Catestatin (CTS) decreases intestinal inflammation in both acute and chronic models of murine colitis through the regulation of macrophages and/or gut microbiota

By

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Thesis abstract

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disease of the gastrointestinal tract with unknown etiology. Although the incidence of IBD is increasing globally, there is no curative treatment for this complex disease. Therefore, revealing the etiology of IBD and identifying an optimal treatment is a major challenge. Mucosal changes in IBD are characterized by transmural inflammation and associated with an alteration in the prohormone chromogranin-A (CHGA) producing enterochromaffin (EC) cells. However, it is not clear whether the change plays any role in immune activation and in regulation of gut inflammation. The aim of this thesis was to identify the impact of catestatin (CTS), a bioactive peptide derived from the conserved C-terminal portion of CHGA on gut inflammation.

In our first study, using an acute model of murine colitis and biopsy samples from ulcerative colitis (UC) patients, we demonstrated that CHGA and CTS were increased during inflammation. Moreover, we identified that administration of human (h) CTS significantly down- regulated gut inflammation. This down-regulation occurred via the modulation of pro-inflammatory cytokine secretion from the macrophage population. Although CTS is known as an antimicrobial peptide, there was no reported *in vivo* study demonstrating its antimicrobial impact in the gut. In our second study, we depicted that hCTS administration altered the gut microbiota composition, associated with a more prominent effect on the fecal microbiota than the mucosa-associated microbiota (MAM). In a separate study, we showed that the chemical agent (dextran sulfate sodium [DSS]),

used in our colitis model, was also able to induce a microbial dysbiosis both in fecal and MAM samples. Finally, considering IBD as a relapsing disease of intestinal inflammation, we investigated the effect of hCTS in the context of intestinal inflammation reactivation using a chronic model of colitis mimicking the natural history of IBD. We observed that hCTS administration could down-regulate the reactivation of colitis through a down-regulation of the M1 but not the M2 macrophages and the gut microbiota.

In conclusion, this Ph.D. thesis work revealed a novel anti-inflammatory effect of hCTS in gut inflammation which in the future might open novel therapeutic avenues for IBD.

Acknowledgement

By the name of Almighty, this is my honor today to deeply express my respect and appreciation to my advisor Dr. Jean-Eric Ghia. It would be impossible to finish this difficult task without your help. Thank you for taking me under your wing since July 2012. It was a tremendous gift for me to receive your guidance, to learn to keep patience both in science and life. You always went beyond your academic duties to keep me on track. Thank you for believing in me.

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Finally, I want to remember my deceased father Dr. Shah Alam, whose vision and guidance helped me to pursue my higher studies. I want to show my deepest respect to my mother Begum Marium whose unconditional affection and love helped me in every step of my life. A special thanks to my wife Shanjida Ahmed, for supporting me in every hurdle of my life. My deepest appreciation to my uncle, Mr. Jabed Iqbal Pathan, for his unconditional support during the hardest part of my life and utmost moral support he provided to pursue my dream. I am also grateful all my family members for their well wish and prayers.

May the Almighty bless us all with happiness and good health.

Dedication

To the loving memory of my father who is my inspiration in every step of my life and to my mother whose sacrifices for me mean no bounds.

Foreword

This thesis was written following a manuscript format and is composed of four published manuscripts. The title of the manuscripts along with the contributing authors are listed below.

Manuscript 1 |

Rabbi MF, Labis B, Boutigue MHM, Bernstein CN, Ghia JE, 2014. Catestatin decreases macrophage function in two mouse models of experimental colitis. Biochemical Pharmacology.

Manuscript 2 |

Rabbi MF, Munyaka P, Nour E, Khafipour E, Ghia JE, 2017. Human catestatin alters gut microbiota composition in mice. Frontiers in Microbiology.

Manuscript 3 |

Rabbi MF, Munyaka P, Khafipour E, Ghia JE, 2016 Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice. Journal of Basic Microbiology.

Manuscript 4 |

Rabbi MF, Munyaka P, Nour E, Khafipour E, Ghia JE, 2017. Reactivation of intestinal inflammation is suppressed by catestatin in a murine model of colitis via M1 macrophages and not the gut microbiota. Frontiers in Immunology.

