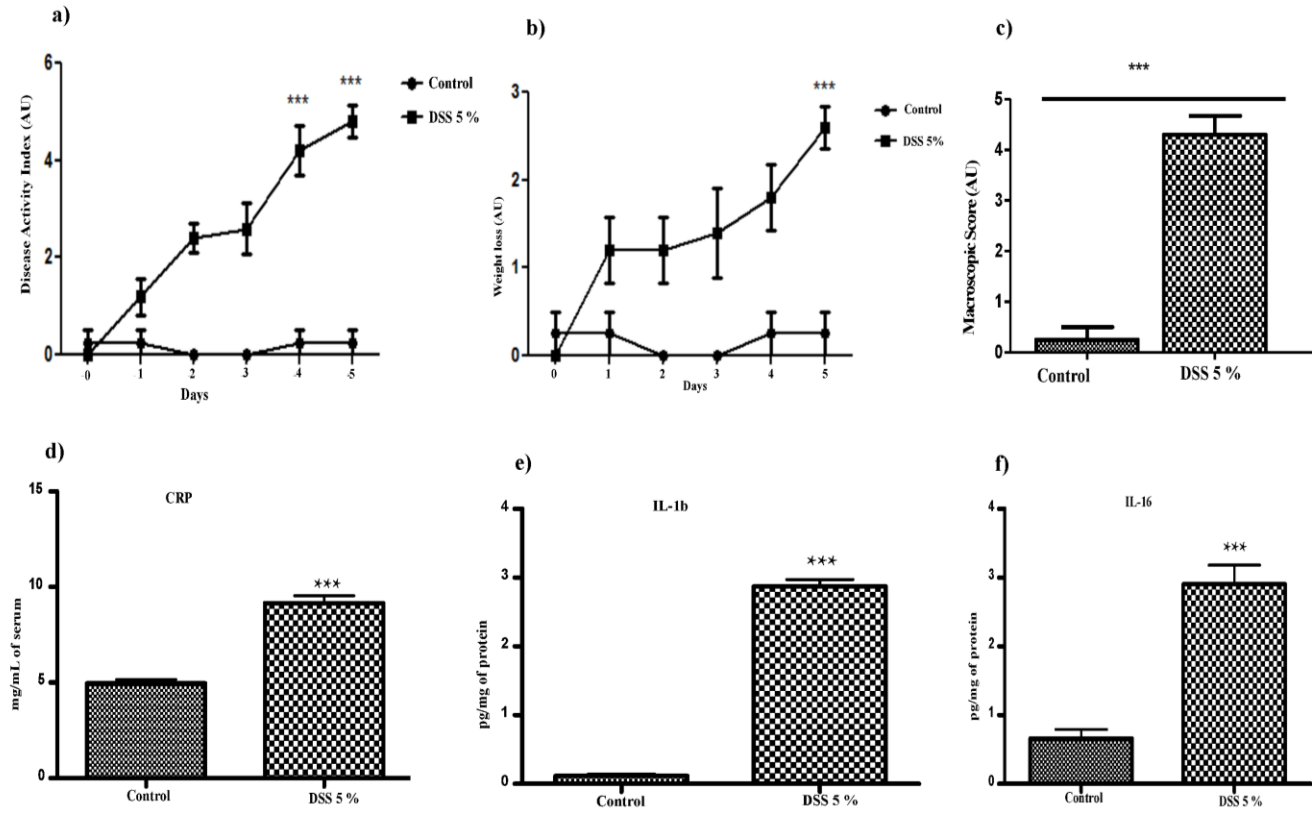

Figure 6.1. Dextran sulfate sodium (DSS)-induced colonic inflammation. Five percent DSS treatment for 5 days induced a significant increase in the; **a)** disease activity index, **b)** weight loss score, **c)** macroscopic scores, **d)** C-reactive protein (CRP), **e)** IL-1β, and **f)** IL-6. Values are shown as the mean ± SEM. AU, arbitrary units.

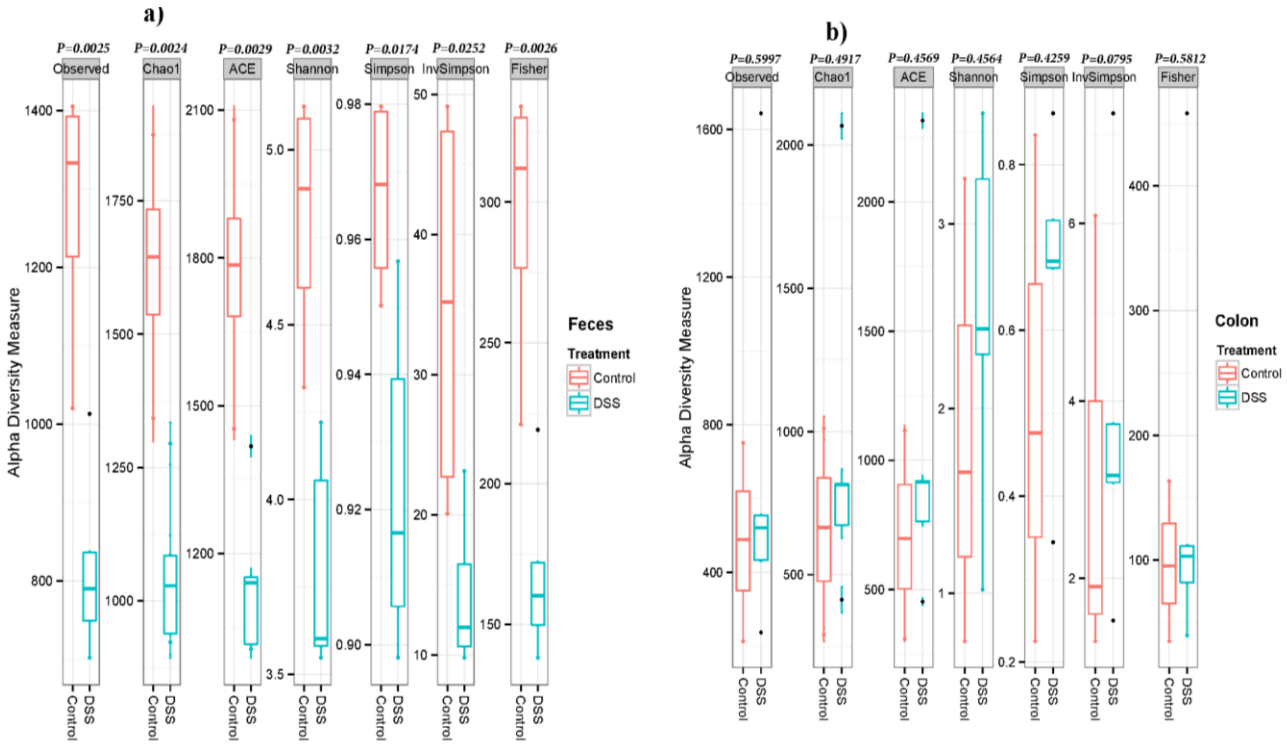

Figure 6.2. Alpha-diversity analysis, a measure of species richness and evenness based on different diversity indices, from mice exposed to dextran sulfate sodium-induced colitis and control group studied at fecal and colon levels. **a)** Fecal colitic samples had a lower bacterial diversity compared to non-colitic samples, suggesting that DSS reduced bacterial species richness. **b)** Colonic mucosal bacterial diversity is not different between colitic and non-colitic samples, suggesting that both sample groups have a similar bacterial species richness and evenness. The *P* values shown on top of each bar were calculated using SAS. DSS = dextran sulphate sodium.

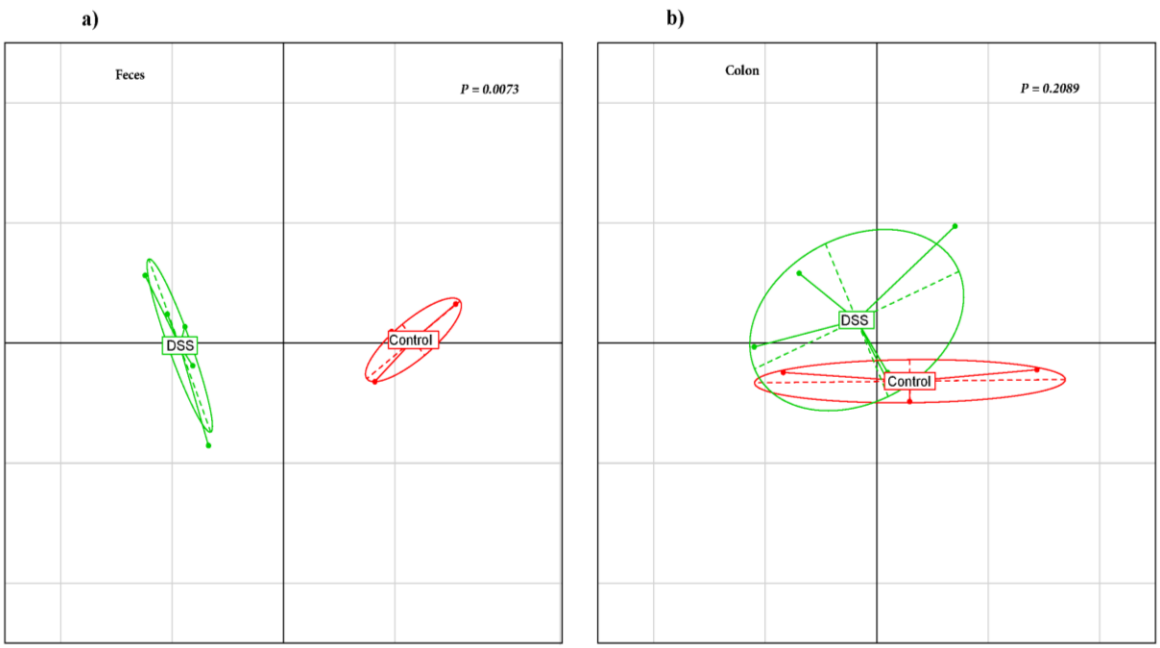

Figure 6.3. Principle coordinate analysis (PCoA) of weighted UniFrac distances, a measure of β-diversity of bacterial community composition. **a)** Fecal samples clustered separately according to treatment status of the mice, suggesting that DSS and control mice samples are composed of distinct bacterial communities; **b)** Colon mucosa samples have shared bacterial communities. The *P* values were determined using PERMANOVA. DSS = dextran sulphate sodium.

communities, suggesting that DSS and control mice colon mucosa samples had shared bacterial communities (P = 0.2) (**Figure 6.3 b**).

6.4.4. Microbiota composition at phylum and lower taxonomic levels in the fecal samples.

A total of 259,126 sequences were generated after quality filtering steps with an average of 28,791 high-quality sequences per sample, which resulted in identification of 11 phyla and 86 taxa. While most taxa were classified at the genus (g.) level, some were only classified at the phylum (p.), class (c.), order (o.), or family (f.). Of the 11 phyla, six were abundant (≥ 1% of community), including Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres, Tenericutes, and Verrucomicrobia. The low-abundance phyla (< 1% of community), included Actinobacteria, Cyanobacteria, Fibrobacteres, Spirochaetes, and TM7. Among the abundant phyla, DSS treatment reduced Bacteroidetes (P = 0.01) while Proteobacteria were increased (P = 0.004) compared to the control. Results of the relative abundances of various phyla are summarized in **Figure 6.4 a**.

Of the 86 taxa, 56 had abundance greater than or equal to 0.01% of community, whilst 30 were below 0.01%. Bacterial taxa with relative abundance of ≥ 0.01% of community were analysed using PLS-DA to identify bacteria that were most characteristic of the DSS or control treatments. As shown in **Figure 6.5 a,** *Bacteroides ovatus, Clostridium perfrigens, Helicobacter hepaticus,* and *Parabacteroides distasonis*; g. *Desulfovibrio, Escherichia, Helicobacter, Clostridium, Turicibacter, Parabacteroides, Bacilli, Allobaculum, Coprobacillus, Bacteroides, rc4-4;*and unclassified members of f. Enterobacteriaceae, Clostridiaceae, Bacteroidaceae; o. RF39, RF32, Bacteroidales, and Erysipelotrichales were positively correlated with the DSS group but negatively correlated with the control group. In addition, g. *Dessulfovibrio, Oscillospira, Odoribacter, Coprococcus, Dehalobacterium, Adlercreutzia, Lactobacillus,*

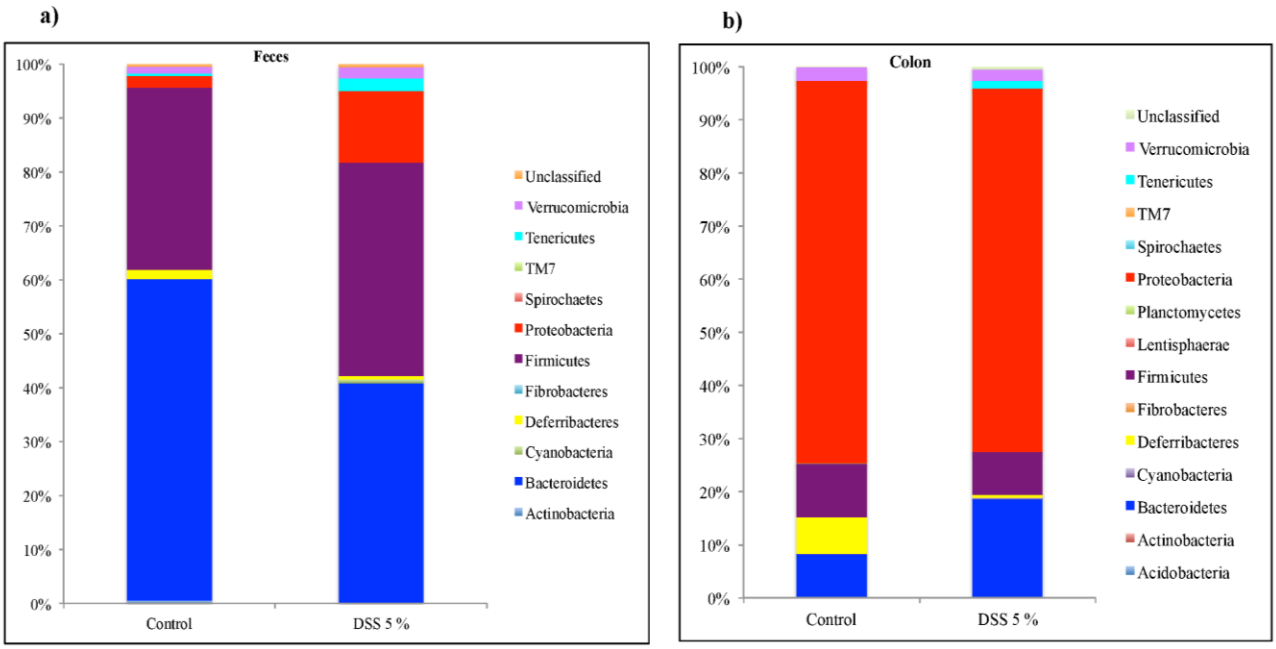

Figure 6.4 Percentage of relative abundances of bacterial phyla for: **a)** fecal samples and, **b)** colon mucosa samples. The DSS significantly decreased Bacteroidetes and increased Proteobacteria in fecal samples but no significant difference was observed in the colonic mucosa samples. DSS = dextran sulphate sodium.

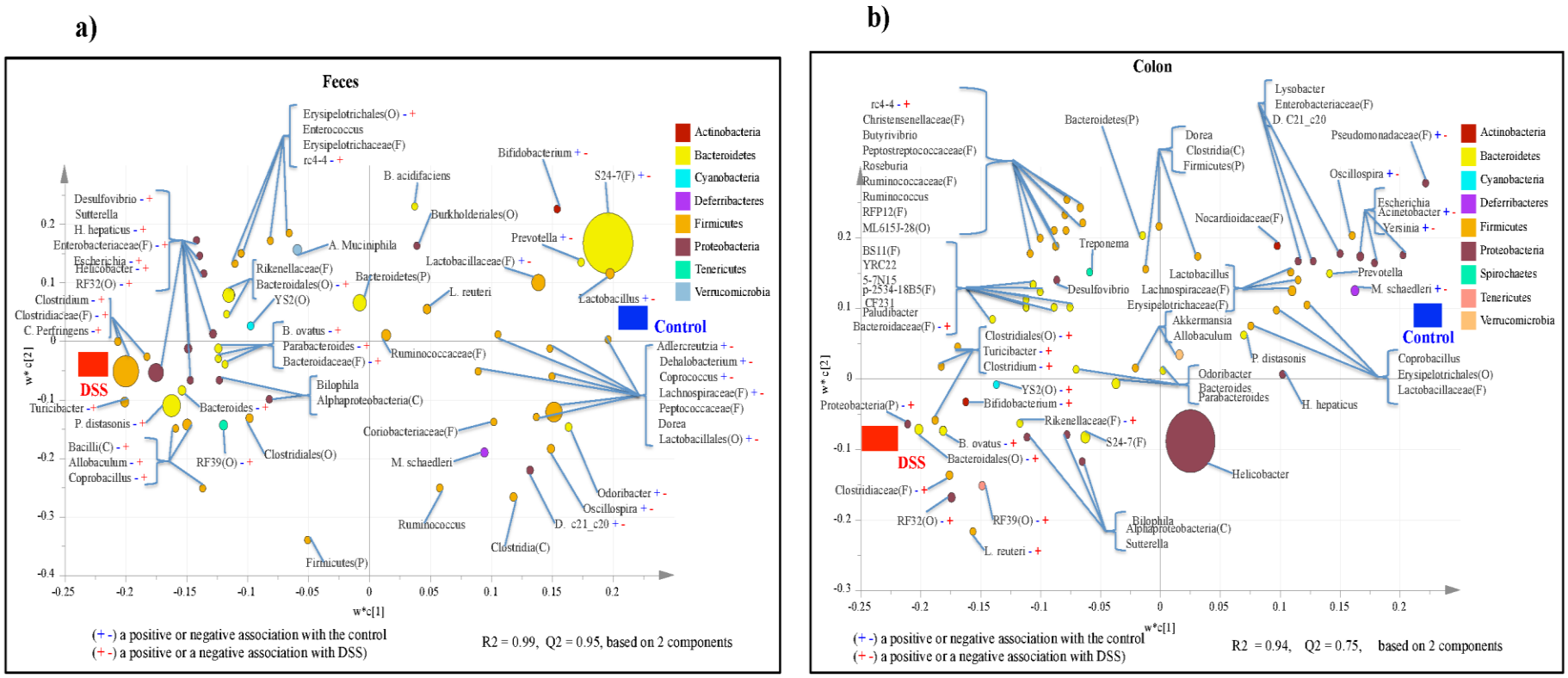

Figure 6.5. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the control or DSS treatments in the mice's **a)** fecal samples and **b)** colon mucosal samples**.** All taxa are coloured based on the phyla to which they belong. While majority of OTUs were classified at the genus (g.) level, some could only be affiliated to phylum (P), class (C), order (O), or family (F) levels. - or + signs are indicative of significant negative or positive association between each taxon and the associated treatment. DSS = dextran sulphate sodium.

Prevotella, Bifidobacterium; unclassified members of f. Lachnospiraceae, Lactobacillaceae, S24-7; and o. Lactobacillales, were positively associated with the control group but negatively associated with the DSS group. **Appendix 1 Supplementary Table 6.1** shows a summary of mean abundances of all the taxa in fecal samples.

6.4.5. Microbiota composition at phylum and lower taxonomic levels in colonic samples.

A total of 199,057 sequences were generated after quality filtering steps with an average of 24,882 high-quality sequences per sample, which resulted in identification of 14 phyla and 151 taxa. Of the 14 phyla, five were abundant (≥ 1% of community), including Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres, and Verrucomicrobia. The low-abundance phyla (< 1% of comunity), included Acidobacteria, Actinobacteria, Cyanobacteria, Fibrobacteres, Lentisphaerae, Planctomycetes, Spirochaetes, TM7, and Tenericutes. No significant difference was observed among the abundant phyla between DSS and control mice ($P > 0.05$). Results of the relative abundances of various phyla are summarized in **Figure 6.4 b**.

Of the 151 taxa, 68 had abundances greater than or equal to 0.01% of community, whereas 83 were below 0.01% of community. Bacterial taxa with relative abundance of ≥ 0.01% of community were analysed using PLS-DA to identify bacteria that were most characteristic of DSS or control treatments. As shown in **Figure 6.5 b**, *Lactobacillus reuteri, Bacteroides ovatus*; g. rc4-4, *Rikenellaceae, Bifidobacterium, YS2, Clostridium*; f. Bacteroidaceae, Clostridiaceae; o. RF32, RF39, Bacteroidales, Clostridales; and p. Proteobacteria, were positively associated with DSS, but negatively associated with the control. Also, *Mucispirillum schaedleri*; g. *Oscillospira, Acinetobacter, Yersinia*; and f. Pseudomonadaceae, were positively associated with the control but negatively associated with the DSS. **Appendix 1 Supplementary Table 6.2** shows a summary of mean abundances of all the taxa in colonic mucosa samples

6.4.6. Functional metagenome of colonic MAM and fecal microbiome

The analysis provided insights into functional shifts in the murine intestinal microbiome. A number of metabolic pathways were highly enriched in the colonic MAM and fecal microbiome in the DSS-treated mice compared to the control. In this context, the fecal microbiome of DSS-treated mice was associated with but not limited to, increased fatty acid biosynthesis, lysine degradation, pyruvate metabolism, propanoate metabolism, replication, recombination and repair proteins, and transcription machinery (**Figure 6.6 a**). In addition, the colonic MAM of the DSS-treated mice was associated with increased DNA repair and recombination proteins, peptidoglycan biosynthesis, DNA replication proteins, alanine, aspartate and glutamate metabolism, ribosome biogenesis, and several other pathways (**Figure 6.6 b**).

6.5. DISCUSSION

The indigenous gut microbiota are thought to play a key role in the pathogenesis of IBD. Part of the evidence for the involvement of intestinal bacteria in IBD comes from studies with murine models of the disease supporting the hypothesis that a deregulated immune response against components of the intestinal microbiota is critically involved in the pathophysiology of IBD (Wirtz and Neurath, 2007). This is supported by the fact that antibiotic administration reduces the severity of disease in these models and rederivation of the mice to the germfree state prevents initiation of disease (Rath et al., 2001; Sartor, 2008). Conversely, some germfree mice have been shown to have a high mortality rate when given DSS in drinking water as compared to conventional mice (Rath et al., 2001), suggesting that different subsets of normal microbiota may play certain roles in terms of the initiation, development or attenuation of disease.

We evaluated the fecal and colonic mucosal bacterial community dynamics, their functional alterations, as well as selected host variables in DSS-treated mice compared to control

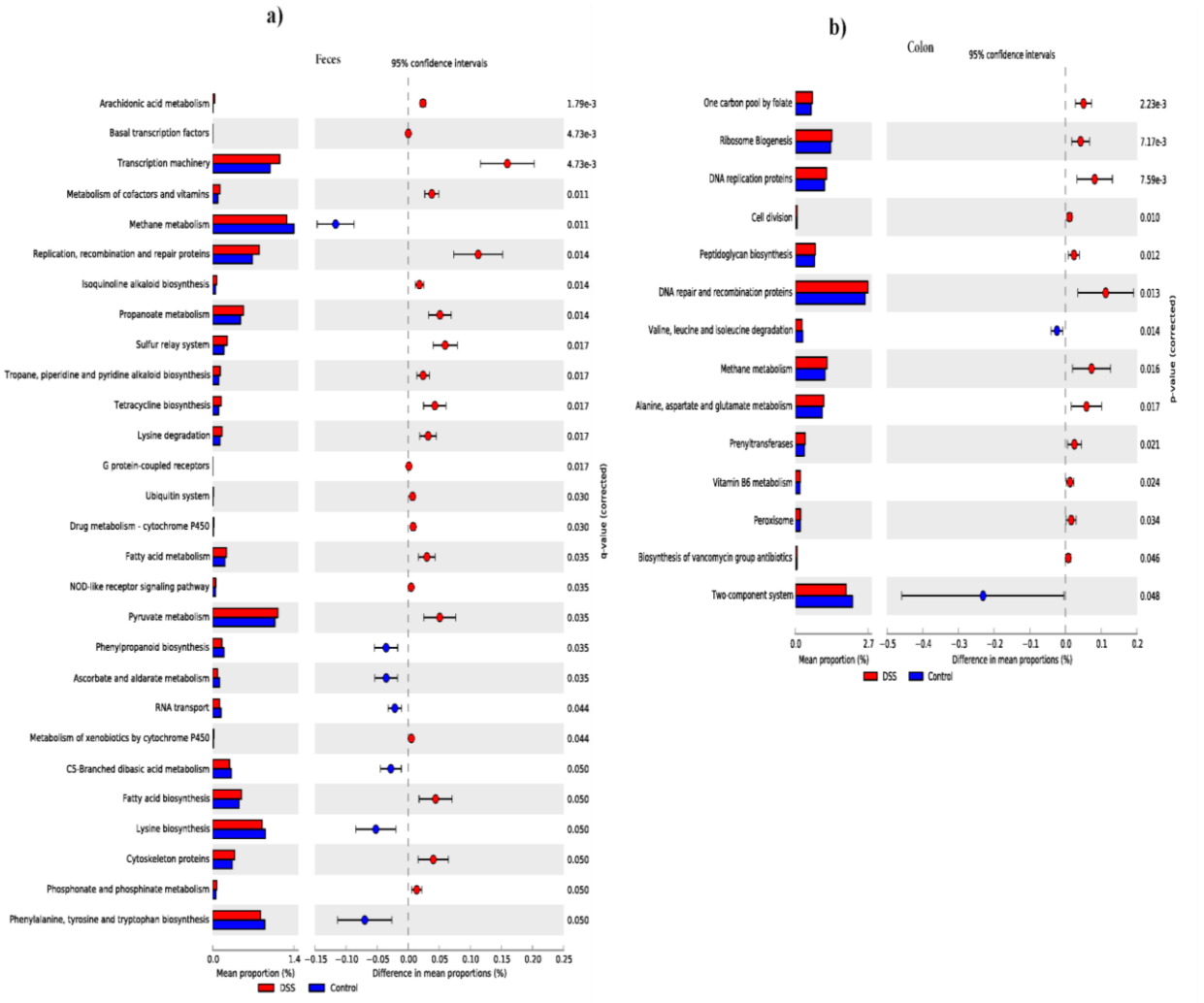

Figure 6.6 a) Subsystems and pathways enriched or decreased within mice fecal samples isolated from colitic and non-colitic groups. Corrected *P* values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the DSS or (control) fecal samples have a positive or (negative) difference between mean proportions and are indicated by red or (blue) coloring, respectively. **b)** Subsystems and pathways enriched or decreased within mice colon samples isolated from the colitic and non-colitic groups. Subsystems or pathways overrepresented in the DSS or (control) mice colon samples have a positive or (negative) difference between mean proportions and are indicated by red or (blue) coloring respectively.

to elucidate which bacteria are most characteristic of DSS treatment, and which bacterial functional activities or metabolic pathways are affected. From the macroscopic assessment of disease severity in the colon, it was apparent that mice in the DSS-induced colitis treatment exhibited overt features of colitis compared to the healthy control group, which is in agreement with previous results where a DSS model was used (Samanta et al., 2012; Rabbi et al., 2014). Also, analysis of the inflammatory markers including serum CRP level, a marker of systemic inflammation, showed significant differences between the two groups as DSS up-regulated this marker. In line with our findings, previous studies reported an extensive accumulation of neutrophils and an increase in the serum CRP level and colonic myeloperoxidase in DSS-colitis (Ghia et al., 2008; Rabbi et al., 2014). We next considered other potential pro-inflammatory mediators that also play central role in the pathogenesis of IBD including UC and CD. The DSS significantly up-regulated inflammatory cytokines IL-1β and IL-6, a condition that has also been reported previously in colon of mice as a result of DSS-induced colitis (Yan et al., 2009; Rabbi et al., 2014).

Decreased richness or diversity of bacterial species has been reported widely in both fecal and intestinal MAM samples of human patients with UC and CD, in intestinal MAM of dogs with IBD, and in fecal samples of rats with DSS-induced colitis (Manichanh et al., 2006; Andoh et al., 2007; Frank et al., 2007; Xenoulis et al., 2008; Samanta et al., 2012; Wills et al., 2014). This is consistent with our observation in fecal samples in which diversity analysis of bacterial species richness of the control and DSS-treated mice showed significant differences between these two groups. Also, our results on shifts in bacterial community composition are in agreement with Berry et al. (Berry et al., 2012), who reported differences in bacterial community composition in a pool of colonic and cecal contents of wild type mice, which was mostly a result

of DSS treatment, although the authors did not find significant differences in α-diversity analysis. Furthermore, a loss of species diversity and a distinctive microbial community composition in intestinal MAM of DSS-treated mice (Nagalingam et al., 2011) and in both fecal and intestinal MAM samples of humans with IBD (Willing et al., 2010) have also been reported. Interestingly, in our study, colonic MAM profiles from DSS-treated mice were not significantly different from bacterial profiles of the control mice in terms of bacterial richness and community composition. This may suggest possible differences in the effect of DSS on the richness and composition of microbiota at different anatomical sites or between mucosal and fecal samples. However, this was only limited to diversity, community composition and changes at phylum level as bacterial differences were observed at lower taxonomical levels in both fecal and colon samples. This indicates that profiling of fecal and colonic bacteria by Illumina sequencing may be sensitive for investigating changes associated with colitis and may reveal bacterial shifts at lower taxonomic levels that could otherwise be missed. In this regard, it is worth noting that more taxa were significantly associated with the DSS treatment in fecal samples compared to the colonic samples; however, several taxa including: *Bacteroides ovatus,* g. *Clostridium*, *rc4-4*; f. Clostridiaceae, Bacteroidaceae; and o. Bacteroidales, RF39, and RF32 were positively associated with the DSS treatment in both fecal and colonic mucosa samples. Some of these taxa have been reported to increase as a result of DSS (Berry et al., 2012), and although they may have different roles, their association with DSS in both colonic mucosa and in feces may indicate that they may play significant roles in the potentiation of the abnormal inflammatory response seen in DSS-treated animals, and could therefore be important as intervention targets against the disease. Lending support to these results, Berry et al. (Berry et al., 2012) also found that the overall abundance of the dominant phyla Firmicutes and Bacteroidetes were not affected by DSS

treatment in mice, but taxa within the two phyla showed clear changes in abundance with respect to DSS treatment.

Members of the Bacteroidetes, such as o. Bacteroidales and f. Bacteroidaceae, have been identified as possible indicators of disease onset in the mouse model of colitis (Schwab et al., 2014), and were also previously shown to be capable of inducing colitis in antibiotic-pretreated mice (Bloom et al., 2011). However, data on members of p. Bacteroidetes are more ambiguous, and thus, inconsistent findings have been reported for their presence in IBD compared to healthy controls (Swidsinski et al., 2002; Ott et al., 2004; Bibiloni et al., 2006; Gophna et al., 2006; Sepehri et al., 2007; Andoh et al., 2011). Moreover, although still under investigations, some bacteria that were characteristic of DSS in the fecal samples, such as *Helicobacter hepaticus* and the f. Enterobacteriaceae, specifically g. *Escherichia*, have been implicated in IBD patients and in animal models of IBD (Morgan et al., 2012; Schwab et al., 2014; Wallace et al., 2014; Wills et al., 2014). Similar findings for increased levels of Enterobacteriaceae have also been reported in murine colitis, and after antibiotic treatment or infection by enteric pathogens (Lupp et al., 2007; Hill et al., 2010; Stecher et al., 2010). Therefore, a variety of conditions seem to drive the increase of Enterobacteriaceae, suggesting that the group may be a general indicator of a disrupted intestinal microbiota, but not necessarily a trigger of colitis (Bloom et al., 2011). However, there is strong data in support of the association of Enterobacteriaceae with IBD, especially CD. Generally, despite these differences in relative abundances of specific phylotypes, there appear to be a consensus in overall decrease in biodiversity in IBD patients and in murine models of colitis (Ott et al., 2004; Manichanh et al., 2006; Nagalingam et al., 2011; Berry et al., 2012).

The mechanisms by which DSS induced stronger bacterial shifts in the feces than in the colon mucosa are not fully understood. A similar observation has also been reported in IBD subjects whereby, changes in microbiome composition were more associated with the sample origin (stool or biopsy), and minor changes were observed in biopsy samples as opposed to stool samples (Morgan et al., 2012). It is possible that because DSS-induced colitis interferes directly with the intestinal epithelium and its barrier function as well as causing crypt damage (Cooper et al., 1993; Ni et al., 1996; Yan et al., 2009; Klag et al., 2013), this may lead to sloughing of mucosal epithelium, thereby decreasing the number of bacteria adhering to the mucosa, and consequently increasing the abundance of bacteria in the fecal matter.

Studies applying metagenomics, metatranscriptomics or metaproteomics investigating functional capacities of gut microbiota in humans (Erickson et al., 2012; Morgan et al., 2012), and in mouse model of colitis (Berry et al., 2012), reported alterations in metabolic pathways that might affect host-microbiota interactions. These findings are in agreement with our results whereby, several alterations in microbial metabolic pathways in both the colon mucosa and fecal samples were observed. Given that the microbial analysis of diversity, community composition and also changes at phylum level for colonic samples did not show any significant difference between the DSS and control mice, the results suggest that analysis at microbial functional level might reveal important colitis-related changes in gut microbiome that are otherwise not noticeable at higher phylogenetic or community levels.

The microbial community shifts associated with IBD are still poorly understood, and intense investigation continues to determine whether these changes are responsible for disease etiology, or alternatively, an indirect consequence of IBD. However, we present a rigorous analysis of DSS-induced colitis, a commonly used animal model of UC that has analyzed

microbial shifts and functional alterations in colonic mucosa and fecal samples. While acute DSS colitis is different from UC, there are some similarities, such as shifts in microbiota composition, reduced species diversity and increment or decrease of specific groups of bacteria; therefore, the mechanistic outcome of this study might be relevant for research on human IBD. In this context, restoring microbial homeostasis by targeting colitis-associated taxa through specific microbiological interventions could form the basis for novel therapeutic strategies for IBD. Together, our data provide important insight into DSS-induced dysbiosis or perturbations in gut microbiota in the colonic and fecal samples, supporting the DSS model as a useful tool to examine the role of different bacteria in the pathogenesis of IBD.

BRIDGE TO CHAPTER 7

In chapter 6 we demonstrated that administration of 5 % DSS for five days induced bacterial dysbiosis in a mouse model of experimental colitis. In this case, normal mice or mice that were not exposed to antibiotics were used. Chapter 7 therefore describes an experiment in which the role of antepartum use of antibiotics in offspring bacterial dysbiosis and consequent susceptibility to DSS-induced colitis was investigated.

Rationale: The use of antibiotics may disrupt neonatal gut microbiota and have profound consequences for later health (Faa et al., 2013). In this context, antibiotic-mediated disturbance of the intestinal microbiota in very early life has been shown to increase the risk of late-onset sepsis in a mouse model (Deshmukh et al., 2014). In addition, various illnesses with onset in childhood such as asthma, allergies, type 1 diabetes, obesity and autism have been hypothesized to be associated with maternal exposure to antibiotics resulting in perturbations of the indigenous microbiota (Noverr and Huffnagle, 2005; Penders et al., 2007a; Murk et al., 2011). However, despite the high association between the impacts of perinatal or neonatal antibiotic use on the microbial colonization and future risk for allergic reactions and disease conditions, the effects of antepartum antibiotic use on the process of intestinal microbiota development and future susceptibility to ulcerative colitis remain elusive or poorly understood.

CHAPTER 7

MANUSCRIPT V

Antepartum antibiotic treatment increases offspring susceptibility to experimental colitis: a role of the gut microbiota

A version of the material presented in chapter seven of this thesis has been published in PLoS One. The authors are: Peris Mumbi Munyaka, N. Eissa, Charles Noah Bernstein, Ehsan Khafipour & Jean-Eric Ghia. PLoS One. 2015. 10(11):e0142536.

7.1. ABSTRACT

Postnatal maturation of the immune system is largely driven by exposure to microbes, and thus the nature of intestinal colonization may be associated with development of childhood diseases that may persist into adulthood. We investigated whether antepartum antibiotic (ATB) therapy can increase offspring susceptibility to experimental colitis through alteration of the gut microbiota. Pregnant C57Bl/6 mice were treated with cefazolin at 160 mg/kg body weight or with saline starting six days before due date. At 7 weeks, fecal samples were collected from male offspring after which they received 4 % dextran sulfate sodium (DSS) in drinking water for 5 days. Disease activity index, histology, colonic IL-6, IL-1β and serum C-reactive protein (CRP) were determined. The V3-V4 region of colonic and fecal bacterial 16S rRNA was sequenced. Alpha-, beta-diversity and differences at the phylum and genus levels were determined, while functional pathways of classified bacteria were predicted. ATB influenced fecal bacterial composition and hence bacterial functional pathways before induction of colitis. After induction of colitis, ATB increased onset of clinical disease, histologic score, and colonic IL-6. In addition, ATB decreased fecal microbial richness, changed fecal and colon microbial composition, which was accompanied by a modification of microbial functional pathways. Also, several taxa were associated with ATB at lower taxonomical levels. The results support the hypothesis that antepartum antibiotics modulate offspring intestinal bacterial colonization and increase susceptibility to develop colonic inflammation in a murine model of colitis, and may guide future interventions to restore physiologic intestinal colonization in offspring born by antibiotic-exposed mothers.

Key words: antibiotic, ulcerative colitis, microbiota, dysbiosis, inflammatory bowel disease, experimental colitis

7.2. INTRODUCTION

The use of antibiotics during pregnancy or around the time of delivery is a common practice in clinical settings in North America, more so due to the fear of newborn colonization with Group B streptococci (GBS) during passage through the birth canal or when the membranes rapture. The GBS is the leading cause of life-threatening neonatal bacterial infections in developed countries (2010). Efforts to prevent GBS infections in newborns (Verani et al., 2010) and to reduce the incidence of postpartum maternal infection after caesarean section (Committee opinion, 2010) have led to the use of pre-delivery antibiotics in a large number of women in labor, resulting in exposure of the unborn fetus to the antibiotics. Although pre-partum antibiotics are generally recommended for premature rupture of membranes or when vaginal colonization by group B streptococci is detected, antibiotics are also frequently used in other clinical situations in which a clear benefit has not been demonstrated (Flenady et al., 2013), and this has raised some concerns (Bedford Russell and Murch, 2006). Also, even though antibiotics are designed to target bacterial pathogens, they often indiscreetly halt commensal human microbiota, allowing pathogens and opportunistic members of the bacterial community to propagate (Gorkiewicz, 2009). Perinatal antibiotics may also influence the initial microbial colonization of the newborn intestine, which is essential for the normal host development (Sommer and Backhed, 2013). Since the early neonatal period represents the most important opportunity for microbiota-induced host-homeostasis (El Aidy et al., 2013). As such, intrapartum antibiotics have been associated with infant gut microbiota dysbiosis (Azad et al., 2015).

Antepartum antibiotics may affect the bacterial composition of the mother's birth canal and or skin, which will be transmitted to the babies during and following delivery (Palmer et al., 2007; Biasucci et al., 2010; Dominguez-Bello et al., 2010). In this context, although antepartum

antibiotics are short-term, they are given at a critical time when newborn acquisition of gut bacteria, which is also known to influence the initial immune system development, is just beginning. Thus, antepartum antibiotic exposure may have far-reaching implications on neonatal immune system maturation (Noverr and Huffnagle, 2005; Renz et al., 2012). The use of broad-spectrum antibiotics in the perinatal period has been shown to alter the expression of genes involved in gastrointestinal (GI) tract development, with major consequences on the architecture and functionality of the intestinal barrier (Westerbeek et al., 2006). Moreover, antibiotics given to pregnant mothers in the days before delivery have been shown to significantly alter the composition of the preterm newborn microbiota, reducing intestinal microbial diversity on the first stool samples (Collignon et al., 2010). It is therefore apparent that changes in the composition of the newborn indigenous microbiota may have the potential to influence childhood development and also their risk of disease (Blaser and Stanley, 2009).

The rapid increase in illnesses that have their onset in childhood (including asthma, allergies, type 1 diabetes, obesity and autism) suggests that an environmental cause could be present (Noverr and Huffnagle, 2005; Penders et al., 2007a; Murk et al., 2011), and the loss of key constituents of the indigenous microbiota after maternal antibiotic exposure could be a contributing factor. For example, the use of antibiotics in the perinatal period have been associated with delayed colonization of neonates gut by several bacteria especially *Bifidobacteria* and *Lactobacillus* species (Westerbeek et al., 2006; Faa et al., 2013), and by decreased numbers of *Bifidobacteria* spp. and *Bacteroides* spp. (Penders et al., 2006). This may have long-term impacts since these species are considered to have beneficial properties and therefore, their absence may predispose to infections. In this regard, increased incidences of atopic diseases, irritable bowel syndrome (de Silva-Sanigorski et al., 2010), and inflammatory

bowel disease (IBD) have all been reported in antibiotic-exposed children (Droste et al., 2000; Shaw et al., 2010; Roberts et al., 2011; Russell et al., 2012; Villarreal et al., 2012; Metsälä et al., 2013; Stensballe et al., 2013).

Despite the high association between the impacts of perinatal or neonatal antibiotic use on the microbial colonization and future risk for asthma, other allergic reactions and disease conditions, the effects of antepartum antibiotic use on the process of intestinal microbiota development and future susceptibility to ulcerative colitis remain elusive or poorly understood. We assessed the susceptibility to colitis and compositional and functional alterations of fecal and colon mucosa-associated microbiota (MAM) in mice that were exposed to antepartum antibiotics and treated with dextran sulfate sodium (DSS) to induce acute colitis later in life.

7.3. MATERIALS AND METHODS

7.3.1. Animals.

Four pregnant C57Bl/6 mice were obtained from University of Manitoba breeding facility and maintained in the animal care facility at the Faculty of Health Sciences, University of Manitoba. These mice were treated with cefazolin (Midwest Veterinary Purchasing Cooperative Ltd, Winnipeg, MB, Canada) at 160 mg/kg/d (ATB group; 2 mice) or with saline (Control group; 2 mice) starting six days before due date. After delivery, the pups were left with their mothers until they were weaned on d 22. Upon weaning, the mice were housed in cages without mixing mice from different mothers and received a standard chow diet. The experimental protocol was approved by the University of Manitoba Animal Ethics Committee (10-073) and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

7.3.2. Induction of DSS colitis

At 7 weeks of age, fecal samples were collected from male offspring from the two groups

(ATB, n=10 and Control, n=10) after which they received DSS in their drinking water for 5 days (ATB-DSS, Control-DSS). The DSS (molecular weight; MW 40 kDa: MP Biomedicals, Soho, OH, USA) was added to the drinking water at a final concentration of 4% (wt/vol). Ten mice were included in each ATB-DSS and Control-DSS groups with five mice from each mother. **Figure 7.1** shows the experimental design and timelines for different activities.

7.3.3. Evaluation of inflammation.

During the period of colitis, the weights of the mice were recorded daily, and were expressed as a percentage of body weight prior to induction of colitis. Disease activity index comprised of the percentage of body weight lost score in combination with stool consistency, and blood in feces scores. The 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, diarrhoea; and bleeding: 0, no blood; 2, presence of blood; and 4, gross blood, and the scores have historically correlated well with the pathological findings in DSS-induced model of colitis (Cooper et al., 1993). The DAI scoring was performed from day 0 to day 5 over the period of DSS treatment. Presence of blood in the stool was assessed using the Hemoccult II test (Beckman coulter, Oakville, ON, Canada).

The colon was opened longitudinally and macroscopic damage was evaluated on the full section of the colon. The macroscopic scoring was performed immediately after the mice were sacrificed using previously established scoring system (Cooper et al., 1993; Khan et al., 2002), and the categories evaluated for macroscopic scores included, rectal bleeding, rectal prolapse, diarrhea and colonic bleeding.

For histology analysis and scoring, formalin (10 %; Sigma, Mississauga, ON, Canada)-fixed colonic segments collected during sacrifice were paraffin (Sigma, Mississauga, ON,

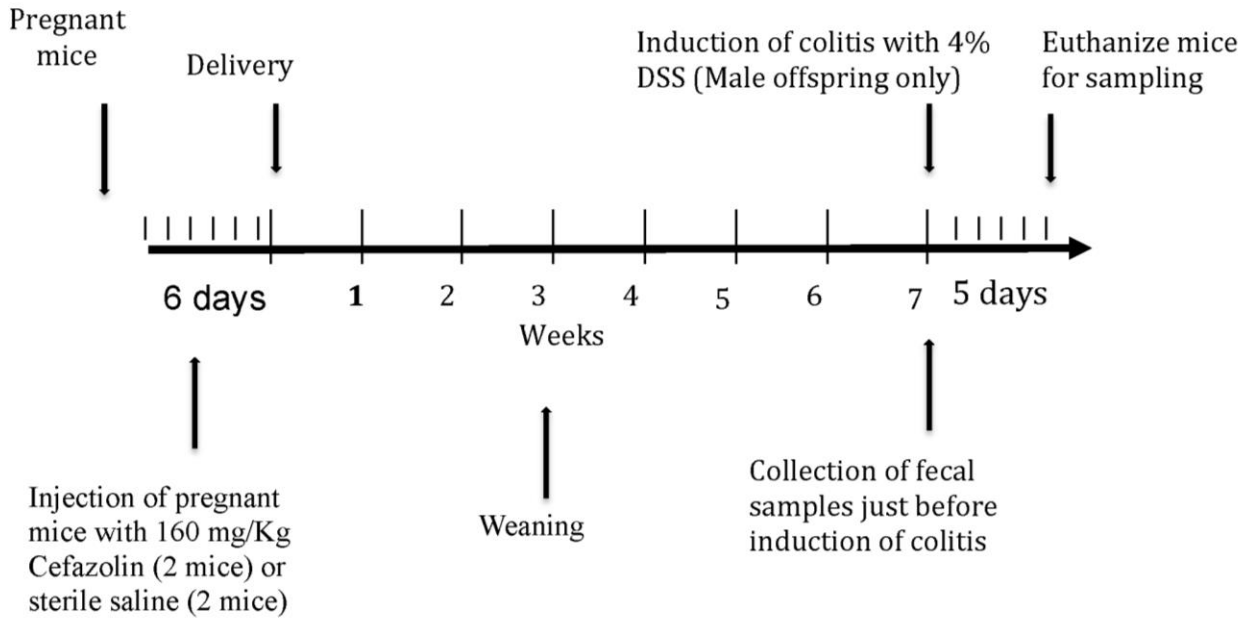

Figure 7.1: Experimental design and timelines for different experimental activities.

Canada)-embedded and 3-mm sections were stained using hematoxylin-eosin (H&E) (Sigma, Mississauga, ON, Canada). Colonic damage was assessed based on a published scoring system that considered loss of architectural, degree of inflammatory cell infiltrate, goblet cell depletion, and crypt abscess (Cooper et al., 1993; Khan et al., 2002).

For the analysis of serum C-reactive proteins, blood was collected by intracardiac puncture under isoflurane (Abbot, Mississaugua, ON, Canada) anaesthesia. Also, in order to assess colonic inflammatory cytokines, colonic samples were homogenized in 700 µl Tris-HCl buffer containing protease inhibitors (Sigma, Mississauga, ON, Canada), centrifuged at 13000 × *g* for 20 min at 4°C and the supernatant was frozen at - 80°C until assay. Serum C-reactive protein (CRP) and colonic cytokine levels (IL-6, IL-1β) were determined using an ELISA commercial kit (R&D Systems, Minneapolis, MN, USA).

7.3.4. DNA extraction and quality control

Approximately 200 mg of each fecal sample were used for DNA extraction using ZR fecal DNA extraction kit (Zymo Research Corp., Orange, CA, USA). For colonic samples, the tissue was cut open and approximately 50 mg of mucosa scrapings were taken. DNA extraction was done using ZR Tissue and Insect DNA kit (Zymo Research Corp., Orange, CA, USA). Both DNA extraction kits had bead-beating step for the mechanical lysis of the microbial cells. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA samples were normalized to 20 ng/µl, and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3'). DNA quality was checked by PCR as described by Khafipour et al.(Khafipour et al., 2009), and amplicons were verified by agarose gel electrophoresis.

7.3.5. Library construction and Illumina sequencing.

The V3-V4 region of the 16S rRNA gene was targeted for PCR amplification using modified F338 primer (5´-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTACTCCTACGGGAGGC AG-3´) for forward primer and modified bar coded R806 as described previously (Caporaso et al., 2012). The PCR reaction for each sample was performed in duplicate and contained 1.0 µl of pre-normalized DNA, 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; this was finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The 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 V3-V4 library was then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (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 Qubit 2.0 Fluorometer (Life technologies, Burlington, ON, Canada). Finally, 15% of the 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 (V4) (5'- TATGGTAATTGTGTGCCAGCMGCCGCGGTAA -3'), read 1 (V3-V4) 5'-TATGGTAATTGTACTCCTACGGGAGGCAG -3' read2 (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT -3') and index read (5'-

ATTAGAWACCCBDGTAGTCCGGCTGACTGACT -3') were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V3 (600-cycle) (Illumina, San Diego, CA, USA). The 150 and 300 paired-end sequencing reactions were 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. The sequencing data are uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed through accession number SRR2728570.

7.3.6. Bioinformatic analyses.

The FLASH assembler (Magoč T and SL., 2011) was used to merge overlapping paired-end Illumina fastq files. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010b). 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. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier and aligned with the Greengenes Core reference database (DeSantis et al., 2006) using PyNAST algorithms (Caporaso et al., 2010a). The phylogenetic tree was built with FastTree 2.1.3. (Price et al., 2010) for further comparisons between microbial communities.

7.3.7. Alpha-, beta-diversity analyses.

Alpha-diversity was calculated using Chao 1(Chao, 1984). Beta-diversity was measured by calculating the unweighted and weighted UniFrac distances using QIIME (Lozupone and Knight, 2005) and the *P* values were calculated using PERMANOVA analyses of Bray-Curtis

distances (Anderson, 2005).

For the alpha-diversity analysis, an even depth of approximately 27,000 sequences per sample for fecal samples collected before induction of colitis (ATB and Control groups), and 14,000 and 16,000 sequences per sample for colon mucosa and fecal samples of colitic mice (ATB-DSS and Control-DSS), respectively, was used for the calculation of diversity indices.

7.3.8. Partial least square discriminant analysis (PLS-DA)

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 14, Umetrics, Umea, Sweden) was performed on genus data to identify the effects of antepartum antibiotic treatment on the offspring as described previously (Li et al., 2012). PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the variable on X. In this case, X variables were bacterial taxa and Y was observations of different treatments compared together. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus, and genera with VIP < 0.5 were removed from the final model. R^2 estimates then were used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. Data was presented in loading scatter plots and the PLS-DA regression coefficients were used to identify taxa that were positively or negatively correlated with each treatment group.

7.3.9. Prediction of functional metagenome.

The open source software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), a computational approach for prediction of functional metagenome of bacterial communities using marker gene data and a database of reference genomes, was used on the Greengene picked OTUs to generate metagenomic data, and derive relative Kyoto Encyclopedia of Genes and Genomes (KEGG)

Pathway abundance. KEGG data was analyzed using open source software STAMP (STatistical Analysis of Metagenomic Profiles) (Parks and Beiko, 2010).