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List of Abbreviation

ARG1	Arginase 1
BMDM	Bone marrow-derived macrophages
CD	Crohn's disease
CHGA	Chromogranin A
CRP	C-reactive protein
CTS	Catestatin
D	Day
DAI	Disease activity index
DF	Dietary fiber
DNBS	2, 4 dinitrobenzene sulfonic acid
DSS	Dextran sodium sulphate
EC	Enterochromaffin cells
IBD	Inflammatory bowel diseases
IBS	Inflammatory bowel syndrome
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
MAM	Monocyte associated microbiota
MCP1	Monocyte chemo attractant protein-1

MPO	Myeloperoxidase
OTU	Operational taxonomic unit
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PLS-DA	Partial least square discriminant analysis
STAMP	Statistical analysis of metagenomic profiles
STAT3	Signal transducer and activator of transcription 3
TNF	Tumor necrosis factor
UC	Ulcerative colitis
VIP	Variable influence on projection value
YM1	Chitinase-like 3

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- Chapter 4: 4.2. Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice.

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Chapter 01. Part one. Introduction

1.1 Overview of inflammatory bowel disease

Inflammatory bowel diseases (IBD) are idiopathic chronic, recurrent intestinal disorders of complex pathogenesis, which include Crohn's disease (CD) and ulcerative colitis (UC) [1]. The clinical symptoms of IBD are characterized by abdominal pain, diarrhea, blood in the stool and weight loss [1]. Mucosal changes in IBD are characterized with transmural inflammation in the intestine, infiltration of monocytes producing an aberrant level of cytokines and proteolytic enzymes against luminal antigens causing collateral damage and ulceration [1, 2]. Being considered as a Western disease, the disease is in progress worldwide but still much more common in North America [3, 4]. To date, the exact etiology of IBD is unknown and although drugs are available to suppress the symptoms [5, 6], there is no curative treatment available for IBD. Therefore, research needed much attention to find new effective drugs.

1.2 Clinical characterization of Crohn's disease and ulcerative colitis

In UC, various degrees of inflammation and underlying morphological changes can be seen at the level of the colon [1]. In general, 95% of UC patients' rectum depict ulceration, edema, hemorrhage, which are generally limited to the mucosa. Other morphological changes that occur in UC patients include crypt abscesses, damage to the mucosal glands, thinning of the mucous layer due to a lower number of goblet cells and inflammation caused by neutrophils and monocytes [1].

Conversely, in CD, the whole gastrointestinal tract can be affected, from mouth to rectum. The inflamed segments in CD are discontinuous in nature and bordered by normal segments, generally known as “skip areas.” In CD, the entire bowel wall can be inflamed, which results in the development of fistula. The areas mostly affected by CD are the ileocecal region and terminal ileum [1].

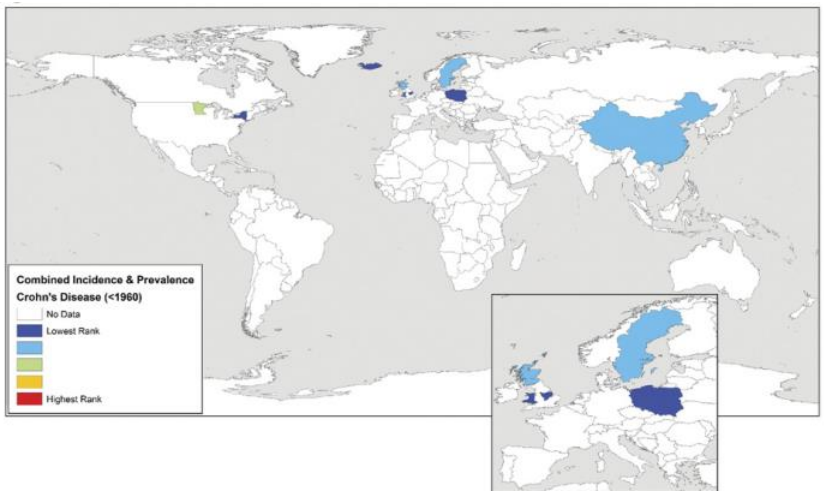
The most classical clinical feature of UC is a bloody diarrhea and the presence of blood in the stool, and these features are used to clinically differentiate UC patients from inflammatory bowel syndrome (IBS) [1]. UC patients also suffer from pain mainly in the lower abdomen which can progress to pancolitis. On the other hand, the clinical symptoms of CD largely vary on the location, extent and severity of inflammation. For example, if the duodenum is affected, clinical manifestations will include nausea, epigastric pain, dysphagia [1]. However, when the small bowel is affected CD patients will demonstrate abdominal pain, diarrhea and weight loss. CD patients who suffer from inflammation of the large intestine exhibit symptoms nearly like UC including blood and mucus in stool and lower abdominal pain. Despite dissimilarities both in UC and CD, the clinical symptoms can be continuous or the patients can suffer from several flares of inflammation including relapse (flare ups) and remission stage (asymptomatic)[1]. .