7.3.10. Other statistical analysis.

Alpha-diversity and phylum percentage data were used to assess the effect of treatment using MIXED procedure of SAS (SAS 9.3). Disease activity index, weight loss, rectal bleeding and stool consistency were analyzed by applying two-way ANOVA followed by Sidak multiple comparison post hoc. Student *t* test was used to compare the macroscopic scores, histological scores and inflammatory markers between antibiotic and control groups using Graphpad Prism 5.0c (Graphpad Prism, La jolla, CA, USA). The significance level was adjusted at level 0.05.

7.4. RESULTS

7.4.1. Fecal microbiota alterations before DSS treatment

As described below our results showed that antepartum use of antibiotics modified the ecology of offspring's indigenous microbiota, and the effects persisted up to and possibly beyond seven weeks of age.

7.4.1.1. Alpha-diversity.

As shown in **Figure 7.2**, there was no difference between ATB and control groups.

7.4.1.2. Beta-diversity.

Figure 7.3 present the three-dimensional PCoA of unweighted and weighted UniFrac distances. Fecal samples were distinctly clustered according to their treatment group when plotted and analyzed using unweighted UniFrac (*P = 0.0003*). The clustering was not as distinct

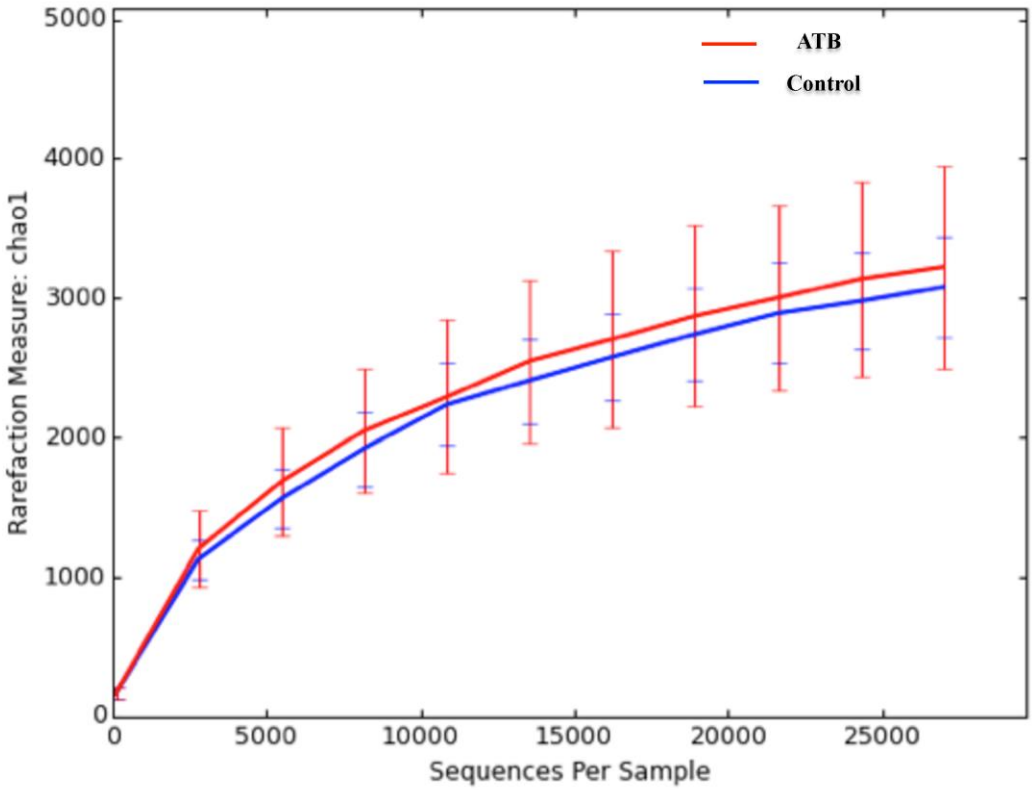

Figure 7.2. Alpha-diversity analysis on Chao 1, a measure of species richness based on operational taxonomic unit (OTU) for fecal samples collected before induction of colitis with DSS.

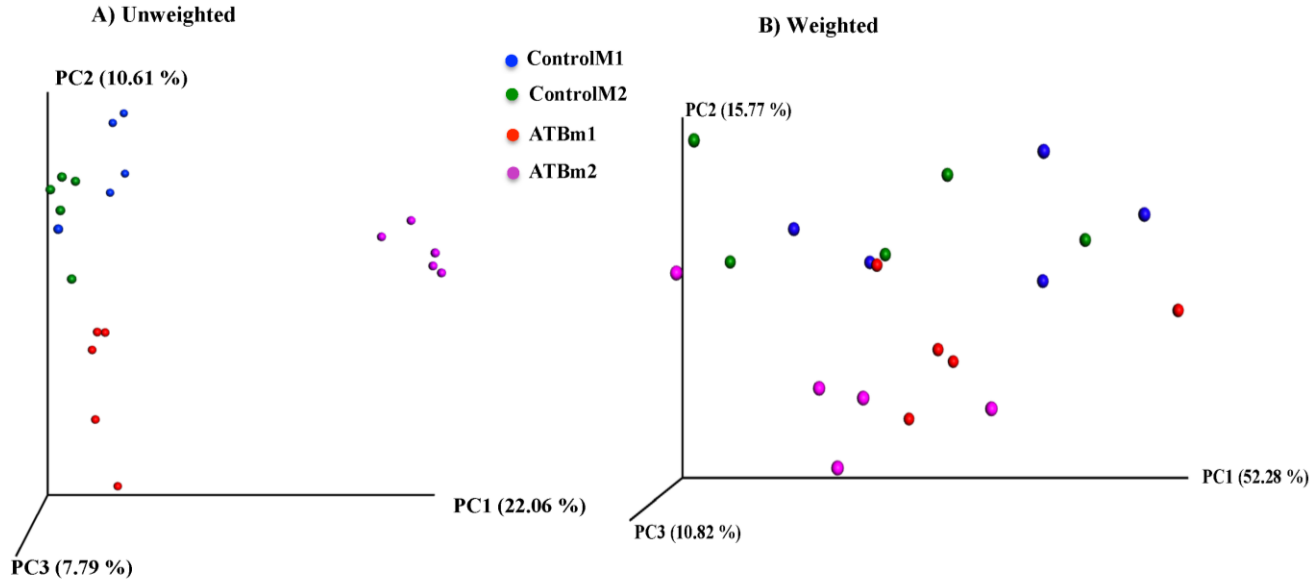

Figure 7.3. Principle coordinate analysis (PCoA) of (A) Unweighted (*P = 0.0003*) and (B) Weighted (*P* = 0.06) UniFrac distances, an OTU based measure of beta-diversity in fecal samples collected before induction of colitis.

(*P = 0.06*) when PCoA and PERMANOVA analyses were performed on weighted UniFrac distances. The mother also influenced the clustering pattern of fecal samples and offspring from each mother clustered closer together in both ATB and Control groups. (*P* = 0.001).

7.4.1.3. Microbiota composition.

A total of 9 phyla were identified, of which 3 were abundant (≥ 1% of community), including Firmicutes, Bacteroidetes and Proteobacteria. The low-abundance phyla (< 1% of community) included Actinobacteria, Cyanobacteria, Deferribacteres, Tenericutes, Verrucomicrobia and TM7. Among the abundant phyla, Firmicutes and Proteobacteria populations were lower (*P* = 0.01, and 0.04, respectively), while Bacteroidetes population was higher (*P* = 0.007) in the antibiotic group compared to the control (**Figure 7.4**).

Classification of the OTUs at the lower taxonomical levels resulted in identification of 102 taxa. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels. Of the 102 taxa, 40 had abundances greater or equal to 0.01% of community, while 62 were below 0.01% of community. Bacterial taxa with relative abundance of ≥ 0.01% of community were analyzed using PLS-DA to identify bacteria that were most characteristic of the control or antibiotic groups. As shown in **Figure 7.5**, g. *Allobaculum*, *Bacteroides acidifaciens*, *Suterella*, *Prevotella*, *rc4-4*, and unclassified members of f. S24-7; and o. RF32 were positively associated with antibiotic (ATB) group but negatively associated with control group. In addition, g. *Odoribacter*, *Bacteroides*, *Enterococcus*, *Desulfovibrio*, *Helicobacter*, *Dehalobacterium*, *Mucispirillum*; and unclassified members of f. Rikenellaceae, Helicobacteraceae, Lachnospiraceae, and Peptococcaceae were positively correlated with the control group but negatively associated with ATB treatment. **Appendix 1 Supplementary Table 7.1** shows a summary of mean abundances of all the taxa.

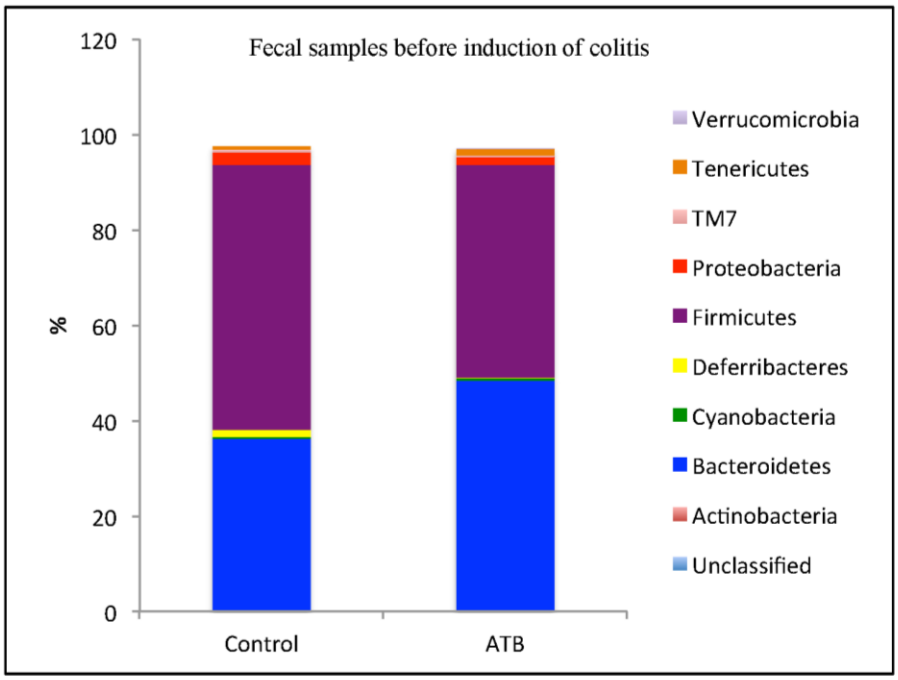

Figure 7.4. Relative abundances of bacterial phyla in fecal samples collected before induction of colitis.

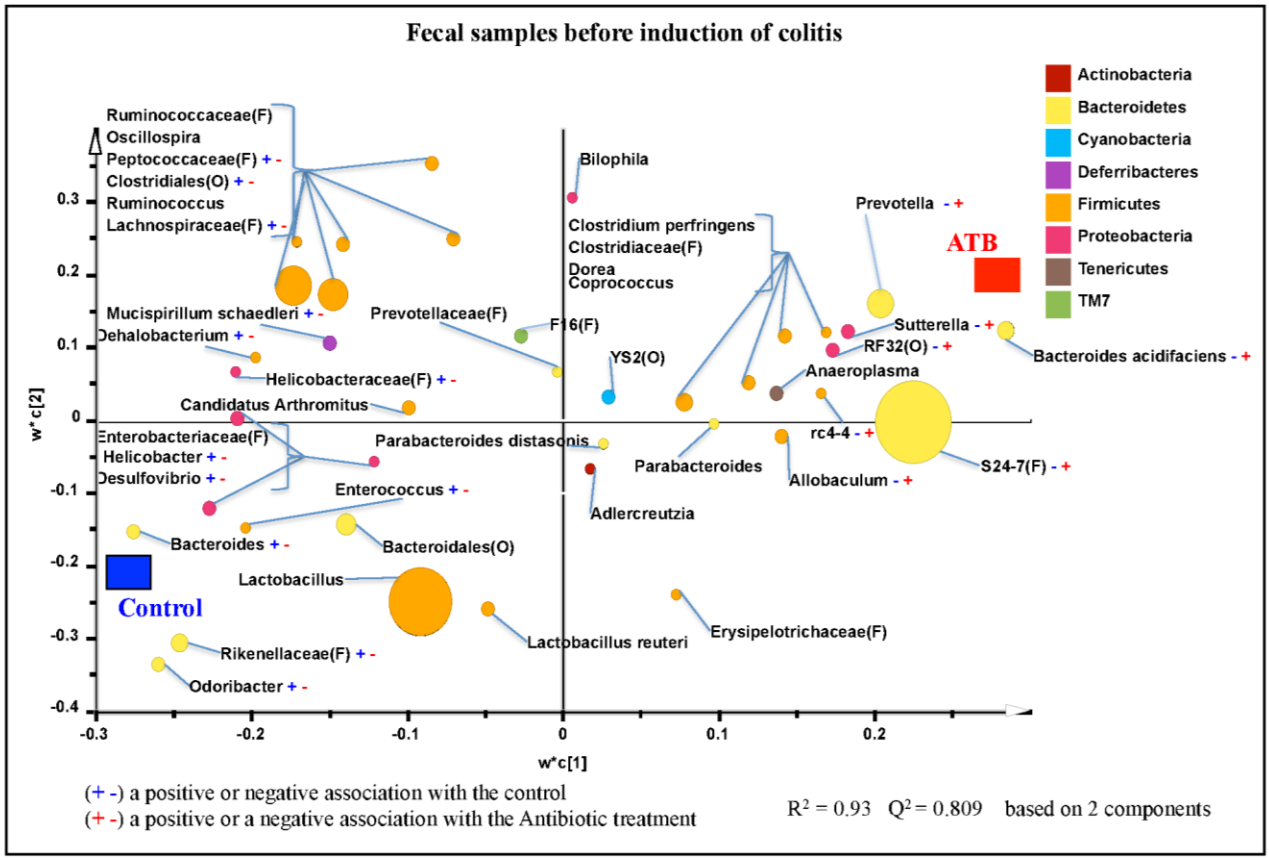

Figure 7.5. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the Control or ATB treatments in the fecal samples collected before induction of colitis.

7.4.1.4. Functional metagenome of fecal microbiome.

As shown in **Figure 7.6**, several pathways including oxidative phosphorylation, folate biosynthesis, pantothenate and CoA biosynthesis, energy metabolism, alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism and histidine metabolism were highly enriched in the fecal microbiome of the ATB group compared to Control. In contrast, flagellar assembly and secretion system were highly enriched in the Control group compared to the ATB group.

7.4.2. Impact of antepartum antibiotics on development of colitis

7.4.2.1. Disease activity index, macroscopic and histological scores.

Following DSS treatment, the onset of clinical disease, as assessed by disease activity index (stool consistency, weight loss and rectal bleeding) on d 2, 4, and 5 of the study increased in the ATB-DSS compared to the Control-DSS group (**Fig 7.7-A**). **Appendix 1 Supplementary Figure 7.1** shows the stool consistency, weight loss and rectal bleeding. As shown in **Figure 7.7-B**, DSS treatment increased colonic macroscopic damage score at d 5 in the ATB-DSS compared to the Control-DSS (P = 0.03). In addition, mucosal inflammation and infiltration was assessed through histological scoring as shown in **Figures 7.7-C and 7.7-D**, antepartum antibiotic exposure increased the severity of colitis associated with the loss of tissue architecture and increased immune cell infiltration.

7.4.2.2. IL-6, IL-1β, and C-reactive protein.

Antepartum antibiotic exposure increased IL-6 levels (P = 0.04; **Figure 7.7-E** in the colon of ATB-DSS mice compared to the Control-DSS, but did not affect IL-1β (**Figure 7.7-F**), and reduced CRP levels (P = 0.002) (**Figure 7.7-G**) in ATB-DSS compared to the Control-DSS.

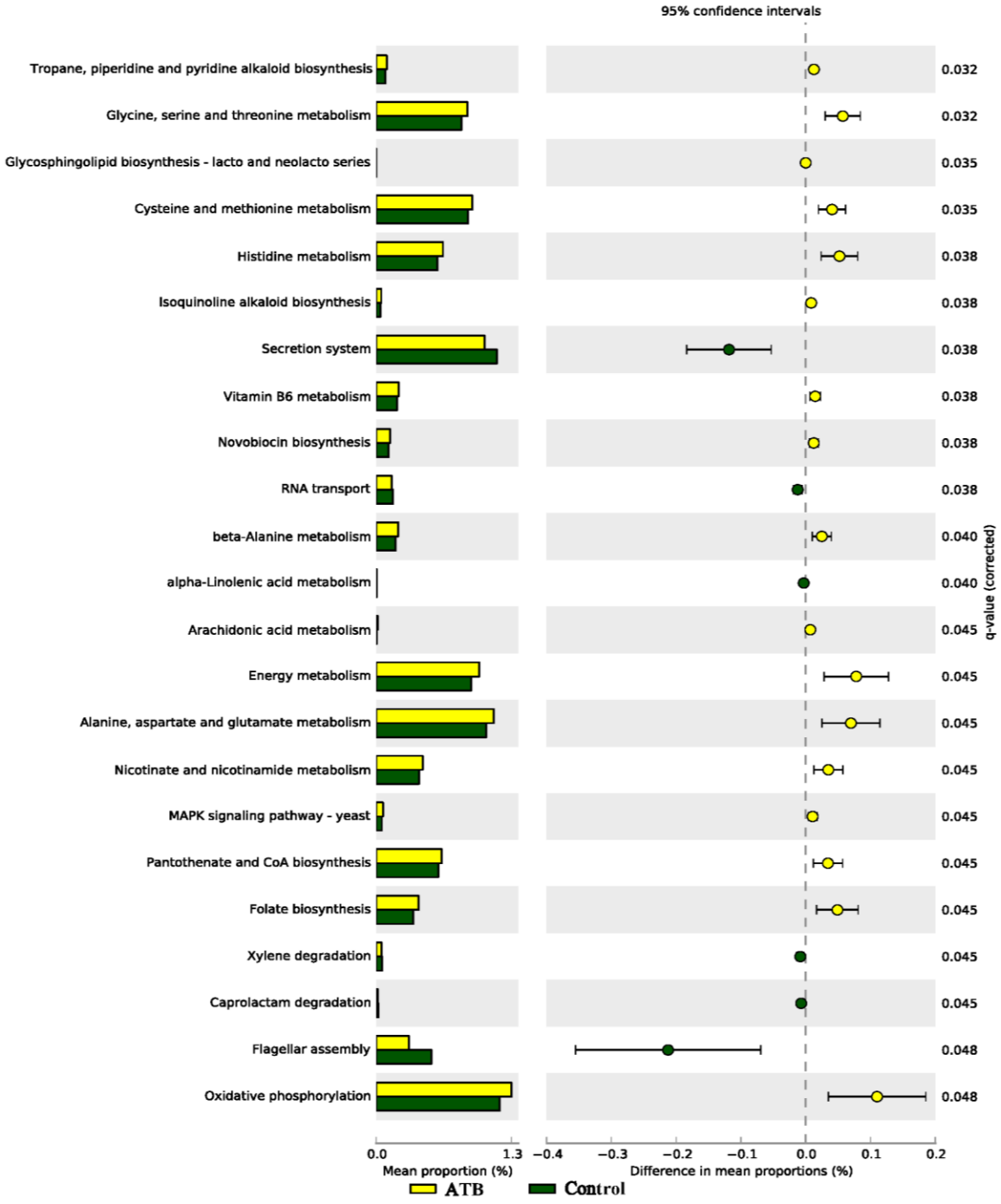

Figure 7.6. Subsystems and pathways enriched or decreased within fecal microbiome from Control and ATB groups before induction of colitis.

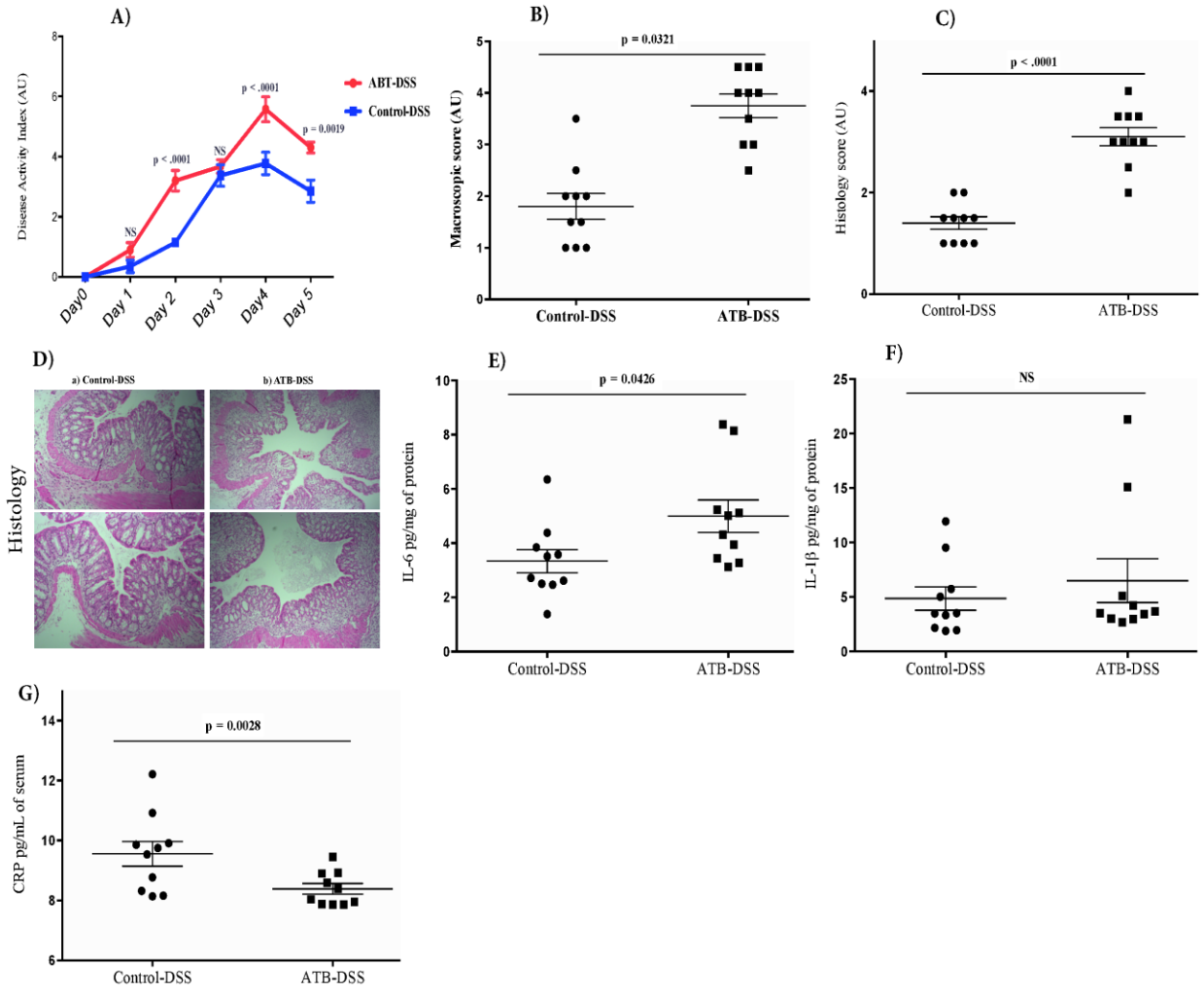

Figure 7.7. Impacts of dextran sulfate sodium (DSS) and antepartum antibiotics on colitis induction and disease severity.

7.4.3. Colonic and fecal microbial alterations following DSS treatment

7.4.3.1. Colon and fecal alpha-diversity.

Antepartum antibiotic exposure did not influence colonic alpha-diversity (**Figure 7.8-A**), but decreased fecal species richness (**Figure 7.8-B**), 5 d after induction of colitis.

7.4.3.2. Colonic beta-diversity

Figure 7.9 shows a three-dimensional PCoA of unweighted and weighted uniFrac distances. Colonic samples clustered separately according to their treatment group when plotted and analyzed using unweighted UniFrac (P = 0.0004). The clustering was not as distinct (P = 0.06) on weighted UniFrac distances. The mother also influenced the clustering pattern of colon samples and offspring from each mother clustered closer together ($P < 0.001$), which was more evident in the ATB-DSS group compared to the Control-DSS group (P = 0.009).

7.4.3.3. Fecal beta-diversity

As shown in **Figure 7.10**, fecal samples clustered distinctly according to treatment group for both unweighted and weighted UniFrac distance analysis (P = 0.0001). The mother also influenced the clustering as offspring samples from each mother clustered closer to each other (P = 0.001) in both the ATB-DSS and the Control-DSS groups. Two samples were omitted from the analysis due to very low sequencing depth.

7.4.3.4. Microbiota composition at phylum level in the colon and fecal samples.

A total of 10 phyla were identified in the colon mucosa samples, of which 4 were abundant (≥ 1% of community), including: Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. The low-abundance phyla (< 1% of community) included Actinobacteria, Cyanobacteria, Tenericutes, Thermi, Verrucomicrobia and TM7. No difference was observed between the antibiotic and the control group among the abundant phyla (**Figure 7.11-A**).

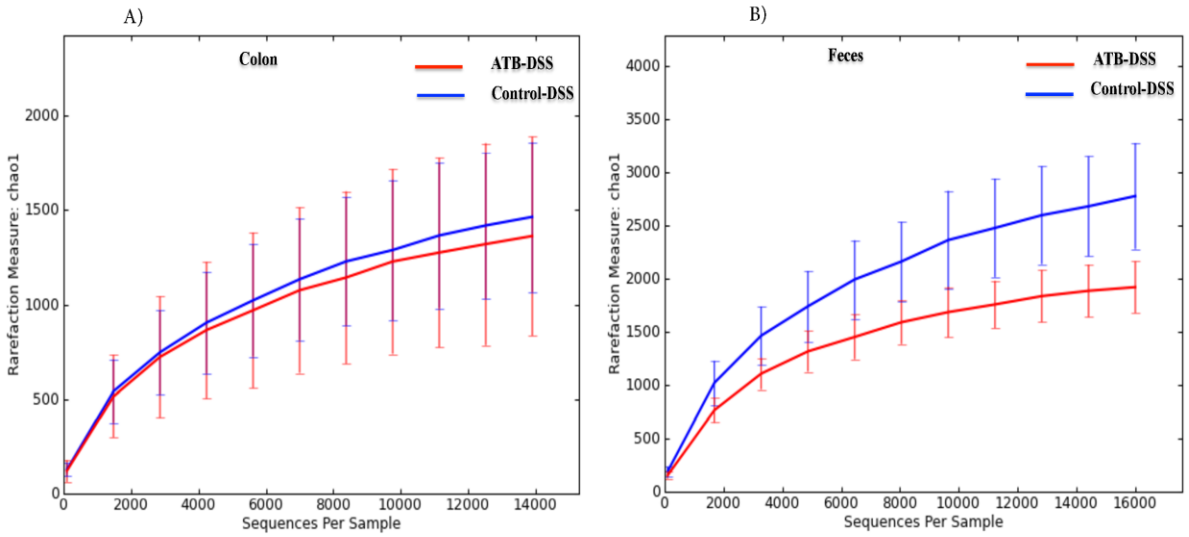

Figure 7.8. Rarefaction analysis on Chao 1, a measure of species richness based on operational taxonomic unit (OTU), following DSS treatment for the Control-DSS and ATB-DSS mice colon mucosa-associated (A) and fecal microbiota (B). Control-DSS and ATB-DSS had similar richness in the colon mucosa-associated microbiota; however, ATB-DSS had a lower fecal richness compared to the Control-DSS.

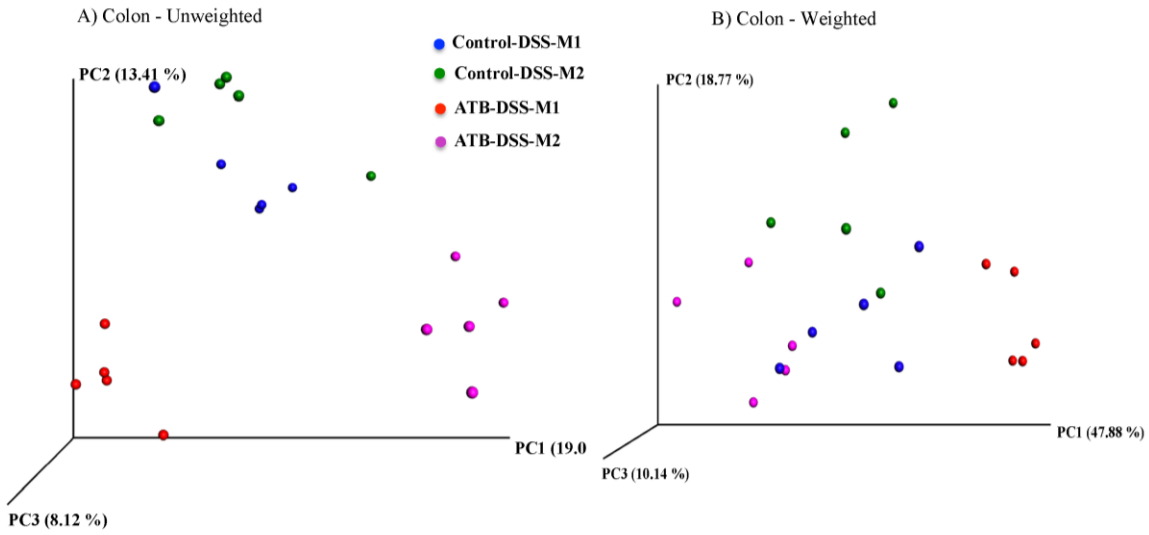

Figure 7.9. Principle coordinate analysis (PCoA) of (A) Unweighted (P = 0.0004) and (B) Weighted (P = 0.06) UniFrac distances in colon mucosa-associated microbiota (MAM) after induction of colitis.

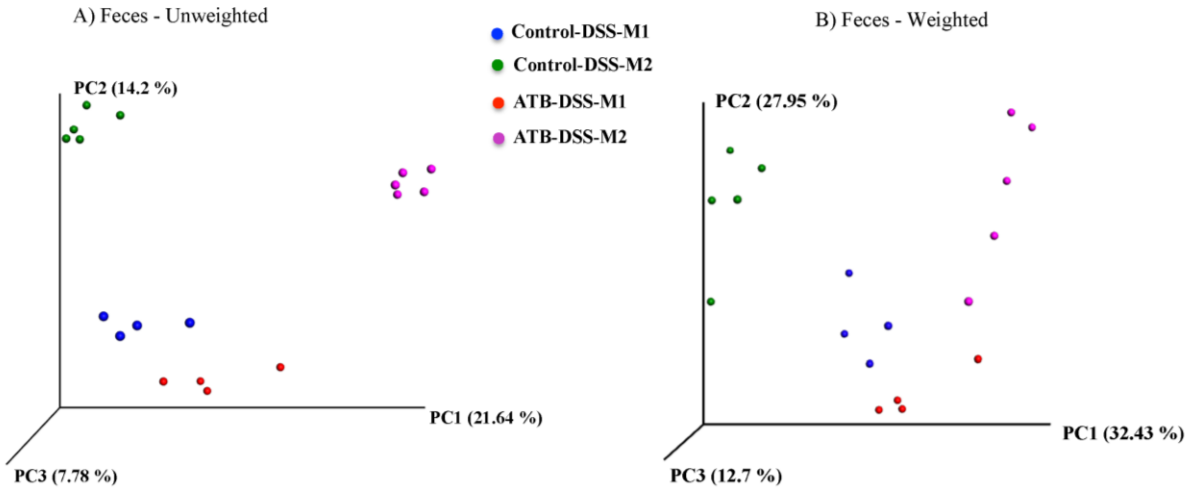

Figure 7.10. Principle coordinate analysis (PCoA) of (A) Unweighted (P = 0.0001) and (B) Weighted (P = 0.0001) UniFrac distances in fecal microbiota after induction of colitis.

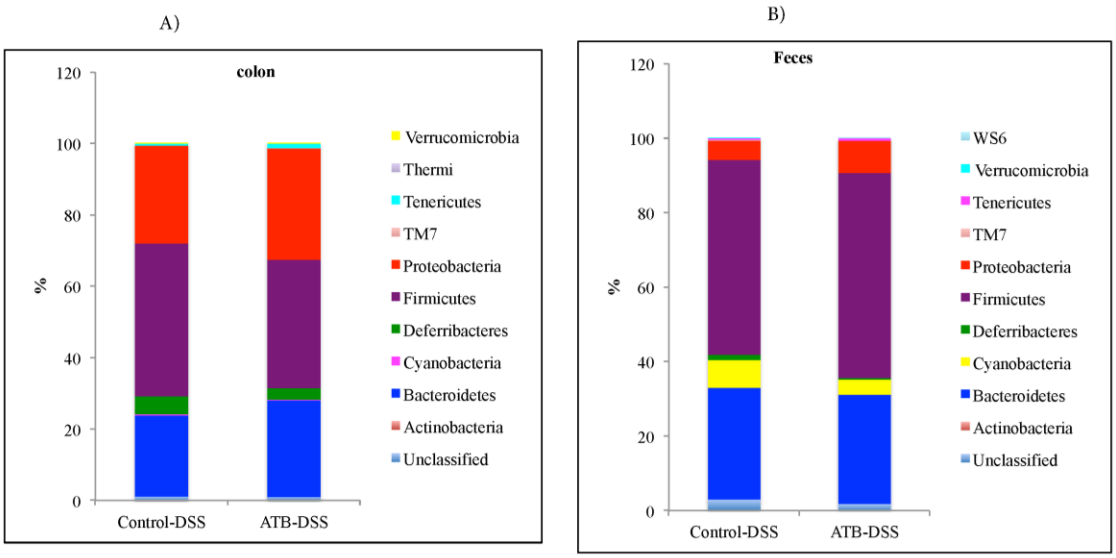

Figure 7.11. Relative abundances of bacterial phyla after induction of colitis in the colon mucosa (A) and in the feces (B).

In the fecal samples, a total of 10 phyla were identified, of which 4 were abundant (≥ 1% of community), including: Firmicutes, Bacteroidetes, Proteobacteria, and Cyanobacteria. The low-abundance phyla (< 1% of community), included Actinobacteria, Deferribacteres, Tenericutes, WS6, Verrucomicrobia and TM7. No significant difference was observed between the ATB-DSS and the Control-DSS group among the abundant phyla (**Figure 7.11-B**).

7.4.3.5. Microbiota composition at lower taxonomic levels in the colonic samples.

Classification of the OTUs at the lower taxonomical levels resulted in identification of 93 taxa. Of the 93 taxa, 60 had abundance grater than or equal to 0.01% of community, while 33 were below 0.01%. Bacterial taxa with relative abundance of ≥ 0.01% of community were analyzed using PLS-DA to identify bacteria that were most characteristic of the Control-DSS or ATB-DSS groups. As shown in **Figure 7.12-A**, g. *Clostridium, Allobaculum, Bacteroides acidifaciens, Parabacteroides distasonis, Clostridium perfrigens, rc4-4*; and unclassified members of f. S24-7 were positively associated with the ATB-DSS group but negatively associated with the Control-DSS group. In addition, g. *Bacteroides, Coprobacillus, Odoribacter, Desulfovibrio, Gnavus, Dehalobacterium, Oscillospira, Desulfovibrio C21_c20*; and unclassified members of o. RF32, YS2, Erysipelotrichales, and Bacteroidales were positively associated with the Control-DSS group but negatively associated with the ATB-DSS group. **Appendix 1 Supplementary Table 7.2** shows a summary of mean abundances of all the taxa.

7.4.3.6. Microbiota composition at lower taxonomic levels in the fecal samples

Classification of the OTUs at the lower taxonomical levels resulted in identification of 87 taxa. Of the 87 taxa, 55 had abundances greater than or equal to 0.01% of the community whilst 32 taxa were below 0.01% of the community. Bacterial taxa with relative abundance of ≥ 0.01% were analyzed using PLS-DA to identify bacteria that were most characteristic of the control or

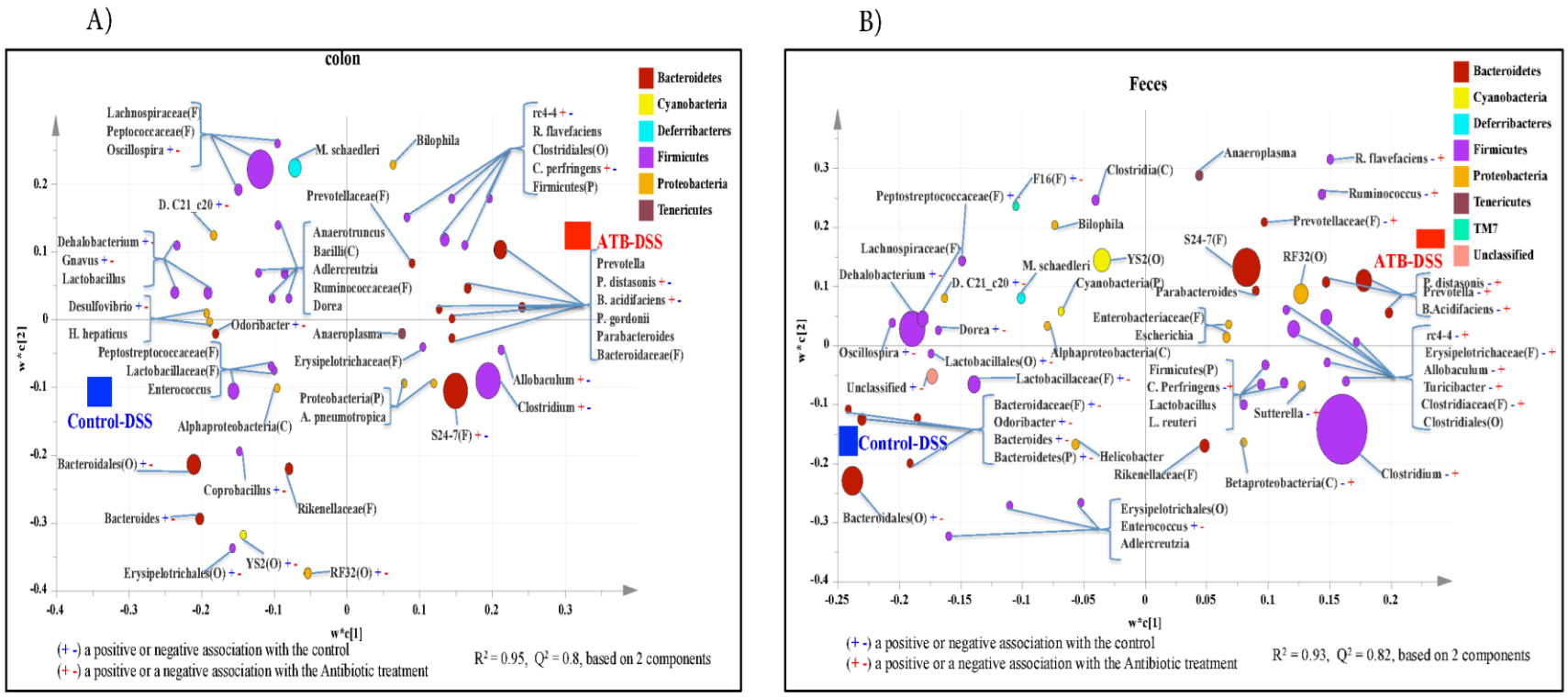

Figure 7.12. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the Control-DSS or ATB-DSS treatments in the A) Colon mucosa, and B) Feces

antibiotic groups. As shown in **Figure 7.12-B**, g. *Clostridium, Betaproteobacteria, Suterella, Clostridium perfrigens, Turicibacter, Allobaculum, rc4-4, B. acidifaciens, Prevotella, Parabacteroides distasonis, Ruminococcus, Ruminococcus flavefacien;* unclassified members of f. Prevotellaceae, Erysipelotrichaceae, Clostridiaceae; and o. Clostridiales were positively associated with the ATB-DSS group but negatively associated with the Control-DSS group. Also, g. *Enterococcus, Bacteroides, Odoribacter, Dorea, Dehalobacterium, Desulfovibrio C21_c20*; unclassified members of f. Bacteroidaceae, Lactobacillaceae, Peptostreptococcaceae, F16; o. Lactobacillales; and p. Bacteroidetes were found to be positively correlated with the Control-DSS but negatively correlated with the ATB-DSS group. **Appendix 1 Supplementary Table 7.3** shows a summary of mean abundances of all the taxa.

7.4.3.7. Functional metagenome of colonic and fecal microbiome.

As shown in **Figures 7.13-A and B**, several metabolic pathways including: arachidonic acid metabolism, butanoate, ribosome biogenesis metabolism, ribosome, peptidoglycan biosynthesis, carbohydrate digestion and absorption, arginine and proline metabolism, glycine, serine and threonine metabolism, were highly enriched in the mucosal microbiota of colon and in the feces of the ATB-DSS group.

7.5. DISCUSSION

The use of antibiotics may disrupt neonatal gut microbiota and have profound consequences for later health (Faa et al., 2013). In this context, antibiotic-mediated disturbance of the intestinal microbiota in very early life has been shown to increase the risk of late-onset sepsis in a mouse model (Deshmukh et al., 2014). In addition, various illnesses with onset in childhood such as asthma, allergies, type 1 diabetes, obesity and autism have been hypothesized to be associated with maternal exposure to antibiotics resulting in perturbations of the indigenous

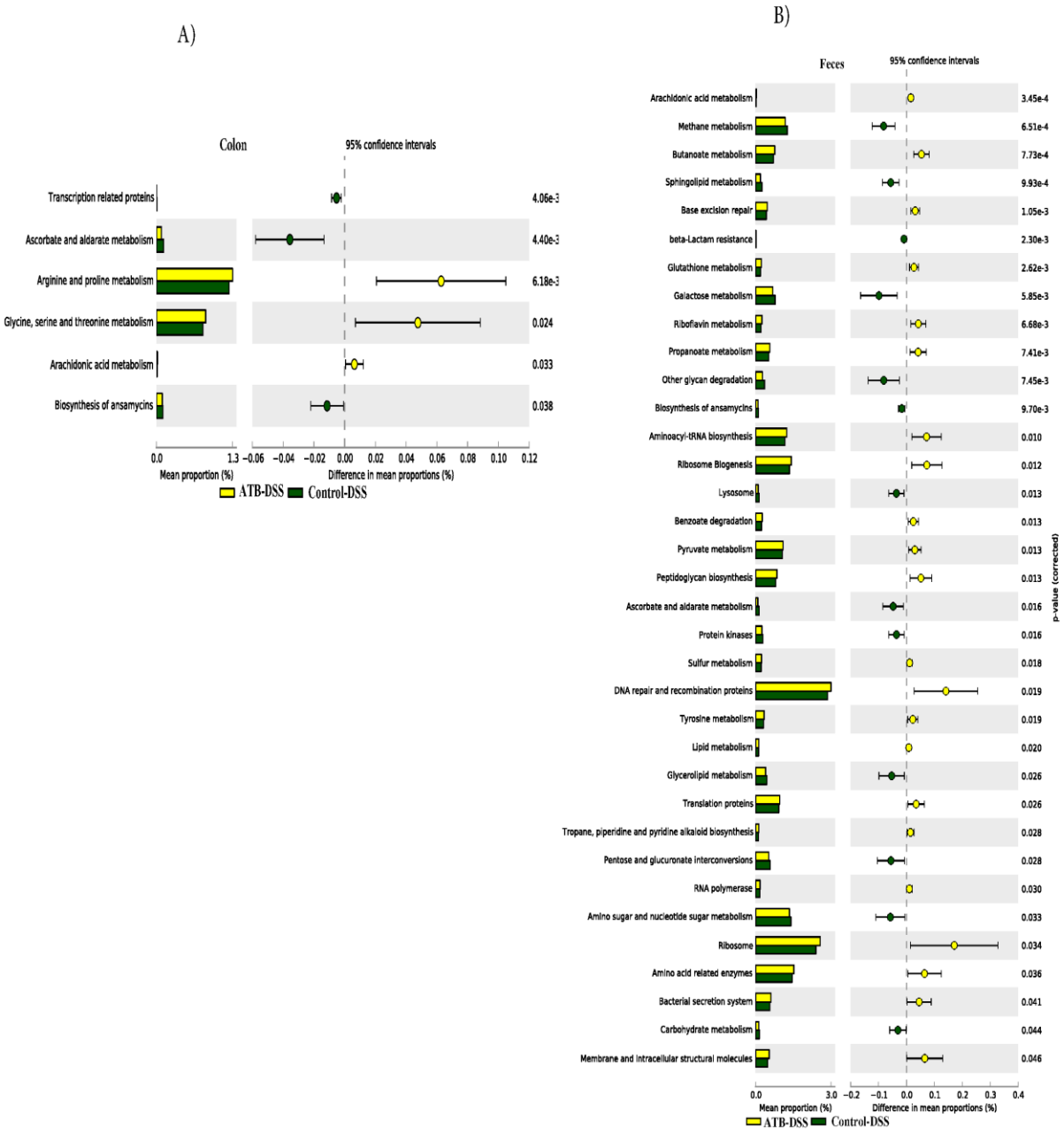

Figure 7.13. Subsystems and pathways enriched or decreased within (A) Colon muscosa-associate microbiome, and (B) Fecal microbiome after induction of colitis in Control-DSS and ATB-DSS.

microbiota (Noverr and Huffnagle, 2005; Penders et al., 2007a; Murk et al., 2011). Here, we assessed the effects of antepartum antibiotic (cefazolin) exposure of the mother on the gut microbiota composition of the offspring without or with exposure to experimental colitis later in life. Cefazolin belongs to the beta-lactam class and have a bactericidal effect inhibiting cell envelope synthesis (Perez-Cobas et al., 2013a; Mohammad, 2015), and it was recently shown to be among the commonly administered intrapartum antibiotics in retrospective and prospective cohort studies conducted in Canada (Persaud et al., 2014b; Azad et al., 2015).

Despite the smaller sample size in our study, we have demonstrated that at seven weeks of age, antepartum antibiotic treatment altered offspring fecal bacterial diversity, its composition at the phylum and lower taxonomical levels, and its predicted functional genome content. In addition, the characterization of the disease progress and severity demonstrated increased susceptibility to colitis in mice whose mothers were exposed to antepartum antibiotics compared to the mice whose mothers did not receive antibiotics. Similarly, the pattern of fecal microbiota was more profoundly altered following colitis induction in ATB-DSS mice compared to Control-DSS. It is important to note that all mice from the ATB and Control groups remained with their mothers and were exclusively suckling until the time of weaning (d 22). In addition, the mice from each mother were caged separately (i.e., mice from different mothers within the same group were not mixed at any point). Exclusive suckling, interactions with the mother, and the separate caging are all confounding factors, which have a direct influence on the nature of microbial colonization and might therefore complicate the interpretation of the data. Nevertheless, this may explain our observation on beta-diversity of fecal microbiota where both the antibiotic status and the mother seemed to influence sample clustering, suggesting that in addition to the effect of the antepartum antibiotics, mice from different mothers had distinct bacterial composition. In

support of this, intrapartum antibiotics have been associated with infant gut microbiota dysbiosis, and breastfeeding was found to modify the effects (Azad et al., 2015). With respect to colon MAM, the effect of mother on bacterial composition was not as apparent in the Control-DSS mice compared to the ATB-DSS mice. Although the differences between colon MAM and fecal microbiota composition are well established (Morgan et al., 2012), the reasons why colon MAM in the Control-DSS was not influenced by the mother as opposed to that of fecal microbiota are not clear and remain to be explained.

Mice whose mothers received antepartum antibiotics had an increased disease activity index on d 2, 4 and 5 of DSS treatment, as well as increased macroscopic score on d 5 and an increased level of colonic IL-6 compared to the DSS mice with no antibiotics. The histological score also revealed destruction of the colonic wall characterized by a loss of crypts. However, IL-1β level was not modified whereas serum CRP, a marker of systemic inflammation decreased in the antibiotic group. These results suggest an increased activity of DSS because of antepartum antibiotic exposure, as assessed by disease severity and colonic damage, compared to the control. This is in agreement with other studies where the use of broad-spectrum antibiotics in the antepartum period was shown to alter expression of genes involved in gastrointestinal tract development, particularly the architecture and functionality of the intestinal barrier (Westerbeek et al., 2006). It is important to emphasize, however, that the course of systemic inflammation as measured by CRP showed different response as the ATB-DSS group had lower level of CRP compared to that of Control-DSS group, which is in contrast to histological and disease severity indices, suggesting a different mode of action. Nevertheless, although this phenomenon is not clear to us, intravenous administration of cefazolin was previously shown to lower CRP to normal levels in a patient with elevated levels of CRP (Perez-Cobas et al., 2013b).

Our data showed that fecal bacterial species richness did not differ between the Control and ATB mice before induction of colitis although microbiota composition was different; however, after 5 days of DSS administration, ATB-DSS mice had a lower fecal bacterial richness, suggesting that the antepartum antibiotic exposed mice were more susceptible to colitis compared to the control mice that were equally treated with DSS. Also, both colon MAM and fecal microbiota differed in ATB-DSS mice compared to Control-DSS. Direct exposure to antibiotics is known to affect intestinal colonization by suppressing commensal bacteria and causing the emergence of pathogens such as *Clostridium difficile* (Murgas Torrazza and Neu, 2011). Research shows that antibiotic use in the immediate period after birth can severely alter gut microbiota in infants (Penders et al., 2006; Tanaka et al., 2009), and evidence from long-term studies suggests that these perturbations could last for months, if not years (Jernberg et al., 2007; Dethlefsen et al., 2008b). Indirect exposure is also relevant, because gut microbial diversity was reduced in infants born to mothers who received antibiotics during pregnancy or while breastfeeding (Fallani et al., 2010), which is in agreement with our results. The finding that fecal bacterial species richness did not differ before induction of colitis but the antibiotic group had a lower species richness following administration of DSS, suggests a role of antepartum antibiotics in susceptibility to DSS-induced microbial dysbiosis.

Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria are usually the most abundant phyla in a healthy gut and in most cases Firmicutes and Bacteroidetes are depleted whereas Actinobacteria and Proteobacteria substantially become more abundant in IBD patients compared to healthy controls (Ley, 2006). Although we did not observe differences between antibiotic and non-antibiotic groups at the phylum level 5 days after induction of colitis, we found that specific taxa were associated with each group at lower taxonomical levels. However,

data on the indirect impact of antepartum antibiotic use on offspring gut microbiota colonization is not consistent. In this regard, previous studies did not observe effect of maternal antibiotics during pregnancy upon infant gut microbiota (Penders et al., 2006), whereas others reported an effect of maternal perinatal antibiotics use on fecal microbiota or first stool sample, such as reduced intestinal microbial diversity and shifts in abundance of specific bacteria in both full-term and pre-term infants (Collignon et al., 2010; Fallani et al., 2010; Arboleya et al., 2014). In agreement with our observations, intrapartum antimicrobial prophylaxis was also found to have an equal or even higher effect on intestinal microbiota in infants during the first days of life compared to direct antibiotic administration, even when the mothers received only a single dose of ampicillin (Arboleya et al., 2014).