Beside the gastrointestinal tract, about 40% of all IBD patients suffer from extra-intestinal manifestations affecting almost all organs which increase the morbidity and distress in the patients. Most common cases include peripheral and axial arthritis, skin rashes, psoriasis, and erythema nodosum [7].

1.3 Epidemiology

Since 2012, May 19th is the official day to raise awareness of IBD around the world [8]. Historically, IBD is prevalent in developed countries compared to the developing low industrialized countries [3, 9]. In fact, when the world was experiencing a rapid industrialization development in the middle of last century, the incidence and prevalence of IBD have rapidly increased (**Figure 1-1)** and by the beginning of this century, IBD has been considered as one of the most prevalent gastrointestinal diseases (*i.e.* highest incidence and prevalence of IBD are observed in Europe, Great Britain and North America) [9-12]. Recent epidemiological data suggest that the incidence of IBD is still progressing in developing countries in parallel to the modernization and Westernization of their lifestyle [9, 11, 12]. Approximately, five million people are now affected by IBD, and Canada has one of the highest incidence and prevalence rates for around the globe with an estimated prevalence of 5/1000 persons. Cost for Canadian economy is nearly USD 2.8 billion per annum [13, 14].

A



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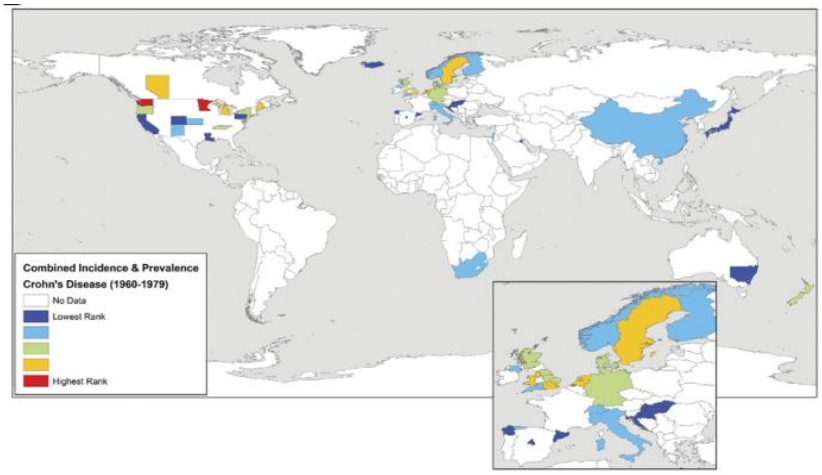

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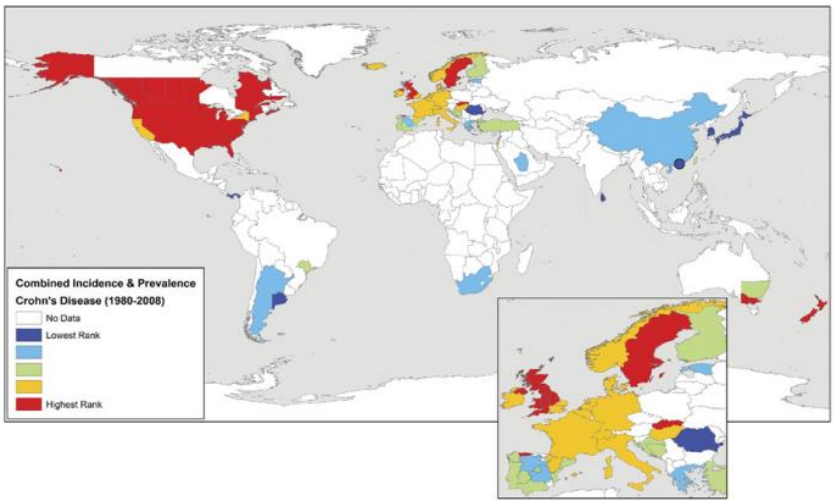