The analysis of microbiota profile in fecal samples before the induction of colitis provided an opportunity to address a relevant question regarding antepartum antibiotics. In this context, the results support the hypothesis that antepartum antibiotics indeed do perturb the initial establishment of gut microbiota in offspring and therefore, provide an opportunity to further establish whether these perturbations have any role to play in susceptibility to colitis. The fecal bacterial community composition was altered as a result of antepartum antibiotics and these alterations were even more pronounced after treatment with DSS. The observed microbial changes because of the antepartum use of Cefazolin could be driven by the presence/absence and enrichment/depletion of specific taxa within certain phyla. In this regard, several genera including *Clostridium*, *Allobaculum*, and *rc4-4*, and species, such as *Bacteroides acidifaciens*, *Parabacteroides distasonis*, *Clostridium perfrigens* were positively associated with the antibiotic group in both colon MAM and fecal microbiota. There were several other taxa that were positively associated with the antibiotic group either only in the colon (unclassified members of

f. S24-7), or in the feces (g. *Ruminococcus flavefaciens, Betaproteobacteria, Sutterella, Prevotella, Ruminococcus* and *Turicibacter;* unclassified members of o. Clostridiales; f. Clostridiaceae, Erysipelotrichaceae, and Prevotellaceae). Of particular interest, g. *Bacteroides acidifaciens, Allobaculum, rc4-4, Prevotella*, and *Sutterella*, and f. S24-7 were also enriched in the feces of the ATB group before induction of colitis indicating their strong association with antepartum exposure to cefazolin which persisted even after DSS treatment, and therefore, it is speculated that these taxa may play significant roles in the susceptibility to colitis. For example, *Sutterella* is associated with low levels of IgA in the gut and just recently it was shown to have the capacity to degrade secretory IgA (Moon, 2015) that protects the mucosa and regulates microbial attachment in the mucosa. As such, degradation of secretory IgA by *Sutterella* which was more enriched both in ATB and ATB-DSS mice compared to Control and Control-DSS mice, may imply a less protected mucosa in mice whose mothers were exposed to antepartum antibiotics, and thus, render the mucosa susceptible to colitis.

Persaud et al. (Persaud et al., 2014a) examined the effects of antibiotic exposure in the perinatal period on the gut microbiota of 184 infants enrolled in the Canadian Healthy Infant Longitudinal Development Study. The study showed that infants had an increased relative abundance of *Clostridium* (Persaud et al., 2014a), emphasizing on the association of *Clostridium* with prenatal antibiotic use. Most members of Clostridium, including *Clostridium perfrigens* and *Clostridium difficile,* are known to be pathogenic and or opportunistic bacteria that are able to take advantage of gut microbiota dysbiosis following antibiotic exposure, facilitating their proliferation, and ability to occupy ecological niches previously unavailable for them (Biasucci et al., 2008). Also, perinatal antibiotics have been shown to increase the abundance of Enterobacteriaceae family in infants (Arboleya et al., 2014). In addition, antibiotic administration

during the first hours of life increased the levels of Enterobacteriaceae as analyzed during the first 2 months of life (Tanaka et al., 2009; Fouhy et al., 2012). Similarly, incomplete recovery of the gut microbiota after a 5 d antibiotic administration has been demonstrated in adults aged 22-43 years (Dethlefsen et al., 2008b). These results suggest a lasting effect of antibiotic administration on gut microbiota composition that is likely to influence disease risk, which is in agreement with our observations. Based on our results, we can speculate that the taxa that were associated with the antibiotic group may have specific roles in the susceptibility to colitis. However, it is important to note that different bacteria exhibit redundancy in their functions and some may appear or disappear from the community depending on the existing conditions. Also, different antibiotics may exhibit different effects depending on which members of the commensal bacteria are targeted by the antibiotics.

Metagenome prediction revealed functional shifts in the murine intestinal microbiome, with different metabolic pathways enriched in the colon mucosa-associated microbiome and the fecal microbiome in the ATB group compared to the Control, although the range of functions that were impacted were greater in fecal compared to the colon mucosa-associated microbiome. Microbial functions related to arachidonic acid metabolism may play important roles in inflammatory responses through production of prostaglandins (Ricciotti and FitzGerald, 2011), while butanoate metabolism could be associated with the integrity of the colonocytes. Other functional pathways including: ribosome biogenesis and metabolism, carbohydrate digestion and absorption, arginine and proline metabolism, glycine, serine and threonine metabolism may be important in nutrient availability and synthesis of proteins, which may directly or indirectly influence the host. Apart from glycine, serine and threonine metabolism that was common in the antibiotic group before and after DSS treatment, the fecal microbial functional and metabolic

activities in the ATB group were majorly different before and after induction of colitis suggesting that DSS further modified the community functional potential although this may also be influenced by interactions between indirect effects of cefazolin and DSS.

In this study, we used the antibiotic cefazolin as a tool to indirectly induce shifts in the intestinal microbiota and model an altered colonization state later in life. We have demonstrated a microbiota-driven, specific increase in susceptibility to experimental murine colitis and have provided data that suggests these effects could be mediated by changes in microbial colonization through antepartum antibiotic exposure. Although the mechanisms involved in cefazolin-mediated susceptibility of murine colitis remain to be directly elucidated, the data presented here provide new insights and offer avenues for future work. This new knowledge has extended our understanding on impact of antepartum antibiotics with respect to microbial dysbiosis and colitis and may form a basis for designing intervention strategies targeting the gut microbiota. This data also suggests that antepartum antibiotics may predispose offspring to colitis and thus, the potential deleterious effects upon gut microbiota composition may need to be considered when deciding on maternal antibiotic use.

CHAPTER 8

GENERAL DISCUSSION

As highlighted in the literature review of this thesis (Chapter 2), inflammatory bowel disease (IBD) is an idiopathic chronic inflammatory condition of the gastrointestinal tract (GIT) encompassing two main clinical entities: Crohn's disease (CD) and ulcerative (UC) colitis (Strober et al., 2007; Cosnes et al., 2011; Manichanh et al., 2012). Both CD and UC are associated with multiple pathogenic factors including environmental changes, an array of susceptibility gene variants, a qualitatively and quantitatively abnormal gut microbiota and a broadly dysregulated immune response (Strober et al., 2002; Nagalingam et al., 2011; Manichanh et al., 2012; Molodecky et al., 2012). However, in spite of this realization and the identification of seemingly pertinent contributory factors, a full understanding of IBD pathogenesis is still out of reach and, consequently, treatment is far from optimal. This calls for more research to further investigate on the perceived factors that might play significant role in the initiation or advancement of IBD, and to explore alternative therapeutic measures that can add to, partially replace or supplement the existing measures. Therefore, the overall objective of the research described in this thesis was to investigate the role of adherent invasive *E. coli (*AIEC) strain UM146, and antepartum use of antibiotics on gut microbiota profile and immune responses in pig and mice models of experimental colitis, and therapeutic role of prebiotics (resistant starch) and probiotics (*E.coli* UM2 and 7).

Degraded carrageenan (CG) has been used previously as a chemical model of colitis in other species including mice, rats, guinea pigs (Watt et al., 1979; Tobacman, 2001b), but not in domestic pig, which is thought to be closer to humans in terms of gastrointestinal physiology

compared to other species (Miller and Ullrey, 1987; Heinritz et al., 2013). In addition, dextran sulphate sodium (DSS) has been utilized extensively in the past majorly in rodents such as mice and rats, and to lesser extends in pigs, to model colitis. However, most of the previous studies using these models have explored the changes in the gut mucosal histomorphology and immune response (Kitajima et al., 2000; Wirtz et al., 2007; Melgar et al., 2008; Yan et al., 2009), and the role of antimicrobial peptides (Shi, 2007), but very few studies have looked at changes in gut microbiota in relation to experimental colitis. Therefore, the main effects studied in the experiments described in this thesis were focused on the changes in the microbiota profile, local inflammation, and their interactions with different dietary additives that can modulate the gut microbiota.

The research described in this thesis was conducted in 4 phases to achieve the overall objective. In phase I, weaner piglets were exposed to 1% degraded carrageenan gum (CG) in drinking water for 21 days, and the piglets were inoculated with adherent invasive *E. coli (*AIEC) strain UM146 on day 8 of the study, in order to evaluate the efficacy of CG in inducing colitis and microbial dysbiosis in pigs, and the causative or consequent role of AIEC in IBD. In phase two, weaner piglets exposed to CG-induced colitis for 21 days (as shown in Phase I) received *E. coli* strain UM2 &7 probiotics, and prebiotics (resistant starch; RS) from day 8 of the study to evaluate therapeutic role of the probiotics and prebiotics. A further study was conducted in this phase to evaluate the protective and therapeutic role of RS (MSPrebiotic) in pigs exposed to CG-induced colitis, but for a relatively longer period of time (40 days). In phase 3, male mice (6 - 7 weeks of age) received 5 % dextran sodium sulphate (DSS) for 5 days to investigate the role of DSS-induced colitis in bacterial dysbiosis. Lastly, in phase 4, pregnant mice received antibiotics during the last 6 days of pregnancy, and the offspring were exposed to DSS-induced colitis at 7

weeks of age, to investigate the impact of antepartum use of antibiotics on offspring initial gut microbiota colonization, and future susceptibility to colitis.

As reported in manuscript I, in line with previous findings in IBD patients, CG-induced bacterial dysbiosis through a decrease in bacterial diversity, change in the structure of the microbiota as well as an increament in the levels of members of Proteobacteria and Defferibacteres but a decrease in Firmicutes, Actinobacteria, and Bacteroidetes at phylum and lower taxonomical levels, and alterations in the microbiome metabolic capacity. However, the role of *E. coli* strain UM146 alone, or in combination with CG was transient or still not clear. As indicated in manuscript I, the reason for the lack of effect by the *E. coli* strain UM146 could be due to the one time inoculation that was employed in our study, which may not have been strong enough to colonize the gastrointestinal tract and hence, cause a significant impact. Also, *E. coli* strain UM146 was initially isolated from a human CD patient and therefore, this might have compromised the ability of the strain to successfully and competitively colonize the pig's gastrointestinal tract. In addition, resident enteric bacteria have been shown to be necessary for the development of spontaneous colitis through immune system activation in IL-10 deficient mice (Sellon et al., 1998), and transient colonization with AIEC was found to be sufficient in triggering chronic intestinal inflammation in mice lacking Toll-like receptor 5 (TLR5) (Chassaing et al., 2014b). Therefore, AIEC role in IBD could be important in genetically susceptible hosts. Nevertheless, re-culturing an infectious agent used for inoculation from the inoculated individual(s) is an important aspect in studies where association or/ and causality are being investigated, and hence, there was need to culture *E. coli* UM146 from the luminal contents or tissue mucosa of the pigs in order to verify our results. However, due to technical issues, we were unable to culture *E. coli* strain UM146 from samples collected during and at the

end of the study and therefore, this was a major weakness in this study that could further complicate the interpretation of the data. Therefore, our model could be improved to ensure and confirm successful colonization by AIEC before a concrete conclusion can be arrived at.

The experiment described in manuscript II was conducted simultaneously with the study described in manuscript I, and here, we investigated therapeutic role of resistant starch (RS) and *E. coli* UM 2 & 7 probiotics or their combination in a pig model of experimental colitis. Although CG-induced colitis caused bacterial dysbiosis, the effects of the additives in modulating gut microbiota were very minimal as indicated by slight modification of bacterial structure and downregulation of proinflammatory cytokines. As mentioned in the discussion section of manuscript II, the lack of great impact could be due to the short duration that the prebiotics and probiotics were administered to the colitic pigs; however, while this may be true especially for the prebiotics, other factors could account for this as well. The timing of application and the dosage are major factors that might greatly influence the success or failure of these products, and therefore, titration studies to determine the dosage are necessary, as well as studies to evaluate different modes and timing of application. In addition, the comparatively low number and diversity of bacterial species available in a typical probiotic preparation in comparison with the gut microbiota, is a major confounding factor of the probiotic approach. Therefore, probiotic bacterial strains may not be able to compete effectively against the complex interactions of an established and adapted indigenous gut microbial community. As in the case of the *E. coli* UM146 described above, another important factor that also lacked in our study was the confirmation of the presence in the GIT or shedding of the *E. coli* UM2&7 probiotics in feces, before the start of their administration, during the period of administration and at the end of the study. This could have partially explained further or clarified the results with respect to the

effect of the probiotics. However, due to technical issues our attempts to culture the probiotics were unsuccessful and therefore, this was a key factor that should be addressed in future studies.

By combining both the prebiotics and probiotics (synbiotic), it was expected that the pigs in this group would have an added advantage over the other pigs; however, the synbiotic did not improve microbiota composition relative to the pigs receiving prebiotics or probiotics. The lack of synbiotic enhanced effect on bacterial composition could not only be ascribed to the inability of the probiotics to effectively colonize the gut due to the lack of clear details on the dosage and also the duration of administration, but also the inability of the probiotics to utilize resistant starch as a substrate, a condition that is expected to give them a competitive advantage. However, it is possible that the changes in gut microbiota emanating from the CG-induced dysbiosis also compromised the ability of commensal gut microbiota to ferment resistant starch, thereby interfering with their capacity to proliferate and modulate the effects of CG.

To address the shorter period and timing of application with respect to the administration of prebiotics as mentioned in manuscript II, we evaluated the protective and therapeutic role of RS (MSPrebiotic) in a pig model of experimental colitis for a relatively longer period of time (manuscript III). This was guided by the fact that a longer period of time may be required for the intestinal microbiota to adapt to a load of fermentable substrate (Gorvers et al., 1999; Martinez-Puig et al., 2003). The results showed that RS could have both protective and therapeutic effects in a pig model of experimental colitis, although in some cases the therapeutic effects were more prominent compared to the protective effects. Different studies have evaluated therapeutic effects of RS but not its protective effects and therefore, this was a strong part of our study, and could as well help in establishing a relatively optimal time of application. The implication of this study was that RS could ameliorate dysbiosis, inflammatory responses, as well as histological mucosa

damage during colitis and therefore, might aid in inducing remission. However, the results also suggest that RS my protect healthy individuals from developing colitis, although the protection may not be so strong based on the strength of the disease at onset. The data from this research suggest two possible explanations for the observed protective and therapeutic effects of resistant starch. First, whereas RS supplementation both as protective or preventive measure did not greatly differ in terms of bacterial diversity and structure, the protective measure was less effective in protecting against diarrhea and histological damage especially in ascending colon, relative to the therapeutic measure. This could indicate that the pigs had established a relatively stable environment in the gut; however, the abrupt introduction of CG broke the stability and severely compromised the mucosal barrier integrity thereby interfering with water reabsorption and consequently reducing fecal consistency. Secondly, why the histological structure particularly in the ascending colon showed less protective effects of RS could suggest that the microbiota in ascending colon could be less effective in utilizing RS. Also, phylogenetic comparisons of ascending colon mucosa-associated microbiota showed no significantly differentially abundant taxa in the RSP group (protective), again supporting the lack of clear effects of RS when used as a protective measure. However, this remains a question warranting further investigations.

Because most of the studies utilizing DSS as a model of inducing colitis have concentrated much on intestinal ulceration and immune responses (Kitajima et al., 2000; Wirtz and Neurath, 2007; Melgar et al., 2008; Yan et al., 2009), it was hypothesized that DSS- induced colitis will cause bacterial dysbiosis, which may exacerbate the disease course (manuscript IV). Also, since many factors may contribute to susceptibility to DSS-induced colitis, it was further hypothesized that antepartum use of antibiotics may negatively influence the nature of offspring

initial bacterial colonization, consequently predisposing them to colitis later in life (manuscript V). We have demonstrated that in addition, to the already known increased disease activity, histological damage, and inflammatory responses, DSS also induced bacterial dysbiosis, although the changes were more pronounced in fecal samples compared to the colonic samples. This is possible since passage of fecal matter through the colon may cause sloughing off of mucus debris and hence, the microbiota attached to it, thereby increasing bacterial load in the feces. Other studies have also reported changes in microbiota composition including a decrease in diversity, following DSS-induced colitis in mice (Nagalingam et al., 2011; Berry et al., 2012), and this also agrees with observations made in IBD patients. In manuscript IV (Munyaka et al., 2016 a), we demonstrated that antepartum exposure to antibiotics affects the nature of offspring initial bacterial colonization and consequently predispose to colitis later in life. However, while the results are quite informative, the sample size in this study was small and therefore, a larger sample size may strengthen the conclusions drawn from this study. Nevertheless, the results are in agreement with previous studies in which administration of antibiotics to pregnant mothers reduced the neonates' microbial diversity (Collignon et al., 2010), which can be explained by the fact that antepartum antibiotics may affect the bacterial composition of the mother's birth canal and or skin, which will be transmitted to the babies during and following delivery (Palmer et al., 2007; Biasucci et al., 2010; Dominguez-Bello et al., 2010). This may further have implications on neonatal immune system maturation (Noverr and Huffnagle, 2005; Renz et al., 2012), that play a major role in disease resistance or susceptibility.

Generally, studies using animal models are prone to several confounding factors that could influence the response. In this context, both CG and DSS were administered in drinking water and therefore, this may influence the results as the intake may vary among individuals,

thereby skewing the results, with higher consumption translating to great impact. However, this was not a problem in mice studies as their water consumption is more uniform and they also don't waste the drink (based on our experience), but it could be a major problem with pigs due to their large size. Monitoring consumption rate could be difficult as pigs tend to play around with water nipples and therefore, much of the drink might be going to waste and get mixed up with urine, making it difficult to account for the wastage. Also, in the first three manuscripts, the pigs were used immediately after weaning and during their first days after separation from their mothers, which in itself might be a cofounding factor especially in microbial studies. In addition, at this stage of life, pigs grow very fast and therefore, the rapid physiological changes taking place might influence the experimental effects. This can be overcome by using pigs at their adult stage where the rate of growth and physiological changes have a minimum effect, however, handling becomes a major draw back for adult domestic pigs. On another note, bacteriology in both CD and UC may differ based on age or stage of the disease, and on the nature of treatment and therefore, this may complicate interpretation of data from different studies, and between human and animal models. Moreover, the commonly known beneficial bacteria in humans for example *Bifidobacterium* and *Lactobacillus* may differ from those found in mice and pigs in terms of their abundance and available sub-species (Heinritz et al., 2013), and this may be true to many other bacteria. This could therefore significantly influence the interpretation of the information generated from both humans and animal models, and could also partially explain the inconsistencies reported among microbiota studies in IBD, including the case of probiotics. Nevertheless, some specific taxa including members of Enterobacteraceae that have been previously and repeatedly associated with IBD, were also associated with CG-induced colitis supporting the validity of our model. Interestingly, these taxa and others such as members of

Deferribacteres and Fusobacteria that were associated with CG-induced colitis in our studies, were either low in abundance or completely not associated with the therapeutic measures employed in our studies, suggesting merit in the use of the therapeutic measures used in this thesis particularly the prebiotics (RS; MSPrebiotics).

On another note, the fact that we tend to associate a specific taxa or a group of microbiota with similar characteristics with a specific health condition (beneficial or pathogenic) is questionable as these taxa may contain specific members that are actually beneficial while others are pathogenic. For example, even though family Enterobacteraceae particularly *E. coli* is commonly associated with IBD conditions, we also know that there are specific *E. coli* strains that are not harmful and others actually have probiotic effects. In this regard, besides the probiotic strains used in this study, other members of *Escherichia coli* have been found to have probiotic effects including *E. coli* Nissle 1917 and *E. coli* strain HS (Maltby et al., 2013; Sassone-Corsi and Raffatellu, 2015). Similarly, genera *Lactobacillus* is generally known for it's beneficial effects; however, *Lactobacillus* was enriched in the colon of the colitic pigs, and similar results have been reported in colonic mucosal biopsy samples of patients with active IBD (Wang et al., 2014). The species of *Lactobacillus* are phylogenetically diverse (Goh and Klaenhammer, 2009), and therefore, characterization of *Lactobacillus* genus at lower levels may help to elucidate on specific members that could be associated with IBD conditions during active phase and their possible roles. This therofore challenges the use of a broad classification and it might need to be revised especially with the improvement in laboratory techiques that can aid in identifying specific species or strains.

In the pig experiments (manuscript I, II and III), we examined intestinal tissue samples from the ileum, cecum, ascending and descending colon. This was important since the specific

section of the intestinal tract that would be more affected by CG-induced colitis is not clearly established. In this context, although some bacterial changes and inflammation were observed in the ileum, the effects were not very strong and therefore, most of bacterial changes resulting from CG-induced colitis or the intervention measures used were observed in the large intestines. However, it should be noted that even in the large intestines, the results were not very consistent as in some cases less changes were observed in the ascending colon compared to the cecum and descending colon (manuscript I), and in other cases, mixed responses were observed (manuscript II and III). The results may therefore suggest that the impact of CG is majorly confined in the large intestines, which mimics UC as opposed to CD, whereas the inconsistencies observed in the large intestines with regard to microbiota changes, may be partially explained by the rate of movement of luminal contents in different sections of the large intestines, and hence the amount of time spent, and it could also be due to differences in microbiota composition in each intestinal section.

Overall, results of the studies described herein suggest that both CG- and DSS-induced colitis caused bacterial dysbiosis, and that, exposure to antepartum antibiotics increased susceptibility to DSS-induced colitis later in life. However, inoculation of pigs with *E. coli* UM146 was not able to induce or exacerbate dysbiosis and inflammation, and neither did the administration of *E. coli* UM2 & 7 probiotics and RS for two weeks greatly influence bacterial composition in colitic pigs. Nevertheless, RS showed both protective and therapeutic effects when administered for a relatively longer period of time, although the therapeutic effects were more prominent relative to the protective effects. The results provide insight into the use of domestic pigs to model human colitis and partially explain why the use of prebiotics may yield varying results based on the duration of administration. In addition, the studies have

demonstrated the need to improvise on experimental techniques to verify successful colonization by infectious inoculants and probiotic preparations in order to be able to make more informative conclusions with respect to their role in experimental colitis. Furthermore, the studies provide useful information that may aid in decision-making regarding the use of antibiotics around the time of delivery.

CHAPTER NINE

CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

9.1. CONCLUSIONS

The following conclusions can be drawn based on the studies described in this thesis:

1. Administration of 1 % CG for 21days induced colitis and consequently caused bacterial dysbiosis in a pig model of experimental colitis. CG can therefore be used to mimic human ulcerative colitis.

2. Inoculation of pigs with *E. coli* UM146 alone did not cause inflammation or bacterial dysbiosis in the gut and neither did its combination with CG exacerbate the state of colitis or bacterial dysbiosis. This could be due to the one point inoculation that was used in our study, and therefore, the amount of inoculant could have been weak to compete with the existing microbiota.

3. Administration of *E. coli* UM 2&7 probiotics and RS or their combination for 2 weeks in colitic pigs had minimal effects in attenuating bacterial dysbiosis. This could be due to the short period of administration that was employed in our study, the dose, and or the inability of the probiotics to utilize RS as a substrate.

4. Administration of RS both as a protective or therapeutic measure was effective in ameliorating bacterial dysbiosis and local inflammation when used for a relatively longer period of time (25 - 40 days). However, in some cases, the therapeutic effects were more prominent relative to the protective effects an observation that warrants further investigations.

5. Administration of 5% DSS for five days induced colitis and consequently caused bacterial dysbiosis in mice model of experimental colitis.

6. Antepartum exposure to antibiotics influenced the nature of offspring initial bacterial colonization consequently predisposing them to colitis later in life.

9.2. SIGNIFICANCE

The work described in this thesis constitutes a significant contribution to the field of IBD pathogenesis and management/treatment. Firstly, it expounds on the role of both DSS- and degraded carrageenan gum-induced colitis on gut microbiota dysbiosis. Most of the previous studies using DSS or CG colitis models explored changes in the gut mucosal histomorphology and immune responses (Kitajima et al., 2000; Wirtz et al., 2007; Melgar et al., 2008; Yan et al., 2009), and the role of antimicrobial peptides (Shi, 2007), but very few studies have looked at changes in gut microbiota in relation to experimental colitis. The work described in this thesis has highlighted an important aspect regarding the development of IBD that could be associated with interactions between inflammation in the gut and changes in the composition, distribution and functional activities of gut microbiota. This may partially explain the contributions of the gut microbiota in IBD and may present gut microbiota as an important therapeutic target in the management of IBD.

Another important contribution of the research described in this thesis is the characterization of the impact of antepartum antibiotic (ATB) treatment on the offspring intial gut microbiota colonization and future susceptibility to colitis. The use of antibiotics around the time of delivery is critical especially in mothers suspected to have group B Streptococci (GBS) infection that could be transmitted to the newborn during birth. Interestingly, very little attention has been directed to the possible detrimental effects that the antibiotics might have on the

newborn regarding IBD development. This work has therefore validated the deleterious effect of ATB treatment in the development of IBD on the offspring. Also, future identification of management/treatment strategies that can mitigate the impact of ATB on offspring gut microbiota may lead to novel public recommendations to decrease incidences of paediatric IBD, which could be of significant importance to mothers who have a diagnosis of GBS. In addition, the research may open avenues to explore viable solutions to reverse microbiota dysbiosis in mothers exposed to ATB, thus attenuate offspring gut microbiota dysbiosis and susceptibility to colitis later in life.

Finally, there are a lot of inconsistencies associated with the use of prebiotics in the management of IBD. This work highlights an important aspect in the importance of resistant starch in modulating gut bacterial dysbiosis, inflammation and histological damage during active colitis and provides insights regarding the duration and timing of application of resistant starch, a dietary product that does not pose any serious health risk. This could positively contribute in the management of IBD particularly in patients with active ulcerative colitis. It also holds implications not only for IBD but also several other gastrointestinal infections which ae driven by inflammation and or gut microbiota dysbiosis.

9.3. FUTURE DIRECTIONS

1. Because the response of *E. coli* UM146 inoculation was not clear, and unsuccessful colonization was suspected, there is need to improvise the protocol used in this thesis in order to confirm the absence of this *E. coli* before the start of the experimental procedures, and to confirm successful/unsuccessful colonization following inoculation.

2. The impact of the *E. coli* UM 2&7 probiotics used in this thesis was not clear, therefore further research is needed in order to titrate the administration of these probiotics so as to

establish the correct dose, and to establish the best method of administration for a successful colonization.

3. With the modifications mentioned in 1 and 2 above, there is need to replicate the pig studies described in this thesis with a larger sample size, but using miniature pigs. Miniature pigs are smaller in size and relatively lighter at adult stage, and therefore this will overcome the challenges of handling, rapid growth and physiological changes. Also, animals are likely to exhibit inter individual differences in response to different factors and therefore, permissiveness and resistance are factors that might need to be considered especially in response to inoculation with infectious agents and administration of probiotics/prebiotics, and even though it reflects what might as well happen in human subjects, a larger sample size may minimize the impact of these kinds of variations.