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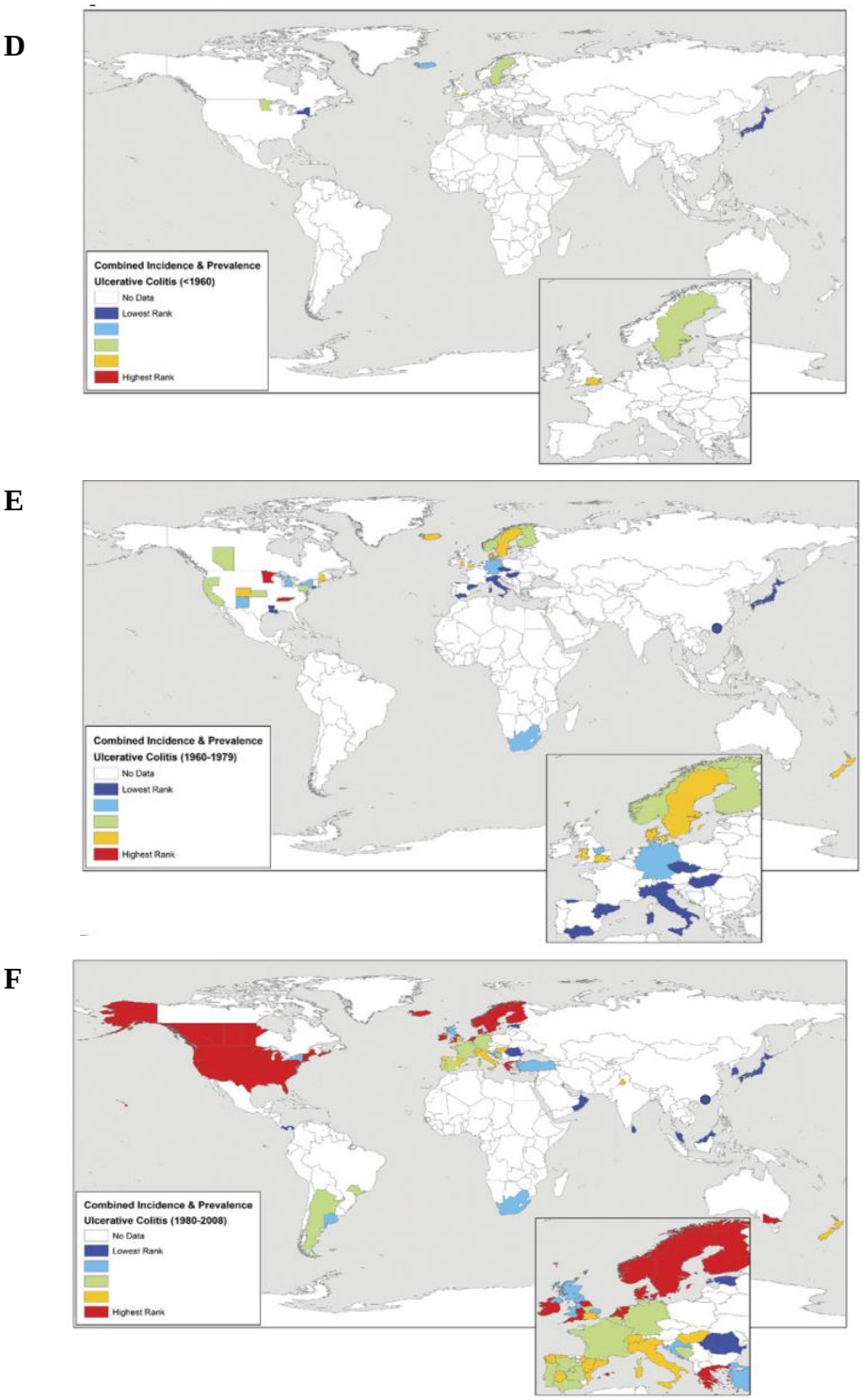

Figure 1-1. Global IBD incidence/prevalence rate.
A, B and C are representing Crohn's disease (CD) incidence/prevalence rate at (A) before 1960, (B) from 1960 to 1979 and (C) after 1980 respectively. D, E and F represents Ulcerative colitis (UC) incidence/prevalence rate at (D) before 1960, (E) from 1960 to 1979 and (F) after 1980 respectively. The figure is retrieved from Molodecky, N.A., *et al*.2012 [9]**.**

1.4 Etiology

The exact etiology of IBD is still unknown. Although it is thought to be multifactorial and probably involves an aberrant immune response to environmental antigens in genetically predisposed individuals [2].