4. In our mice studies, acute DSS-induced colitis was used. There is need to characterize bacterial changes in response to a chronic model of DSS-induced colitis.

5. There is need to replicate the antepartum antibiotic use and susceptibility to colitis later in life using a larger sample size, and to investigate alternative therapeutic measures that can be used to modify gut microbiota following exposure to antepartum antibiotics, and hence, protect against colitis later in life.

6. In both the pig and mice studies described in this thesis, male animals were used and therefore, there is need to employ both genders and establish whether there would be any differences in terms of colitis susceptibility, microbiota changes, and response to probiotics and prebiotics.

CHAPTER TEN

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CHAPTER 11

APPENDICES

APPENDIX 1

Supplementary Table 3.1. Analysis of the most abundant phyla in each intestinal segment of pigs treated with degraded carrageenan gum (CG)[1] and infected with adhered invasive *E. coli* strain UM146[2]

Items	Treatments					
	UM 146	**CG**	**CGUM146**	**Control**	**SED**	***P value***
Ileum						
Firmicutes	76.8695	93.3542	69.9675	73.6225	12.7	0.2588
Bacteroidetes	7.6663	2.0287	9.4842	2.0326	4.3	0.1382
Proteobacteria	8.4468	6.4346	15.0357	10.3429	2.9	0.2312
Tenericutes	0.02587	0.0579	0.2372	0.0899	0.08	0.1217
Cecum						
Firmicutes	42.0656	48.0226	36.0631	34.9073	10.8	0.5668
Bacteroidetes	35.1657^{a}	4.7135^{c}	14.7126^{abc}	28.961^{ab}	7.8	**0.002**
Proteobacteria	26.2615	52.8395	51.0098	43.4782	13.5	0.3568
Deferribacteres	0.1138	0.9278	0.7182	0.8592	0.45	0.3257
Ascending Colon						
Firmicutes	77.044^{a}	48.67^{b}	40.854^{b}	55.326^{ab}	8.5	**0.0070**
Bacteroidetes	17.444^{ab}	5.027^{cb}	27.3776^{a}	26.3016^{a}	0.3	**<.0001**
Proteobacteria	3.3882^{b}	24.2887^{a}	18.366^{a}	11.048^{b}	0.2	**0.001**
Spirochaetes	0.4752	0.5688	1.1368	1.1202	0.6	0.5348
Deferribacteres	0.9962^{b}	2.4868^{a}	4.246^{a}	0.3614^{b}	0.3	**0.0393**
Descending Colon						
Firmicutes	37.4655^{ab}	30.2227^{b}	31.0306^{b}	49.9345^{a}	5.6	**0.0055**
Bacteroidetes	57.268^{a}	18.967^{b}	56.162^{a}	42.38^{a}	5.6	**<.0001**
Proteobacteria	3.2064^{b}	35.8847^{a}	7.7043^{b}	4.2591^{b}	0.4	**<.0001**

Tenericutes	0.688[ab]	0.282[b]	0.5027[b]	0.9955[a]	0.1	**0.0047**
Deferribacteres	0.05434	0.8774	1.2915	0.03921	0.5	0.1201

[1]CG was administered to the designated groups from d 1 of the experiment.

[2]*E. coli* UM146 infection was administered on d 8 of the experiment. Pigs in designated groups received 100 mL of an overnight *E. coli* UM146 culture (108 cfu/mL) in feed.

CG= pigs received 1% carrageenan gum only in drinking water on daily basis, UM146=pigs were infected with *E. coli* UM146 on d 8 of the study, CGUM146=pigs received 1 % CG from d 1 of the study and were infected with *E. coli* UM146 on d 8 of the study.

Supplementary Table 3.2. A summary showing mean relative abundances of taxa in **Ileal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
----------Greater than or equal to 0.01%----------				
g. Corynebacterium	0.001	0.033	0.009	0.071
g. Kocuria	0.047	0.033	0.000	0.002
g. Bifidobacterium	0.033	0.056	0.001	0.046
o. Bacteroidales	0.351	1.776	0.184	1.099
g. 5-7N15	0.000	0.010	0.053	0.010
g. Bacteroides	0.009	0.586	0.042	2.388
g. Parabacteroides	0.006	0.149	0.039	0.015
g. Prevotella	1.531	4.255	1.305	5.523
f. Rikenellaceae	0.000	0.008	0.013	0.036
f. S24-7	0.090	0.560	0.208	0.296
g. CF231	0.018	0.230	0.000	0.025
f. Chitinophagaceae	0.013	0.041	0.055	0.058
g. Hymenobacter	0.000	0.000	0.109	0.000
o. YS2	0.015	0.008	0.013	0.015
o. CAB-I	0.451	0.046	0.196	0.013
o. Streptophyta	0.001	0.005	0.003	0.095
g. Mucispirillum	0.022	0.393	0.010	0.137
p. Firmicutes	0.069	0.015	0.026	0.066
f. Staphylococcaceae	0.000	0.043	0.290	0.003
o. Lactobacillales	0.014	0.037	0.009	0.006
g. Enterococcus	0.000	0.626	0.000	0.010
f. Enterococcaceae	0.655	0.120	0.868	1.037
f. Lactobacillaceae	0.084	0.037	0.198	0.147
g. Lactobacillus	28.560	62.010	77.631	34.800
f. Streptococcaceae	2.880	0.470	1.750	0.524
g. Streptococcus	0.756	4.859	0.485	0.304
g. Turicibacter	0.029	0.015	0.033	0.019
c. Clostridia	5.987	0.063	0.192	0.700
o. Clostridiales	0.092	0.151	0.162	0.462
f. Christensenellaceae	0.009	0.006	0.089	0.026
f. Clostridiaceae	0.254	0.282	0.094	16.969
g. Clostridium	0.356	0.000	0.006	0.013
g. Sarcina	12.519	0.037	1.191	0.071
f. Lachnospiraceae	0.092	0.348	0.625	0.634
g. Blautia	0.206	0.512	0.567	0.492
g. Butyrivibrio	0.015	0.012	0.007	0.007
g. Coprococcus	0.022	0.039	0.007	0.101

Supplementary Table 3.2. A summary showing mean relative abundances of taxa in **Ileal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Dorea	0.076	0.165	0.337	0.137
g. Lachnospira	0.007	0.017	0.000	0.102
g. Oribacterium	0.013	0.070	0.002	0.057
g. Roseburia	0.307	0.533	0.169	0.397
f. Lachnospiraceae	0.141	0.141	0.309	0.517
f. Peptostreptococcaceae	9.219	0.044	0.378	0.143
f. Ruminococcaceae	0.209	0.582	0.450	0.754
f. Ruminococcaceae	0.297	0.408	0.629	0.484
g. Faecalibacterium	0.174	0.278	0.216	0.482
g. Oscillospira	0.078	0.239	0.069	0.174
g. Ruminococcus	8.490	0.445	0.685	1.432
f. Veillonellaceae	0.181	0.450	0.522	0.605
g. Acidaminococcus	0.006	0.000	0.000	0.048
g. Anaerovibrio	0.107	0.509	0.261	0.177
g. Dialister	0.693	0.448	0.734	1.877
g. Megasphaera	0.138	1.985	0.657	4.663
g. Mitsuokella	0.057	0.158	0.006	0.506
g. Phascolarctobacterium	0.015	0.031	0.010	0.079
g. Selenomonas	0.003	0.155	0.000	0.048
g. Veillonella	0.493	0.274	0.023	0.347
f. Coriobacteriaceae	0.024	0.023	0.100	0.024
f. Erysipelotrichaceae	0.002	0.002	0.014	0.118
g. Allobaculum	0.021	0.004	0.223	0.007
g. Bulleidia	0.058	0.033	0.019	0.106
f. Erysipelotrichaceae	0.076	0.107	0.356	0.133
o. Erysipelotrichales	0.001	0.000	0.039	0.000
g. Catenibacterium	0.083	0.000	0.147	0.063
g. Sharpea	0.000	0.010	0.117	0.006
f. Fusobacteriaceae	0.000	0.000	0.003	0.206
o. RF32	0.009	0.128	0.026	0.019
f. Bradyrhizobiaceae	0.002	0.002	0.005	0.027
g. Methylobacterium	0.000	0.000	0.002	0.035
f. mitochondria	0.003	0.002	0.022	0.263
g. Sphingobium	0.040	0.000	0.186	0.094
g. Sphingomonas	0.001	0.012	0.022	0.009
c. Betaproteobacteria	0.017	0.077	0.334	0.019
g. Sutterella	0.052	0.263	0.086	0.793
f. Comamonadaceae	0.006	0.012	0.061	0.041

Supplementary Table 3.2. A summary showing mean relative abundances of taxa in **Ileal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Comamonas	0.007	0.041	0.077	0.000
g. Hylemonella	0.000	0.026	0.000	0.036
f. Oxalobacteraceae	0.008	0.019	0.317	0.036
g. Desulfovibrio	0.050	0.360	0.018	0.187
g. Campylobacte	1.691	0.482	0.366	4.433
g. Helicobacter	0.065	0.581	0.024	1.906
f. Succinivibrionaceae	0.006	0.000	0.008	0.073
g. Succinivibrio	0.049	0.128	0.141	0.112
f. Enterobacteriaceae	0.014	0.391	0.571	3.634
g. Enterobacter	0.000	0.004	0.030	0.172
g. Escherichia	0.281	5.409	0.417	2.319
f. Pasteurellaceae	10.343	5.803	0.904	0.199
g. Actinobacillus	0.028	0.025	0.014	0.002
g. Acinetobacter	0.032	0.019	0.271	0.028
f. Pseudomonadaceae	0.269	0.356	1.330	0.251
g. Pseudomonas	0.004	0.004	0.026	0.017
f. Xanthomonadaceae	0.051	0.050	0.780	0.217
g. Treponema	0.000	0.271	0.000	0.000
c. Mollicutes	0.000	0.066	0.000	0.029
g. RFN20	0.008	0.023	0.000	0.015
g. Mycoplasma	10.547	0.005	0.000	4.491
o. RF39	0.056	0.138	0.224	0.097
Unclassified	0.049	0.043	0.055	0.212
----------Less than 0.01%----------				
o. Acidimicrobiales	0.0000	0.0000	0.0032	0.0024
o. Actinomycetales	0.0017	0.0000	0.0000	0.0094
g. Microbispora	0.0000	0.0186	0.0000	0.0118
g. Mycobacterium	0.0000	0.0000	0.0000	0.0040
f. BS11	0.0000	0.0020	0.0010	0.0024
g. Paludibacter	0.0000	0.0000	0.0000	0.0028
g. Porphyromonas	0.0009	0.0000	0.0032	0.0000
g. Odoribacter	0.0000	0.0062	0.0000	0.0033
f. Flavobacteriaceae	0.0028	0.0013	0.0000	0.0073
f. Pedobacter	0.0100	0.0000	0.0134	0.0012
c. Bacilli	0.0028	0.0000	0.0000	0.0008
f. Bacillaceae	0.0029	0.0000	0.0063	0.0000
g. Geobacillus	0.0028	0.0000	0.0072	0.0019
f. Planococcaceae	0.0000	0.0012	0.0010	0.0000

Supplementary Table 3.2. A summary showing mean relative abundances of taxa in **Ileal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Staphylococcus	0.0000	0.0103	0.0065	0.0000
g. Lactococcus	0.0028	0.0000	0.0016	0.0000
f. Catabacteriaceae	0.0166	0.0000	0.0000	0.0017
g. Peptoniphilus	0.0000	0.0000	0.0058	0.0000
g. Anaerostipes	0.0000	0.0000	0.0099	0.0167
g. Lachnobacterium	0.0017	0.0000	0.0000	0.0033
g. Peptococcus	0.0017	0.0103	0.0167	0.0023
g. rc4-4	0.0006	0.0116	0.0000	0.0000
o. Coriobacteriales	0.0000	0.0000	0.0000	0.0049
g. Collinsella	0.0024	0.0021	0.0000	0.0072
g. p-75-a5	0.0055	0.0000	0.0016	0.0000
g. Coprobacillus	0.0048	0.0000	0.0000	0.0165
f. Caulobacteraceae	0.0078	0.0000	0.0072	0.0123
o. Ellin329	0.0018	0.0000	0.0000	0.0049
g. Bradyrhizobium	0.0006	0.0000	0.0070	0.0047
g. Hyphomicrobium	0.0012	0.0000	0.0164	0.0023
f. Rhodospirillaceae	0.0028	0.0000	0.0000	0.0008
o. Sphingomonadales	0.0041	0.0000	0.0000	0.0000
g. Acidovorax	0.0116	0.0037	0.0000	0.0000
g. Janthinobacterium	0.0009	0.0000	0.0130	0.0094
g. Ralstonia	0.0009	0.0000	0.0000	0.0094
c. Gammaproteobacteria	0.0000	0.0000	0.0000	0.0035
g. Anaerobiospirillum	0.0000	0.0000	0.0000	0.0254
g. Erwinia	0.0006	0.0128	0.0000	0.0000
g. Trabulsiella	0.0000	0.0041	0.0000	0.0144
g. Yersinia	0.0083	0.0000	0.0065	0.0052
g. Alkanindiges	0.0000	0.0012	0.0261	0.0000
f. Sinobacteraceae	0.0000	0.0062	0.0020	0.0017
g. Stenotrophomonas	0.0000	0.0413	0.0000	0.0035
g. Candidatus cloacamonas	0.0000	0.0054	0.0000	0.0071
g. Akkermansia	0.0000	0.0041	0.0020	0.0000

* Mean values only, no statistics

Supplementary Table 3.3. A summary showing mean relative abundances of taxa in **Cecal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
----------Greater than or equal to 0.01%----------				
g. Bifidobacterium	0.017	0.039	0.303	0.007
o. Bacteroidales	0.344	0.347	0.026	0.088
o. Bacteroidales	1.546	0.952	0.261	0.608
g. Bacteroides	0.014	0.017	0.424	4.329
g. Parabacteroides	0.147	0.084	0.038	0.050
g. Prevotella	12.610	16.819	1.138	7.683
f. S24-7	3.570	0.587	0.185	0.308
g. Butyricimonas	0.154	0.000	0.000	0.000
g. CF231	1.238	0.541	0.240	0.124
o. Bacteroidales	9.293	10.022	2.180	2.964
o. YS2	0.031	0.009	0.015	0.014
g. Mucispirillum	2.067	0.097	1.836	2.739
p. Firmicutes	0.136	0.154	0.149	0.113
o. Lactobacillales	0.008	0.006	0.058	0.007
f. Lactobacillaceae	0.080	0.089	0.469	0.327
f. Lactobacillaceae	0.013	0.025	0.110	0.062
g. Lactobacillus	5.845	24.427	24.886	8.046
f. Streptococcaceae	0.044	0.065	0.734	0.250
f. Streptococcaceae	0.000	0.000	0.064	0.004
g. Streptococcus	0.028	0.058	0.209	0.143
g. Turicibacter	0.020	0.025	0.036	0.006
c. Clostridia	0.175	0.162	0.076	0.105
c. Clostridia	0.041	0.105	0.051	0.172
o. Clostridiales	0.112	0.127	0.075	0.064
f. Christensenellaceae	0.007	0.021	0.010	0.007
f. Clostridiaceae	0.056	0.058	0.131	0.061
f. Clostridiaceae	0.096	0.116	0.138	0.223
g. Clostridium	0.029	0.002	0.006	0.006
g. Sarcina	0.957	0.032	0.158	0.088
f. Lachnospiraceae	0.545	0.757	0.550	0.560
f. Lachnospiraceae	0.046	0.110	0.067	0.089
g. Blautia	0.812	1.083	0.818	0.525
g. Butyrivibrio	0.007	0.015	0.026	0.012
g. Coprococcus	0.169	0.116	0.144	0.228
g. Dorea	0.204	0.378	0.274	0.171
g. Lachnospira	0.020	0.062	0.024	0.030
g. Oribacterium	0.089	0.074	0.023	0.069

Supplementary Table 3.3. A summary showing mean relative abundances of taxa in **Cecal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Roseburia	1.250	1.644	1.984	1.559
f. Lachnospiraceae	0.608	0.620	0.561	0.529
g. Peptococcus	0.026	0.046	0.033	0.030
f. Peptostreptococcaceae	0.142	0.206	0.333	0.069
f. Ruminococcaceae	4.286	4.060	3.313	3.728
g. Anaerotruncus	0.019	0.002	0.010	0.009
g. Faecalibacterium	3.227	2.887	1.155	0.786
g. Oscillospira	1.077	0.410	0.768	0.540
g. Ruminococcus	1.213	0.934	0.649	1.523
f. Veillonellaceae	1.891	3.925	1.183	1.217
g. Acidaminococcus	0.689	0.408	0.174	0.129
g. Anaerovibrio	0.541	0.317	0.507	0.639
g. Dialister	3.106	3.954	1.508	3.382
g. Megasphaera	0.996	2.593	1.652	4.136
g. Mitsuokella	0.357	0.219	0.162	0.152
g. Phascolarctobacterium	0.181	0.318	0.057	0.153
g. Selenomonas	0.005	0.108	0.023	0.013
g. Veillonella	0.005	0.005	0.005	0.026
f. Coriobacteriaceae	0.721	0.013	0.466	0.025
g. Collinsella	0.029	0.010	1.484	0.018
g. Slackia	0.013	0.000	0.110	0.000
g. Bulleidia	0.100	0.170	0.101	0.075
f. Erysipelotrichaceae	0.361	0.282	0.934	0.554
g. p-75-a5	0.041	0.012	0.029	0.011
g. Catenibacterium	0.334	0.038	0.213	0.275
f. Fusobacteriaceae	0.004	0.000	0.000	0.107
f. Mitochondria	0.000	0.001	0.013	0.056
g. Sutterella	0.114	0.115	0.047	0.556
g. Hylemonella	0.000	0.000	0.085	0.000
f. Desulfovibrionaceae	0.006	0.017	0.027	0.009
g. Desulfovibrio	0.896	0.819	0.685	0.721
o. Campylobacterales	0.070	0.063	0.230	0.146
g. Campylobacter	4.640	12.429	7.898	23.385
f. Helicobacteraceae	0.013	0.004	0.005	0.029
g. Helicobacter	31.140	4.443	36.639	23.696
f. Succinivibrionaceae	0.002	0.002	0.025	0.035
g. Anaerobiospirillum	0.082	0.034	0.041	0.017
g. Succinivibrio	0.029	0.279	0.068	0.070

Supplementary Table 3.3. A summary showing mean relative abundances of taxa in **Cecal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
f. Enterobacteriaceae	0.012	0.005	0.030	0.328
g. Escherichia	0.101	0.049	0.043	0.137
f. Pasteurellaceae	0.140	0.018	0.011	0.001
f. Pseudomonadaceae	0.008	0.049	0.036	0.016
f. Xanthomonadaceae	0.006	0.015	0.018	0.002
g. Treponema	0.151	0.186	0.015	0.045
g. RFN20	0.059	0.047	0.139	0.022
g. Mycoplasma	0.007	0.000	0.000	0.147
o. RF39	0.244	0.236	0.156	0.230
Unclassified	0.271	0.267	0.165	0.225
----------Less than 0.01%----------				
g. Kocuria	0.0018	0.0038	0.0075	0.0000
f. Nocardioidaceae	0.0011	0.0000	0.0025	0.0008
p. Bacteroidetes	0.0128	0.0044	0.0014	0.0078
f. BS11	0.0025	0.0000	0.0026	0.0007
f. Porphyromonadaceae	0.0117	0.0013	0.0000	0.0000
g. Paludibacter	0.0018	0.0016	0.0010	0.0000
f. Prevotellaceae	0.0026	0.0000	0.0000	0.0000
f. Rikenellaceae	0.0068	0.0000	0.0007	0.0007
g. YRC22	0.0032	0.0020	0.0015	0.0029
f. Flavobacteriaceae	0.0004	0.0000	0.0088	0.0004
f. Chitinophagaceae	0.0010	0.0000	0.0012	0.0000
g. Pedobacter	0.0032	0.0000	0.0049	0.0004
f. Chlamydiaceae	0.0000	0.0000	0.0020	0.0021
o. CAB-I	0.0000	0.0023	0.0031	0.0012
o. Streptophyta	0.0000	0.0000	0.0105	0.0028
f. Elusimicrobiaceae	0.0009	0.0024	0.0000	0.0000
c. Bacilli	0.0000	0.0000	0.0003	0.0033
f. Planococcaceae	0.0000	0.0023	0.0021	0.0000
g. Lactococcus	0.0021	0.0023	0.0000	0.0000
f. Catabacteriaceae	0.0059	0.0000	0.0117	0.0004
g. Anaerostipes	0.0038	0.0132	0.0052	0.0021
g. Lachnobacterium	0.0018	0.0000	0.0100	0.0008
o. Clostridiales	0.0037	0.0000	0.0000	0.0000
g. Olsenella	0.0063	0.0000	0.0015	0.0024
g. Allobaculum	0.0177	0.0015	0.0136	0.0008
g. L7A_E11	0.0021	0.0000	0.0003	0.0000
g. cc_115	0.0021	0.0000	0.0013	0.0000

Supplementary Table 3.3. A summary showing mean relative abundances of taxa in **Cecal** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	**UM146**	**CG**	**CGUM146**
g. Coprobacillus	0.0039	0.0000	0.0096	0.0000
g. Sharpea	0.0115	0.0145	0.0096	0.0000
f. Victivallaceae	0.0021	0.0006	0.0000	0.0000
c. OP8_1	0.0008	0.0000	0.0015	0.0000
p. Proteobacteria	0.0144	0.0040	0.0013	0.0108
o. RF32	0.0148	0.0000	0.0030	0.0008
g. Methyloba	0.0000	0.0000	0.0005	0.0008
g. Paracoc	0.0042	0.0000	0.0046	0.0000
f. Sphingomonadaceae	0.0000	0.0000	0.0050	0.0017
g. Sphingobium	0.0000	0.0008	0.0200	0.0008
g. Sphingomonas	0.0000	0.0000	0.0074	0.0008
c. Betaproteobacteria	0.0014	0.0029	0.0005	0.0000
c. Betaproteobacteria	0.0034	0.0000	0.0012	0.0000
o. Burkholderiales	0.0004	0.0008	0.0000	0.0059
f. Comamonadaceae	0.0011	0.0000	0.0050	0.0000
g. Acidovorax	0.0011	0.0000	0.0060	0.0008
f. Oxalobacteraceae	0.0034	0.0000	0.0062	0.0000
g. Oxalobacter	0.0023	0.0045	0.0024	0.0000
g. Ralstonia	0.0000	0.0000	0.0000	0.0000
c. Deltaproteobacteria	0.0000	0.0016	0.0103	0.0000
o. Desulfovibrionales	0.0011	0.0000	0.0000	0.0022
o. GMD14H09	0.0204	0.0098	0.0017	0.0017
f. Syntrophaceae	0.0000	0.0000	0.0012	0.0000
f. Campylobacteraceae	0.0000	0.0000	0.0007	0.0018
c. Gammaproteobacteria	0.0000	0.0000	0.0000	0.0015
g. Enterobacter	0.0000	0.0000	0.0006	0.0229
g. Yersinia	0.0000	0.0023	0.0000	0.0004
g. Actinobacillus	0.0046	0.0016	0.0000	0.0014
g. Acinetobacter	0.0000	0.0093	0.0095	0.0000
g. Enhydrobacter	0.0000	0.0006	0.0010	0.0004
g. Brachyspira	0.0013	0.0000	0.0007	0.0019
f. Dethiosulfovibrionaceae	0.0009	0.0058	0.0000	0.0000
c. Mollicutes	0.0052	0.0258	0.0000	0.0036
f. Anaeroplasmataceae	0.0018	0.0000	0.0000	0.0042
f. RFP12	0.0000	0.0000	0.0000	0.0000

* Mean values only, no statistics

Supplementary Table 3.4. A summary showing mean relative abundances of taxa in the **Ascending colon** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
----------Greater than or equal to 0.01%----------				
g. Bifidobacterium	0.121	1.074	0.032	0.100
p. Bacteroidetes	0.030	0.026	0.014	0.006
o. Bacteroidales	2.752	1.649	0.634	1.573
g. Bacteroides	0.124	0.007	0.345	8.367
g. Parabacteroides	0.176	0.057	0.115	0.419
g. Prevotella	8.900	4.936	1.770	6.340
f. S24-7	1.542	3.125	1.980	2.219
g. Butyricimonas	0.039	0.037	0.013	0.005
g. CF231	1.557	0.370	0.115	0.256
o. Bacteroidales	7.419	7.211	3.549	5.127
o. YS2	0.025	0.018	0.001	0.003
o. Streptophyta	0.003	0.004	0.018	0.017
g. Mucispirillum	6.103	0.996	17.015	10.147
p. Firmicutes	0.209	0.312	0.150	0.079
f. Lactobacillaceae	0.221	0.184	0.409	0.432
g. Lactobacillus	11.393	24.692	13.832	9.541
f. Streptococcaceae	0.181	0.031	0.560	0.165
g. Streptococcus	0.237	0.110	0.382	0.229
c. Clostridia	0.353	0.298	0.241	0.564
o. Clostridiales	0.317	0.249	0.260	0.153
f. Catabacteriaceae	0.045	0.052	0.057	0.001
f. Christensenellaceae	0.015	0.031	0.029	0.012
f. Clostridiaceae	0.503	0.446	0.275	0.490
g. Sarcina	2.269	0.004	0.109	0.082
f. Lachnospiraceae	0.965	0.676	0.668	0.640
g. Blautia	1.454	2.027	0.841	0.438
g. Coprococcus	0.251	0.411	0.160	0.180
g. Dorea	0.351	0.480	0.327	0.070
g. Lachnospira	0.030	0.043	0.003	0.015
g. Oribacterium	0.119	0.057	0.012	0.063
g. Roseburia	4.122	1.295	11.812	1.146
f. Lachnospiraceae	0.788	1.060	0.646	0.333
g. Peptococcus	0.071	0.124	0.098	0.023
f. Peptostreptococcaceae	0.047	0.031	0.019	0.003
f. Ruminococcaceae	6.169	12.296	4.988	6.083
g. Anaerotruncus	0.073	0.038	0.034	0.032