1.4.1 Genetic factors

Genome-wide association studies (GWAS) along with other clinical and epidemiological data suggest that genetic factors have a strong effect on the etiology of the disease [15]. However, it is now clear that a single gene model cannot describe this pathology, instead, IBD is now considered as a multi-gene disorder which follows a non-Mendelian paradigm heavily impacted by environmental factors like microbiome and lifestyle. According to GWAS, there are 99 non-overlapping gene loci identified in IBD. Among them, UC and CD are associated with 47 and 71 gene loci respectively of which 28 are shared between two disease types [16]. In line with this genetic influence, twin studies revealed higher concordance of both UC and CD among monozygotic twins compared to dizygotic counterparts [17]. The genes that are found associated with IBD are mainly responsible for maintaining intestinal homoeostasis by regulating epithelial barrier function, immune cell migration, and their induction [15].

Among various gene loci, *NOD2* gene located on chromosome 16 of humans was first to be identified which is associated with CD but not with UC [18, 19]. This gene contains a C-terminus Leucine rich domain (LRRs) which is crucial to activate central nucleotide oligomerization domain (NOD) and toll like receptors (TLRs). These are crucial to

activate immune response against microbial incursions [20, 21]. Mutation in this gene results in an abrogated immune response via hampering signaling pathways like NF-κβ pathway [22]. It is important to know that, although human subjects homozygous for Nod2 variants have 17.1-fold risk for getting CD, this association is mainly true for European descendants but not in the Asian or African population due to an undefined reason [22-24].

Beside, *NOD2* genes, polymorphism of IFN-γ is quite evident with IBD[25]. The gene for IFN-γ is present on human chromosome 12. It has been observed that CD patients have a higher polymorphism of IFN-γ (+874 T/A) [25, 26]. This might explain the reduction of tissue repair and increased migration of fibroblasts in CD patients influenced by IFN-γ [27]. Further, IL-6 is also considered as a candidate gene for IBD [26] and is located on human chromosome 5 [25]. It has been observed that individuals with higher levels of IL-6 are most susceptible to IBD [25, 28, 29]. Beside these classical cytokine genes, it has been observed that polymorphism in major histocompatibility complex (MHC) genes located on chromosome 6 [25] can influence the antigen recognition process of T cells via antigen presenting cells (APC) which can contribute to IBD susceptibility [30]. However, the true significance of this polymorphism needs to be elucidated. Tumor necrosis factor (TNF)-α is a major cytokine involved in IBD pathogenesis, the gene is located in chromosome 6 [25] and investigation has revealed that polymorphism in TNF promoter gene is associated with IBD susceptibility. For example, it has been observed that single nucleotide polymorphism (SNP) in -1031 promoter region of TNF-α was associated with IBD susceptibility in an Iranian cohort

[31]. Further, it has been suggested that the polymorphism observed in the promoter region of TNF-α might influence the treatment outcome which are associated this cytokine inhibitors. For example, it has been observed that in a Spanish cohort, patients who are resistant to TNF-α inhibitors had higher frequency of 308A alleles and 308GA genotype [32]. However, to identify the clinical significance of these polymorphism to IBD outcome, extensive studies are needed to further characterize genetic association of this relatively complex disease.

1.4.2 Environmental attributes

The impact of environmental factors on IBD etiology is supported by the global distribution of the disease prevalence. As mentioned earlier, the Western world has the highest incidence rate of IBD and the progression of the diseases is increasing with other part of the world especially in developing countries transitioning to developed countries. Epidemiological data from developing countries like South Korea [33], China [34, 35], India [36, 37], Iran [38] Lebanon [39] Thailand [40] and the French West Indies [41] show a clear evidence the rate of UC is increasing which is followed by CD due to a yet unknown reason. It is also evident that the first generation of people, coming from an IBD low incidence country to a developed country with a higher incidence of IBD, has higher risk of developing IBD [3, 42, 43]. These evidences support the hypothesis of the importance of environmental factors over genetic and ethnic background. Some of these factors are discussed below.