Supplementary Table 3.4. A summary showing mean relative abundances of taxa in the **Ascending colon** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Faecalibacterium	6.025	7.007	2.036	3.589
g. Oscillospira	1.785	2.768	2.177	3.127
g. Ruminococcus	2.187	1.892	3.029	2.850
f. Veillonellaceae	5.833	5.066	0.603	1.371
g. Acidaminococcus	0.375	0.375	0.138	0.129
g. Anaerovibrio	0.575	0.601	0.552	0.158
g. Dialister	4.845	5.050	0.619	1.338
g. Megasphaera	1.046	1.947	1.014	7.425
g. Mitsuokella	0.461	0.434	0.134	0.221
g. Phascolarctobacterium	0.487	0.413	0.125	0.169
g. Selenomonas	0.012	0.015	0.014	0.015
g. Veillonella	0.020	0.003	0.006	0.028
f. Coriobacteriaceae	0.103	0.718	0.379	0.201
g. Collinsella	0.055	0.451	0.872	0.185
g. Slackia	0.003	0.011	0.054	0.005
g. Bulleidia	0.237	0.416	0.189	0.121
f. Erysipelotrichaceae	0.411	0.563	0.469	0.447
g. p-75-a5	0.033	0.502	0.194	0.171
g. Catenibacterium	0.578	0.145	0.101	0.166
f. Fusobacteriaceae	0.000	0.000	0.013	0.034
c. Betaproteobacteria	0.012	0.005	0.005	0.038
g. Sutterella	0.112	0.108	0.103	0.953
f. Oxalobacteraceae	0.008	0.014	0.017	0.025
g. Ralstonia	0.012	0.024	0.029	0.084
f. Desulfovibrionaceae	0.032	0.019	0.021	0.048
g. Desulfovibrio	3.649	1.266	1.659	6.110
o. GMD14H09	0.032	0.016	0.001	0.003
o. Campylobacterales	0.020	0.000	0.042	0.015
g. Campylobacter	0.846	1.055	3.720	4.775
g. Helicobacter	5.272	0.553	18.068	2.696
f. Succinivibrionaceae	0.009	0.002	0.067	0.047
g. Anaerobiospirillum	0.030	0.002	0.108	0.003
g. Succinivibrio	0.117	0.033	0.022	0.046
f. Enterobacteriaceae	0.029	0.008	0.065	0.417
g. Escherichia	0.447	0.111	0.009	0.232
f. Pasteurellaceae	0.220	0.003	0.004	0.004
g. Acinetobacter	0.005	0.009	0.023	0.148

Supplementary Table 3.4. A summary showing mean relative abundances of taxa in the **Ascending colon** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
f. Pseudomonadaceae	0.076	0.093	0.166	0.566
g. Pseudomonas	0.004	0.002	0.004	0.079
f. Xanthomonadaceae	0.029	0.023	0.044	0.151
g. Treponema	3.892	2.194	0.554	3.307
g. Brachyspira	0.051	0.002	0.003	0.054
g. RFN20	0.041	0.038	0.048	0.073
g. Mycoplasma	0.014	0.000	0.000	0.077
o. RF39	0.261	0.914	0.433	1.490
Unclassified	0.468	0.334	0.250	0.973
----------Less than 0.01%----------				
o. Acidimicrobiales	0.0033	0.0007	0.0010	0.0147
o. Actinomycetales	0.0000	0.0025	0.0000	0.0016
g. Parascardovia	0.0000	0.0000	0.0010	0.0007
g. Corynebacterium	0.0000	0.0000	0.0057	0.0007
f. Geodermatophilaceae	0.0015	0.0000	0.0044	0.0272
f. Micrococcaceae	0.0000	0.0000	0.0053	0.0000
g. Kocuria	0.0003	0.0005	0.0044	0.0000
f. Nocardioidaceae	0.0000	0.0000	0.0027	0.0125
f. BS11	0.0003	0.0062	0.0097	0.0002
f. Bacteroidaceae	0.0005	0.0000	0.0000	0.0062
g. 5-7N15	0.0000	0.0000	0.0015	0.0000
f. Marinilabiaceae	0.0000	0.0015	0.0057	0.0000
f. Porphyromonadaceae	0.0358	0.0004	0.0005	0.0000
g. Paludibacter	0.0036	0.0000	0.0057	0.0000
f. Prevotellaceae	0.0019	0.0009	0.0004	0.0000
f. Rikenellaceae	0.0019	0.0064	0.0075	0.0052
g. YRC22	0.0146	0.0026	0.0099	0.0011
f. p-2534-18B5	0.0009	0.0010	0.0013	0.0000
f. Flavobacteriaceae	0.0005	0.0012	0.0000	0.0070
f. Chitinophagaceae	0.0000	0.0035	0.0018	0.0076
g. Hymenobacter	0.0000	0.0000	0.0032	0.0000
f. Sphingobacteriaceae	0.0000	0.0000	0.0004	0.0010
g. Pedobacter	0.0000	0.0017	0.0087	0.0076
o. Chlamydiales	0.0000	0.0000	0.0030	0.0057
o. CAB-I	0.0014	0.0050	0.0075	0.0000
c. Bacilli	0.0026	0.0012	0.0016	0.0068
g. Staphylococcus	0.0014	0.0000	0.0017	0.0020

Supplementary Table 3.4. A summary showing mean relative abundances of taxa in the **Ascending colon** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
o. Lactobacillales	0.0087	0.0101	0.0103	0.0000
g. Enterococcus	0.0008	0.0017	0.0000	0.0014
g. Leuconostoc	0.0000	0.0000	0.0029	0.0010
g. Lactococcus	0.0005	0.0017	0.0032	0.0007
g. Turicibacter	0.0003	0.0000	0.0003	0.0000
g. Clostridium	0.0042	0.0059	0.0006	0.0077
g. Dehalobacterium	0.0010	0.0038	0.0045	0.0000
g. Anaerostipes	0.0026	0.0291	0.0000	0.0000
g. Butyrivibrio	0.0093	0.0007	0.0018	0.0021
g. Lachnobacterium	0.0114	0.0000	0.0016	0.0000
o. Coriobacteriales	0.0054	0.0007	0.0039	0.0000
g. Olsenella	0.0000	0.0017	0.0015	0.0000
o. Erysipelotrichales	0.0044	0.0021	0.0064	0.0000
g. Erysipelothrix	0.0018	0.0004	0.0000	0.0009
g. Holdemania	0.0003	0.0000	0.0046	0.0031
g. L7A_E11	0.0029	0.0179	0.0039	0.0144
o. Erysipelotrichales	0.0011	0.0000	0.0005	0.0013
g. Sharpea	0.0049	0.0262	0.0054	0.0009
o. ML615J-28	0.0013	0.0056	0.0010	0.0000
c. Gemm-3	0.0000	0.0000	0.0000	0.0040
f. Victivallaceae	0.0008	0.0049	0.0000	0.0000
f. Pirellulaceae	0.0008	0.0035	0.0002	0.0000
p. Proteobacteria	0.0090	0.0024	0.0129	0.0112
f. Caulobacteraceae	0.0000	0.0000	0.0008	0.0005
o. Ellin329	0.0026	0.0000	0.0034	0.0144
o. RF32	0.0132	0.0087	0.0000	0.0025
f. Bradyrhizobiaceae	0.0071	0.0000	0.0046	0.0149
f. Methylobacteriaceae	0.0000	0.0000	0.0000	0.0048
o. Sphingomonadales	0.0005	0.0000	0.0011	0.0024
f. Erythrobacteraceae	0.0000	0.0020	0.0000	0.0000
f. Sphingomonadaceae	0.0014	0.0000	0.0060	0.0009
g. Kaistobacter	0.0000	0.0000	0.0008	0.0020
g. Novosphingobium	0.0008	0.0000	0.0006	0.0000
g. Sphingobium	0.0000	0.0012	0.0089	0.0222
G. Sphingomonas	0.0000	0.0023	0.0050	0.0082
o. Burkholderiales	0.0055	0.0007	0.0008	0.0103
f. Alcaligenaceae	0.0006	0.0000	0.0011	0.0003

Supplementary Table 3.4. A summary showing mean relative abundances of taxa in the **Ascending colon** mucosa samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	**UM146**	**CG**	**CGUM146**
f. Comamonadaceae	0.0027	0.0000	0.0028	0.0244
g. Acidovorax	0.0000	0.0000	0.0010	0.0164
g. Comamonas	0.0021	0.0000	0.0033	0.0166
g. Janthinobacterium	0.0023	0.0000	0.0080	0.0000
g. Oxalobacter	0.0188	0.0057	0.0070	0.0008
c. Deltaproteobacteria	0.0040	0.0048	0.0024	0.0005
f. Bacteriovoracaceae	0.0010	0.0000	0.0011	0.0005
o. Desulfovibrionales	0.0077	0.0009	0.0008	0.0084
g. Bilophila	0.0000	0.0014	0.0000	0.0000
o. Myxococcales	0.0000	0.0000	0.0008	0.0005
f. Campylobacteraceae	0.0000	0.0000	0.0012	0.0002
f. Helicobacteraceae	0.0023	0.0000	0.0066	0.0009
c. Gammaproteobacteria	0.0018	0.0022	0.0032	0.0102
g. Enterobacter	0.0000	0.0000	0.0000	0.0009
g. Erwinia	0.0000	0.0000	0.0000	0.0060
g. Yersinia	0.0003	0.0000	0.0000	0.0108
g. Alkanindiges	0.0000	0.0037	0.0006	0.0060
f. Sinobacteraceae	0.0000	0.0000	0.0014	0.0040
g. Lysobacter	0.0005	0.0000	0.0000	0.0151
c. Spirochaetes	0.0017	0.0000	0.0004	0.0002
g. Sphaerochaeta	0.0003	0.0000	0.0013	0.0004
f. Sphaerochaetaceae	0.0013	0.0007	0.0002	0.0012
g. Candidatus Cloacamonas	0.0000	0.0000	0.0095	0.0235
f. Dethiosulfovibrionaceae	0.0081	0.0076	0.0000	0.0000
f. F16	0.0000	0.0008	0.0032	0.0000
c. Mollicutes	0.0038	0.0199	0.0072	0.0073
f. Anaeroplasmataceae	0.0009	0.0000	0.0004	0.0012
g. Deinococcus	0.0015	0.0000	0.0000	0.0014
f. RFP12	0.0005	0.0004	0.0004	0.0000
g. Akkermansia	0.0000	0.0009	0.0004	0.0000

* Mean values only, no statistics

Supplementary Table 3.5. A summary showing mean relative abundances of taxa in the **Descending colon** mucosal samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
----------Greater than or equal to 0.01%----------				
g. Bifidobacterium	0.034	0.025	0.008	0.019
p. Bacteroidetes	0.031	0.029	0.017	0.011
o. Bacteroidales	11.058	14.144	5.353	7.271
g. Bacteroides	0.061	0.011	3.362	10.584
f. Porphyromonadaceae	0.095	0.004	0.005	0.001
g. Paludibacter	0.016	0.019	0.003	0.004
g. Parabacteroides	0.278	0.260	0.279	0.244
g. Prevotella	23.851	36.997	9.604	22.789
f. S24-7	4.243	3.149	1.433	0.887
g. Butyricimonas	0.040	0.007	0.039	0.011
g. CF231	2.581	2.617	0.857	0.686
g. YRC22	0.021	0.008	0.012	0.011
f. p-2534-18B5	0.088	0.011	0.004	0.006
f. Chlamydiaceae	0.005	0.040	0.007	0.000
o. YS2	0.077	0.110	0.031	0.023
g. Mucispirillum	0.039	0.054	8.050	3.526
p. Firmicutes	0.123	0.164	0.161	0.103
f. Lactobacillaceae	0.109	0.065	0.176	0.400
g. Lactobacillus	7.729	9.847	10.233	8.983
f. Streptococcaceae	0.125	0.080	1.896	0.294
g. Streptococcus	0.017	0.028	0.562	0.212
c. Clostridia	0.398	0.293	0.152	0.533
o. Clostridiales	0.728	0.778	0.275	0.219
f. Catabacteriaceae	0.414	0.201	0.180	0.033
f. Christensenellaceae	0.018	0.019	0.005	0.008
f. Clostridiaceae	0.314	0.418	0.331	0.324
g. Clostridium	0.042	0.022	0.002	0.000
g. Sarcina	0.339	0.004	0.390	0.053
f. Lachnospiraceae	1.985	1.631	1.151	0.980
g. Blautia	1.462	0.808	0.607	0.361
g. Coprococcus	0.244	0.175	0.093	0.127
g. Dorea	0.255	0.247	0.231	0.152
g. Lachnobacterium	0.026	0.011	0.003	0.001
g. Lachnospira	0.071	0.076	0.016	0.038
g. Oribacterium	0.295	0.051	0.032	0.053
g. Roseburia	7.001	2.800	1.578	1.844

Supplementary Table 3.5. A summary showing mean relative abundances of taxa in the **Descending colon** mucosal samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
g. Peptococcus	0.039	0.028	0.026	0.010
f. Peptostreptococcaceae	0.041	0.016	0.040	0.002
f. Ruminococcaceae	7.654	5.369	2.419	2.248
g. Faecalibacterium	2.398	1.583	2.366	1.130
g. Oscillospira	3.156	2.988	0.923	1.661
g. Ruminococcus	2.362	2.350	0.924	2.540
f. Veillonellaceae	3.449	2.164	1.961	1.737
g. Acidaminococcus	0.348	0.166	0.270	0.099
g. Anaerovibrio	0.669	0.185	0.120	0.174
g. Dialister	3.729	0.768	1.171	1.017
g. Megasphaera	2.462	3.269	1.465	1.791
g. Mitsuokella	0.334	0.139	0.127	0.163
g. Phascolarctobacterium	0.296	0.240	0.394	0.091
g. Selenomonas	0.014	0.006	0.012	0.016
f. Coriobacteriaceae	0.125	0.014	0.035	0.021
g. Collinsella	0.025	0.005	0.031	0.010
o. Erysipelotrichales	0.033	0.016	0.006	0.005
f. Erysipelotrichaceae	0.292	0.142	0.250	0.135
g. Bulleidia	0.191	0.076	0.058	0.070
g. L7A_E11	0.041	0.009	0.002	0.001
g. p-75-a5	0.185	0.069	0.071	0.044
g. Catenibacterium	0.266	0.035	0.103	0.117
o. ML615J-28	0.055	0.035	0.075	0.001
f. Fusobacteriaceae	0.000	0.000	0.020	0.101
o. RF32	0.032	0.016	0.014	0.009
g. Sutterella	0.137	0.114	0.239	0.241
g. Oxalobacter	0.028	0.010	0.016	0.003
g. Ralstonia	0.038	0.015	0.004	0.002
f. Desulfovibrionaceae	0.002	0.003	0.022	0.019
g. Desulfovibrio	0.282	0.178	1.609	2.449
o. GMD14H09	0.055	0.059	0.027	0.003
o. Campylobacterales	0.011	0.001	0.215	0.391
g. Campylobacter	2.305	0.905	6.545	9.437
f. Helicobacteraceae	0.002	0.000	0.040	0.008
g. Helicobacter	0.588	0.061	26.478	10.288
f. Succinivibrionaceae	0.012	0.022	0.164	0.909
g. Succinivibrio	0.253	0.634	0.257	0.124

Supplementary Table 3.5. A summary showing mean relative abundances of taxa in the **Descending colon** mucosal samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
f. Enterobacteriaceae	0.020	0.040	0.102	0.151
g. Escherichia	0.222	1.016	0.023	0.069
f. Pasteurellaceae	0.037	0.009	0.003	0.003
f. Pseudomonadaceae	0.117	0.081	0.041	0.048
f. Xanthomonadaceae	0.022	0.005	0.003	0.018
g. Treponema	0.628	0.465	2.993	0.271
g. Brachyspira	0.000	0.000	0.076	0.035
c. Mollicutes	0.022	0.009	0.005	0.005
g. RFN20	0.173	0.135	0.140	0.040
g. Mycoplasma	0.173	0.060	0.004	0.141
o. RF39	1.619	0.481	0.131	0.759
Unclassified	0.482	0.595	0.637	0.410
----------Less than 0.01%----------				
g. Corynebacterium	0.0024	0.0000	0.0000	0.0000
f. BS11	0.0040	0.0013	0.0000	0.0137
f. Bacteroidaceae	0.0000	0.0000	0.0099	0.0230
f. Marinilabiaceae	0.0012	0.0000	0.0000	0.0000
f. Prevotellaceae	0.0011	0.0050	0.0009	0.0009
f. Rikenellaceae	0.0000	0.0011	0.0037	0.0067
f. Chitinophagaceae	0.0009	0.0016	0.0005	0.0000
f. Flexibacteraceae	0.0092	0.0000	0.0000	0.0000
g. Pedobacter	0.0012	0.0000	0.0089	0.0013
o. CAB-I	0.0005	0.0000	0.0000	0.0004
o. Streptophyta	0.0007	0.0000	0.0000	0.0012
f. Elusimicrobiaceae	0.0049	0.0016	0.0009	0.0000
g. Fibrobacter	0.0081	0.0037	0.0027	0.0010
c. Bacilli	0.0000	0.0000	0.0007	0.0013
g. Staphylococcus	0.0005	0.0258	0.0000	0.0000
o. Lactobacillales	0.0058	0.0085	0.0172	0.0069
g. Enterococcus	0.0000	0.0000	0.0000	0.0075
g. Dehalobacterium	0.0241	0.0084	0.0012	0.0010
g. Anaerofustis	0.0013	0.0006	0.0015	0.0000
g. Anaerostipes	0.0082	0.0030	0.0014	0.0027
g. Butyrivibrio	0.0076	0.0031	0.0000	0.0041
g. rc4-4	0.0015	0.0062	0.0000	0.0007
g. Anaerotruncus	0.0002	0.0008	0.0142	0.0005
g. Veillonella	0.0010	0.0000	0.0101	0.0217

Supplementary Table 3.5. A summary showing mean relative abundances of taxa in the **Descending colon** mucosal samples. While majority of taxa were classified at the genus level (g.), some were only classified at the Phylum (p.), Class (c.), Order (o.), or Family (f.) levels.

Taxa	Mean relative abundance*			
	Control	UM146	CG	CGUM146
o. Coriobacteriales	0.0204	0.0052	0.0053	0.0000
g. Slackia	0.0019	0.0000	0.0005	0.0004
g. Allobaculum	0.0000	0.0018	0.0000	0.0006
o. Erysipelotrichales	0.0004	0.0000	0.0000	0.0004
g. Sharpea	0.0023	0.0107	0.0005	0.0012
f. Victivallaceae	0.0025	0.0043	0.0007	0.0004
f. R4-45B	0.0000	0.0013	0.0000	0.0000
f. Pirellulaceae	0.0085	0.0128	0.0036	0.0025
p. Proteobacteria	0.0028	0.0008	0.0175	0.0194
g. Devosia	0.0071	0.0003	0.0000	0.0000
g. Hyphomicrobium	0.0018	0.0000	0.0000	0.0000
g. Sphingobium	0.0012	0.0000	0.0030	0.0000
c. Betaproteobacteria	0.0021	0.0000	0.0021	0.0073
o. Burkholderiales	0.0107	0.0044	0.0072	0.0022
f. Alcaligenaceae	0.0007	0.0003	0.0046	0.0002
f. Comamonadaceae	0.0085	0.0000	0.0000	0.0016
f. Oxalobacteraceae	0.0145	0.0000	0.0020	0.0044
c. Deltaproteobacteria	0.0019	0.0035	0.0026	0.0047
o. Desulfovibrionales	0.0000	0.0003	0.0005	0.0015
f. Campylobacteraceae	0.0003	0.0000	0.0155	0.0020
c. Gammaproteobacteria	0.0035	0.0066	0.0000	0.0102
g. Anaerobiospirillum	0.0000	0.0070	0.0138	0.0011
g. Acinetobacter	0.0062	0.0112	0.0047	0.0054
g. Alkanindiges	0.0010	0.0000	0.0006	0.0012
g. Enhydrobacter	0.0015	0.0015	0.0000	0.0000
g. Pseudomonas	0.0019	0.0000	0.0000	0.0015
g. Sphaerochaeta	0.0004	0.0008	0.0007	0.0000
f. Sphaerochaetaceae	0.0003	0.0008	0.0011	0.0000
g. Candidatus Cloacamonas	0.0004	0.0003	0.0000	0.0000
f. Dethiosulfovibrionaceae	0.0208	0.0049	0.0033	0.0000
o. Anaeroplasmatales	0.0048	0.0023	0.0013	0.0000
f. Anaeroplasmataceae	0.0005	0.0000	0.0000	0.0067
f. RFP12	0.0066	0.0003	0.0003	0.0000

* Mean values only, no statistics

Supplementary Table 6.1. A summary showing mean relative abundances of taxa in fecal samples. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels.

	Mean relative abundance*	
Taxa	**Control**	**DSS**
----------Greater than or equal to 0.01 %----------		
Unclassified	0.497	0.598
g. Bifidobacterium	0.511	0.001
p. Bacteroidetes	0.006	0.018
o. Bacteroidales	2.162	5.102
f. Bacteroidaceae	0.028	0.116
g. Bacteroides	2.762	12.316
g. Bacteroides acidifaciens	0.012	0.009
g. Bacteroides ovatus	0.231	1.253
g. Parabacteroides	0.081	0.151
g. Parabacteroides distasonis	0.492	1.528
g. Prevotella	0.934	0.000
f. Rikenellaceae	4.774	4.941
f. S24-7	47.002	15.267
g. Odoribacter	1.102	0.112
o. YS2	0.010	0.757
g. Mucispirillum schaedleri	1.710	0.493
f. Firmicutes	0.126	0.160
c. Bacilli	0.000	0.021
o. Lactobacillales	0.030	0.004
g. Enterococcus	0.006	0.067
f. Lactobacillaceae	8.380	2.055
g. Lactobacillus	3.554	0.045
g. Lactobacillus reuteri	1.169	1.072
g. Turicibacter	0.000	2.436
c. Clostridia	1.017	0.315
o. Clostridiales	0.385	1.266
f. Clostridiaceae	0.566	23.151
g. Clostridium	0.037	0.489
g. Clostridium perfringens	0.000	0.161
g. Dehalobacterium	0.112	0.005
f. Lachnospiraceae	13.049	2.021
g. Coprococcus	0.086	0.000
g. Dorea	0.115	0.042
f. Peptococcaceae	0.032	0.001
g. rc4-4	0.005	0.265
f. Ruminococcaceae	2.030	2.056
g. Oscillospira	1.538	0.309

Taxa	Mean relative abundance*	
	Control	**DSS**
g. Ruminococcus	0.333	0.230
f. Coriobacteriaceae	0.021	0.010
g. Adlercreutzia	0.224	0.037
o. Erysipelotrichales	0.055	0.228
f. Erysipelotrichaceae	0.090	0.119
g. Allobaculum	0.759	2.989
g. Coprobacillus	0.047	0.129
c. Alphaproteobacteria	0.001	0.244
o. RF32	0.098	10.528
o. Burkholderiales	0.013	0.010
g. Sutterella	0.496	0.850
g. Bilophila	0.049	0.085
g. Desulfovibrio	0.004	0.030
g. Desulfovibrio C21_c20	1.032	0.001
g. Helicobacter	0.492	1.283
g. Helicobacter hepaticus	0.005	0.022
f. Enterobacteriaceae	0.000	0.025
g. Escherichia	0.006	0.102
o. RF39	0.299	2.319
g. Akkermansia muciniphila	1.330	2.088
----------Less than 0.01%----------		
g. 5-7N15	0.0008	0.0000
g. Bacteroides eggerthii	0.0000	0.0007
f. Porphyromonadaceae	0.0000	0.0035
g. Paludibacter	0.0000	0.0013
f. Prevotellaceae	0.0029	0.0000
p. Cyanobacteria	0.0015	0.0000
o. Streptophyta	0.0062	0.0006
g. Fibrobacter succinogenes	0.0019	0.0000
f. Enterococcaceae	0.0023	0.0061
g. Streptococcus	0.0032	0.0122
f. Christensenellaceae	0.0102	0.0000
g. Anaerofustis	0.0011	0.0000
g. Anaerostipes	0.0168	0.0000
g. Roseburia	0.0023	0.0000
p. Proteobacteria	0.0000	0.0146
f. Mitochondria	0.0061	0.0012
c. Betaproteobacteria	0.0079	0.0076
f. Alcaligenaceae	0.0008	0.0000
g. Burkholderia	0.0000	0.0007
g. Salinispora tropica	0.0000	0.0021

	Mean relative abundance*	
Taxa	**Control**	**DSS**
f. Desulfovibrionaceae	0.0034	0.0007
g. Yersinia	0.0000	0.0014
f. Pasteurellaceae	0.0023	0.0139
g. Acinetobacter	0.0008	0.0000
g. Pseudomonas balearica	0.0010	0.0000
g. Treponema	0.0000	0.0028
f. F16	0.0039	0.0014
g. Mollicutes	0.0034	0.0000
g. Anaeroplasma	0.0120	0.0000
f. RFP12	0.0011	0.0000

* Mean abundance only, no statistics

Supplementary Table 6.2. A summary showing mean relative abundances of taxa in colonic samples. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels.

	Mean relative abundance	
Taxa	**Control**	**DSS**
----------Greater than or equal to 0.01%----------		
Unclassified	0.123	0.500
f. Nocardioidaceae	0.095	0.000
g. Bifidobacterium	0.000	0.025
p. Bacteroidetes	0.045	0.053
o. Bacteroidales	0.368	4.870
f. BS11	0.000	0.032
f. Bacteroidaceae	0.005	0.050
g. 5-7N15	0.000	0.311
g. Bacteroides	2.577	3.310
g. Bacteroides ovatus	0.023	0.567
g. Paludibacter	0.000	0.249
g. Parabacteroides	0.022	0.047
g. Parabacteroides distasonis	0.878	0.414
g. Prevotella	0.093	0.009
f. Rikenellaceae	0.284	2.678
f. S24-7	3.585	5.432
o. Bacteroidales	0.000	0.032
g. Odoribacter	0.040	0.039
g. CF231	0.008	0.308
g. YRC22	0.000	0.017
f. p-2534-18B5	0.000	0.028
f. Ekhidnaceae	0.099	0.000
o. YS2	0.017	0.307
g. Mucispirillum schaedleri	6.923	0.541
p. Firmicutes	0.043	0.032
f. Lactobacillaceae	0.500	0.208
g. Lactobacillus	0.031	0.000
g. Lactobacillus reuteri	0.002	0.067
g. Turicibacter	0.000	0.209
c. Clostridia	0.331	0.332
o. Clostridiales	0.071	0.947
f. Christensenellaceae	0.000	0.070
f. Clostridiaceae	0.041	2.106
g. Clostridium	0.000	0.049
f. Lachnospiraceae	6.271	0.712
g. Butyrivibrio	0.000	0.042
g. Dorea	0.019	0.020

	Mean relative abundance	
Taxa	**Control**	**DSS**
g. Roseburia	0.000	0.042
f. Lachnospiraceae	0.144	0.012
g. rc4-4	0.002	0.063
f. Peptostreptococcaceae	0.000	0.225
f. Ruminococcaceae	0.562	1.164
g. Oscillospira	0.639	0.164
g. Ruminococcus	0.193	1.116
f. Coriobacteriaceae	0.002	0.027
o. Erysipelotrichales	0.147	0.041
f. Erysipelotrichaceae	0.095	0.042
g. Allobaculum	0.190	0.230
g. Coprobacillus	0.797	0.041
o. ML615J-28	0.000	0.017
p. Proteobacteria	0.000	0.030
c. Alphaproteobacteria	0.017	0.166
o. RF32	0.090	3.135
g. Sutterella	0.087	0.334
g. Bilophila	0.010	0.025
g. Desulfovibrio	0.000	0.056
g. Desulfovibrio C21_c20	0.186	0.000
g. Helicobacter	67.885	64.175
g. Helicobacter hepaticus	0.171	0.126
f. Enterobacteriaceae	0.119	0.022
g. Escherichia	3.082	0.163
g. Yersinia	0.036	0.002
g. Acinetobacter	0.026	0.002
f. Pseudomonadaceae	0.126	0.015
g. Lysobacter	0.043	0.000
g. Treponema	0.000	0.091
o. RF39	0.037	1.351
f. RFP12	0.000	0.018
g. Akkermansia	2.533	2.099
----------Less than 0.01%----------		
g. Candidatus Solibacter	0.0257	0.0000
c. Actinobacteria	0.0000	0.0014
o. Actinomycetales	0.0000	0.0025
g. Actinobaculum	0.0000	0.0014
g. Dermacoccus	0.0000	0.0014
f. Bifidobacteriaceae	0.0000	0.0014
g. BF311	0.0000	0.0021
g. Bacteroides acidifaciens	0.0033	0.0050

	Mean relative abundance	
Taxa	**Control**	**DSS**
g. Bacteroides fragilis	0.0000	0.0043
f. Porphyromonadaceae	0.0000	0.0034
g. Butyricimonas	0.0000	0.0014
g. Paraprevotella	0.0000	0.0130
f. Flavobacteriaceae	0.0033	0.0000
f. Chitinophagaceae	0.0223	0.0000
f. Flexibacteraceae	0.0041	0.0000
g. Sphingobacterium multivorum	0.0060	0.0000
o. MLE1-12	0.0176	0.0000
o. Streptophyta	0.0050	0.0000
g. Fibrobacter succinogenes	0.0000	0.0103
g. Brevibacillus	0.0083	0.0000
f. Staphylococcaceae	0.0194	0.0000
o. Lactobacillales	0.0017	0.0000
g. Enterococcus	0.0000	0.0007
g. Streptococcus	0.0017	0.0000
g. Clostridium butyricum	0.0000	0.0055
g. Clostridium perfringens	0.0000	0.0148
g. Finegoldia	0.0033	0.0000
f. Dehalobacteriaceae	0.0000	0.0014
g. Dehalobacterium	0.0133	0.0021
f. Eubacteriaceae	0.0000	0.0014
g. Anaerofustis	0.0000	0.0021
g. Anaerostipes	0.0000	0.0041
g. Blautia	0.0000	0.0055
g. Coprococcus	0.0039	0.0075
g. Shuttleworthia	0.0000	0.0021
f. Peptococcaceae	0.0000	0.0068
g. Anaerovibrio	0.0000	0.0062
g. Phascolarctobacterium	0.0000	0.0096
g. Selenomonas ruminantium	0.0000	0.0013
o. Coriobacteriales	0.0000	0.0062
g. Adlercreutzia	0.0039	0.0075
g. Olsenella uli	0.0000	0.0014
g. Bulleidia	0.0000	0.0014
g. p-75-a5	0.0000	0.0014
g. Sharpea p-3329-23G2	0.0000	0.0008
f. Victivallaceae	0.0000	0.0014
c. BD7-11	0.0021	0.0000
f. Planctomycetes	0.0000	0.0014
f. Caulobacteraceae	0.0079	0.0000

	Mean relative abundance	
Taxa	**Control**	**DSS**
o. Ellin329	0.0206	0.0000
f. Bradyrhizobiaceae	0.0061	0.0000
f. Methylobacteriaceae	0.0066	0.0000
f. Rhizobiaceae	0.0033	0.0000
o. Rhodospirillales	0.0021	0.0000
o. Sphingomonadales	0.0000	0.0008
g. Blastomonas natatoria	0.0041	0.0000
g. Sphingomonas	0.0074	0.0000
c. Betaproteobacteria	0.0033	0.0020
g. Pigmentiphaga	0.0033	0.0000
f. Burkholderiaceae	0.0000	0.0015
g. Burkholderia	0.0000	0.0043
g. Salinispora tropica	0.0000	0.0039
f. Comamonadaceae	0.0083	0.0000
g. Hylemonella	0.0062	0.0000
f. Oxalobacteraceae	0.0182	0.0000
g. Ralstonia	0.0197	0.0000
f. Rhodocyclaceae	0.0041	0.0013
c. Gammaproteobacteria	0.0041	0.0000
g. Ruminobacter	0.0000	0.0014
g. Succinivibrio	0.0000	0.0096
f. Pasteurellaceae	0.0000	0.0084
g. Enhydrobacter	0.0039	0.0000
g. Pseudomonas	0.0019	0.0000
f. Xanthomonadaceae	0.0083	0.0000
g. Pseudoxanthomonas mexicana	0.0033	0.0000
g. Stenotrophomonas	0.0000	0.0034
o. M2PT2-76	0.0000	0.0103
o. CW040	0.0000	0.0041
o. Anaeroplasmatales	0.0000	0.0034
f. Anaeroplasmataceae	0.0000	0.0055
g. Anaeroplasma	0.0000	0.0041
f. Mycoplasmataceae	0.0000	0.0014
c. Opitutae	0.0000	0.0014

* Mean abundance only, no statistics

Supplementary Table 7.1. A summary showing mean relative abundances of taxa in fecal samples collected just before induction of colitis. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels.

Taxa	Mean relative abundance*	
	Control	ATB
----------Greater than or equal to 0.01%----------		
g. Adlercreutzia	0.109	0.115
o. Bacteroidales	5.494	2.571
g. Bacteroides	1.342	0.171
g. Bacteroides acidifaciens	0.569	2.852
g. Parabacteroides	0.094	0.203
g. Parabacteroides distasonis	0.165	0.218
f. Prevotellaceae	0.152	0.149
g. Prevotella	3.574	8.411
f. Rikenellaceae	3.219	1.328
f. S24-7	19.864	31.924
g. Odoribacter	1.630	0.425
o. YS2	0.377	0.462
g. Mucispirillum schaedleri	1.426	0.170
g. Enterococcus	0.047	0.014
g. Lactobacillus	24.655	18.634
g. Lactobacillus reuteri	0.967	0.653
o. Clostridiales	14.538	7.442
f. Clostridiaceae	0.208	1.954
g. Candidatus arthromitus	0.776	0.383
g. Clostridium perfringens	0.001	0.034
g. Dehalobacterium	0.397	0.165
f. Lachnospiraceae	9.110	5.138
g. Coprococcus	0.215	5.214
g. Dorea	0.532	0.899
f. Peptococcaceae	0.024	0.006
g. rc4-4	0.000	0.203
f. Ruminococcaceae	1.337	0.889
g. Oscillospira	1.892	1.025
g. Ruminococcus	0.804	0.614
f. Erysipelotrichaceae	0.038	0.052
g. Allobaculum	0.019	1.305
o. RF32	0.101	0.610
g. Sutterella	0.024	0.701
g. Bilophila	0.057	0.060
g. Desulfovibrio	0.936	0.000
f. Helicobacteraceae	0.059	0.007
g. Helicobacter	1.399	0.214

Taxa	Mean relative abundance*	
	Control	ATB
f. Enterobacteriaceae	0.116	0.006
f. F16	0.534	0.460
g. Anaeroplasma	0.644	1.290
Unclassified	0.025	0.030
----------Less than 0.01%----------		
g. Candidatus Microthrix parvicella	0.0004	0.0001
g. Gordonia	0.0002	0.0002
f. Micrococcaceae	0.0005	0.0002
g. Arthrobacter	0.0005	0.0000
g. Mycobacterium	0.0000	0.0003
f. Coriobacteriaceae	0.0019	0.0017
g. Bacteroides ovatus	0.0010	0.0084
g. Parabacteroides gordonii	0.0005	0.0000
g. Butyricimonas	0.0002	0.0001
g. CF231	0.0000	0.0006
f. Flavobacteriaceae	0.0013	0.0000
g. Chryseobacterium	0.0003	0.0003
f. Sphingobacteriaceae	0.0003	0.0001
p. Bacteroidetes	0.0003	0.0002
f. Chitinophagaceae	0.0002	0.0001
f. Saprospiraceae	0.0012	0.0003
o. Streptophyta	0.0102	0.0048
o. Bacillales	0.0005	0.0002
g. Sporosarcina	0.0006	0.0002
g. Staphylococcus aureus	0.0005	0.0000
g. Staphylococcus sciuri	0.0000	0.0019
o. Lactobacillales	0.0005	0.0006
g. Aerococcus	0.0003	0.0002
g. Facklamia	0.0004	0.0000
f. Carnobacteriaceae	0.0016	0.0015
g. Lactobacillus ruminis	0.0005	0.0002
g. Streptococcus	0.0070	0.0001
g. Streptococcus luteciae	0.0033	0.0000
g. Turicibacter	0.0005	0.0023
f. Christensenellaceae	0.0017	0.0013
g. 02d06	0.0010	0.0080
g. Clostridium	0.0018	0.0096
g. SMB53	0.0003	0.0003
g. Anaerofustis	0.0019	0.0019
g. Blautia	0.0004	0.0002
g. Epulopiscium	0.0006	0.0000
g. Roseburia	0.0002	0.0002
f. Veillonellaceae	0.0000	0.0004
g. Phascolarctobacterium	0.0002	0.0001
g. Coprobacillus	0.0103	0.0089
c. Alphaproteobacteria	0.0019	0.0000
f. Caulobacteraceae	0.0003	0.0007
g. Rhodobacter	0.0002	0.0001
g. Novosphingobium	0.0002	0.0001
o. Burkholderiales	0.0000	0.0016
f. Comamonadaceae	0.0002	0.0001
g. Comamonas	0.0005	0.0005

Taxa	Mean relative abundance*	
	Control	ATB
g. Thiobacterales	0.0005	0.0000
f. Desulfovibrionaceae	0.0063	0.0000
o. Myxococcales	0.0000	0.0002
g. Flexispira	0.0005	0.0000
g. Cellvibrio	0.0005	0.0010
g. Aggregatibacter pneumotropica	0.0010	0.0000
g. Acinetobacter	0.0005	0.0011
g. Acinetobacter johnsonii	0.0014	0.0018
g. Enhydrobacter	0.0005	0.0008
f. Pseudomonadaceae	0.0005	0.0000
g. Pseudomonas	0.0006	0.0017
f. Xanthomonadaceae	0.0002	0.0003
g. Stenotrophomonas	0.0000	0.0005
o. RF39	0.0000	0.0005
g. Akkermansia muciniphila	0.0000	0.0062
Unclassified	0.0011	0.0009

* Mean values only, no statistical analysis

Supplementary Table 7.2. A summary showing mean relative abundance of taxa in colon samples collected after induction of colitis. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels.

Taxa	Mean relative abundance*	
	Control-DSS	ATB-DSS
----------Greater than or equal to 0.01%----------		
p. Bacteroidetes	0.026	0.022
o. Bacteroidales	7.558	2.132
f. Bacteroidaceae	0.012	0.031
g. Bacteroides	2.128	0.594
g. Bacteroides acidifaciens	0.060	1.037
g. Parabacteroides	0.079	0.517
g. Parabacteroides distasonis	0.355	0.873
g. Parabacteroides gordonii	0.002	0.021
f. Prevotellaceae	0.018	0.033
g. Prevotella	2.423	5.959
f. Rikenellaceae	1.463	1.096
f. S24-7	8.256	14.656
g. Odoribacter	0.438	0.170
o. YS2	0.305	0.090
g. Mucispirillum schaedleri	4.984	3.328
p. Firmicutes	0.101	0.140
c. Bacilli	0.050	0.017
g. Enterococcus	0.154	0.011
f. Lactobacillaceae	5.130	1.288
g. Lactobacillus	0.146	0.075
g. Lactobacillus reuteri	0.546	0.238
g. Turicibacter	1.476	1.578
c. Clostridia	0.639	0.643
o. Clostridiales	1.475	2.246
f. Clostridiaceae	0.099	0.116
g. Clostridium	6.510	16.759
g. Clostridium perfringens	0.069	0.284
g. Dehalobacterium	0.536	0.080
f. Lachnospiraceae	16.443	8.960
f. Dorea	0.054	0.016
g. Gnavus	2.600	0.339
f. Peptococcaceae	0.025	0.011
g. rc4-4	0.000	0.053
f. Peptostreptococcaceae	3.221	0.008
f. Ruminococcaceae	0.802	0.627
g. Anaerotruncus	0.072	0.002
g. Oscillospira	1.686	0.750
g. Ruminococcus	0.475	0.494
g. Ruminococcus flavefaciens	0.000	0.257
g. Adlercreutzia	0.130	0.096
f. Erysipelotrichaceae	0.009	0.032
g. Allobaculum	0.178	0.594
o. Erysipelotrichales	0.089	0.022
g. Coprobacillus	0.021	0.005
p. Proteobacteria	0.070	0.119
c. Alphaproteobacteria	0.023	0.011
o. RF32	1.157	0.884
c. Betaproteobacteria	0.012	0.015
g. Sutterella	0.367	0.416

Taxa	Mean relative abundance*	
	Control-DSS	ATB-DSS
g. Bilophila	0.085	0.133
g. Desulfovibrio	0.072	0.000
g. Desulfovibrio C21_c20	0.857	0.000
g. Helicobacter	23.729	28.624
H. hepaticus	0.071	0.032
f. Enterobacteriaceae	0.015	0.025
g. Escherichia	0.911	0.937
g. Aggregatibacter pneumotropica	0.006	0.015
f. F16	0.105	0.147
g. Anaeroplasma	0.519	1.178
Unclassified	1.097	1.021
----------Less than 0.01%----------		
f. Geodermatophilaceae	0.0018	0.0000
g. Mycobacterium	0.0000	0.0008
f. Porphyromonadaceae	0.0000	0.0002
p. Cyanobacteria	0.0014	0.0019
f. Bacillaceae	0.0005	0.0009
f. Planococcaceae	0.0014	0.0000
o. Lactobacillales	0.0146	0.0019
g. Trichococcus	0.0009	0.0000
f. Enterococcaceae	0.0027	0.0008
g. Streptococcus	0.0053	0.0000
g. Clostridium butyricum	0.0012	0.0003
g. Oscillospira guilliermondii	0.0005	0.0030
f. Veillonellaceae	0.0000	0.0006
g. Mitsuokella multacida	0.0000	0.0006
f. Coriobacteriaceae	0.0097	0.0051
f. Caulobacteraceae	0.0036	0.0015
g. Phenylobacterium	0.0004	0.0000
o. Burkholderiales	0.0014	0.0005
g. Delftia	0.0003	0.0011
f. Desulfovibrionaceae	0.0027	0.0000
g. Desulfovibrio	0.0004	0.0000
f. Helicobacteraceae	0.0009	0.0007
c. Gammaproteobacteria	0.0008	0.0007
g. Acinetobacter guillouiae	0.0000	0.0008
f. Pseudomonadaceae	0.0005	0.0000
g. Pseudomonas	0.0007	0.0017
g. Stenotrophomonas	0.0016	0.0000
o. CW040	0.0000	0.0003
c. Mollicutes	0.0021	0.0080
o. RF39	0.0000	0.0006
g. Meiothermus	0.0009	0.0000
g. Thermus	0.0003	0.0006
g. Akkermansia muciniphila	0.0011	0.0014

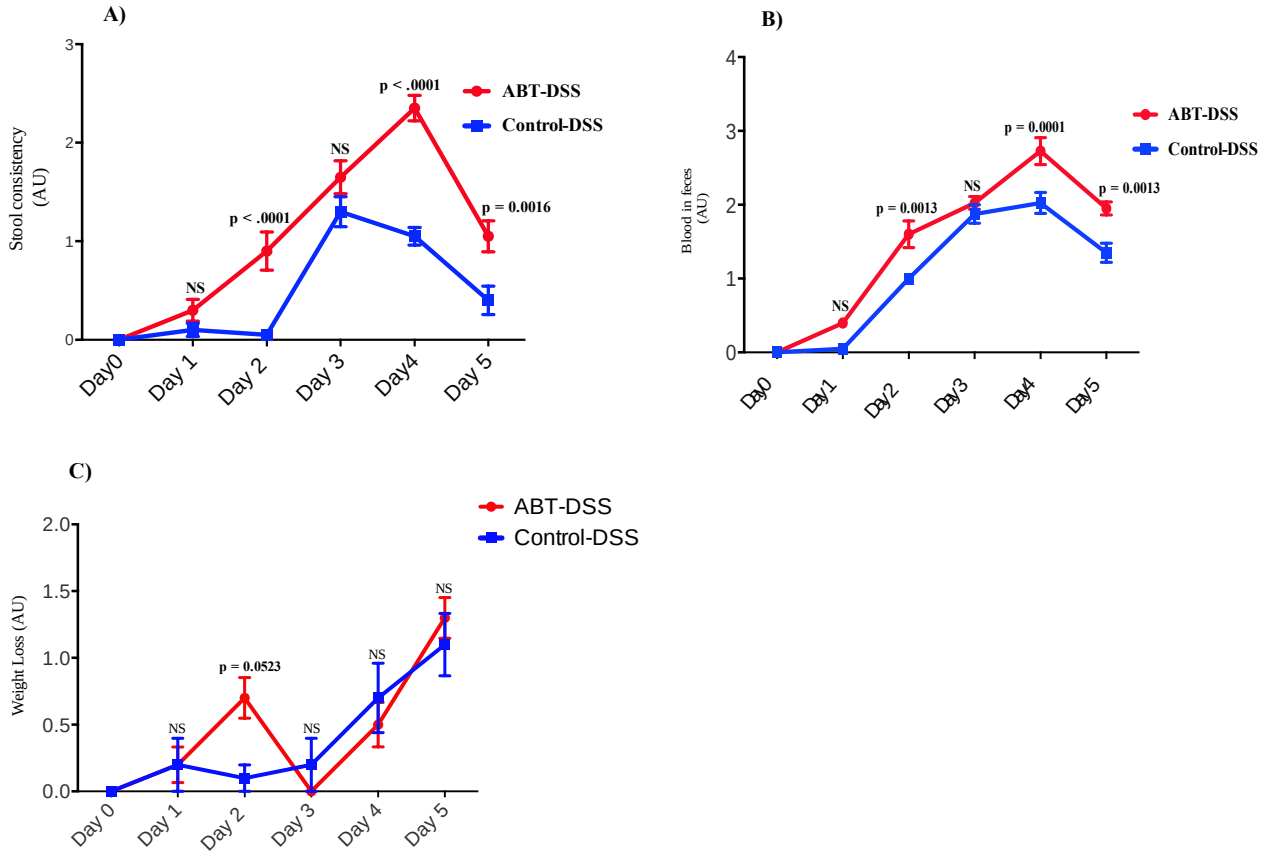
* Mean values only, no statistical analysis

Supplementary Table 7.3. A summary showing mean relative abundance of taxa in fecal samples collected after induction of colitis. Some taxa were only classified at the Phylum (p.), Class (c.), Order (o.), Family (f.), or Genus (g.) levels.

Taxa	Mean relative abundance*	
	Control-DSS	ATB-DSS
----------Greater than or equal to 0.01%----------		
p. Bacteroidetes	0.095	0.041
o. Bacteroidales	12.051	2.822
f. Bacteroidaceae	0.064	0.012
g. Bacteroides	1.907	0.384
g. Bacteroides acidifaciens	0.103	1.470
g. Parabacteroides	0.075	0.845
g. Parabacteroides distasonis	0.317	1.361
g. Parabacteroides gordonii	0.005	0.027
f. Prevotellaceae	0.064	0.056
g. Prevotella	3.594	7.278
f. Rikenellaceae	1.537	2.222
f. S24-7	9.755	13.587
g. Odoribacter	0.345	0.068
p. Cyanobacteria	0.094	0.052
o. YS2	7.427	3.742
g. Mucispirillum schaedleri	1.509	0.402
p. Firmicutes	0.536	1.109
c. Bacilli	0.248	0.230
o. Lactobacillales	0.023	0.005
g. Enterococcus	0.023	0.007
f. Lactobacillaceae	4.298	1.993
g. Lactobacillus	0.544	1.205
g. Lactobacillus reuteri	0.341	0.790
g. Turicibacter	1.586	5.058
c. Clostridia	0.848	0.502
o. Clostridiales	2.181	3.495
f. Clostridiaceae	0.247	0.276
g. Clostridium	18.895	29.706
g. Clostridium perfringens	0.293	0.803
g. Dehalobacterium	0.326	0.028
f. Lachnospiraceae	14.267	5.411
g. Dorea	0.034	0.003
g. rc4-4	0.000	0.163
f. Peptostreptococcaceae	4.735	0.070
f. Ruminococcaceae	0.608	0.598
g. Oscillospira	0.986	0.215
g. Ruminococcus	0.553	0.707
g. Ruminococcus flavefaciens	0.144	0.541
g. Adlercreutzia	0.043	0.032
o. Erysipelotrichales	0.065	0.049
f. Erysipelotrichaceae	0.036	0.163
g. Allobaculum	0.208	0.875
p. Proteobacteria	0.111	0.099
c. Alphaproteobacteria	0.250	0.140
o. RF32	3.195	5.737
c. Betaproteobacteria	0.056	0.162
g. Sutterella	0.215	0.869
g. Bilophila	0.042	0.012
g. Desulfovibrio C21_c20	0.232	0.000

Taxa	Mean relative abundance*	
	Control-DSS	ATB-DSS
g. Helicobacter	0.749	0.600
f. Enterobacteriaceae	0.029	0.093
g. Escherichia	0.335	1.232
f. F16	0.150	0.064
g. Anaeroplasma	0.520	0.662
Unclassified	3.011	1.859
----------Less than 0.01%----------		
g. Kocuria	0.0000	0.0008
g. Bacteroides helcogenes	0.0058	0.0000
f. Porphyromonadaceae	0.0010	0.0071
o. Streptophyta	0.0002	0.0005
o. Bacillales	0.0036	0.0016
g. Bacillus	0.0014	0.0016
g. Brevibacillus	0.0005	0.0005
g. Jeotgalicoccus	0.0000	0.0006
. Trichococcus	0.0002	0.0000
f. Enterococcaceae	0.0026	0.0000
g. Lactobacillus ruminis	0.0015	0.0016
f. Streptococcaceae	0.0034	0.0000
g. Clostridium butyricum	0.0013	0.0010
g. Butyrivibrio	0.0009	0.0010
g. Coprococcus	0.0037	0.0016
f. Peptococcaceae	0.0084	0.0000
g. Anaerotruncus	0.0146	0.0016
g. Oscillospira guilliermondii	0.0016	0.0008
g. Ruminococcus callidus	0.0007	0.0027
f. Coriobacteriaceae	0.0037	0.0038
g. Coprobacillus	0.0099	0.0039
f. Caulobacteraceae	0.0002	0.0005
o. Burkholderiales	0.0006	0.0016
f. Comamonadaceae	0.0000	0.0010
g. Desulfovibrio	0.0075	0.0000
g. Helicobacter hepaticus	0.0078	0.0044
c. Gammaproteobacteria	0.0018	0.0026
f. Pasteurellaceae	0.0012	0.0023
g. Aggregatibacter pneumotropica	0.0048	0.0161
g. Mollicutes	0.0043	0.0093
g. Akkermansia muciniphila	0.0006	0.0000
o. WCHB1-15	0.0000	0.0009

* Mean values only, no statistical analysis



Supplementary Figure 7.1. Impacts of dextran sulfate sodium (DSS) and antepartum antibiotics on colitis induction and disease severity. In the ATB group, DSS caused a significant increase in diarreah (A), Blood in the feces (B) especially on day 2, 4, and 5, but did not significantly influence weight loss (C).