1.4.2.1 Socioeconomic

The higher socioeconomic groups practice more cleanliness which might prevent exposure to certain enteric bacteria. This is crucial especially in the early years of life when limited exposure to enteric bacteria due to improved hygiene and sanitation might result in an impaired immune system development. This phenomenon is widely known as "hygiene hypothesis" and might be the backbone of the increased risk of IBD among children from the higher economic part of the society [3, 44, 45]. *Helicobater pylori* infection, that generally occur in early childhood, is negatively associated with both UC and CD [44]. The mechanism behind this can be explained by the role played by regulatory T (Tregs) cells in the context of maintaining intestinal homoeostasis. Tregs are required to abrogate the immune response against commensal microbes, and their development largely depends on infection stimuli especially during the early childhood[44, 46]. Beside the "hygiene hypothesis", family size, siblings age and birth order might also impact the development of IBD. For example, smaller family size [47], older siblings in the family [48] and reduced birth rate is associated with an increased risk of IBD [44, 49].

1.4.2.2 Lifestyle

Although IBD was first considered as a disease of the Western world, recent epidemiological data suggests that the gap between Western and Eastern world in terms of IBD prevalence is narrowing down. One hypothesis behind this is the adaptation of Western culture by the Eastern population. This statement is further strengthened by migrant studies where it shows that the second generation of migrants in the Western

world are prone to develop chronic inflammatory disorders compared with their parents [3, 42, 43].

One of the important environmental factors is the diet which is postulated to have a strong relationship with the etiology of IBD. Although extensively studied, unfortunately, until today no clear definite association has been found. However, studies show that sugar and fat rich diet are associated with increased risk of CD [4, 50]. Studies led by a Japanese group confirmed that the association of fatty food with elevated risk of CD dominates over the genetic variation (low *NOD2* prevalence in Japan compared to North America) [51]. Beside these, it has been observed that red meat consumption might increase UC flare ups [52].

Another predominant factor associated with IBD is smoking. Epidemiological data suggest that smoking is a risk factor for CD prevalence and also worsen disease symptoms by developing host resistance against drugs and increasing flare ups [53]. Surprisingly, smoking shows somewhat protective association with UC [53]. Although the mechanism behind the role of smoking in modulating immune response is not well-understood yet, it is thought that nicotine, an essential compound in cigarettes, controls T cells function *via* nicotinic acetylcholine receptors (nAChRs) [54]. Nicotine was found to have an inhibitory effect on T-helper 2 (Th2) function which is crucial in UC outcomes [55]. Considering this, clinical trials have been carried out using nicotine as therapeutic approach in UC, but the benefits were modest suggesting that nicotine might not be the

only factor in UC protection [56]. Furthermore, smoking alters autophagy which is crucial for IBD especially in CD [57].

Another dominant environmental factor in IBD is breastfeeding. Like other immune disease, breastfeeding has been found protective in IBD [44, 58-60]. Although the exact mechanism is not known, it is thought that breastfeeding allows the immune system to develop better and faster by mounting a tolerance against commensal microbiota and food antigens [59]. Further lactoferrin, which is exclusively found in breast milk, has antibacterial and anti-inflammatory properties [61].

Physical activity has also been found protective in IBD. For example, IBD prevalence was found low in people who held outdoor and physically demanding jobs compared to clerical position [62]. In line with this, mortality in IBD patient is also higher in professionals who has desk jobs compared to construction workers [62]. The exact protective mechanism of physical activity in IBD warrant further investigation.

1.4.3 Gut microbiota

As discussed above, food intake plays an important factor in the development of IBD. The human body is a large reservoir of microbes and the gastrointestinal tract is heavily colonized with microbial population to digest this food (**Figure 1-2**). On average 10^{14} microbes of 1100 species are present within the human gastrointestinal tract, a number 10 times more than the total cell numbers of the human body [63]. Recently, a European cohort study revealed that, on average every human contains 600,000 microbial

genes in their gastrointestinal tract among which 300,000 microbial genes are common within 50% participants in the cohort [64]. Microbial colonization starts from birth and the infant gather microbes from maternal as well as surrounding environment [65, 66].

The initial colonization in infants is highly variable. This "chaotic" microbial environment remains for quite a sustainable period before a stable gut microbiota formed by the age of three [67, 68]. The major phyla [Phyla is a taxonomic category which is placed after the kingdom and above class] belong to Firmicutes and Bacteroidetes [69, 70]. The microbial profile in an individual is largely determined by the person's birth mode, age, diet, nutrition, geographical location [67]. People with the same cultural background living in a same geographical location seem to have less variable microbiota [67]. For example, Westernized population tends to show less variation in gut microbiota at the phylum level [67]. Diet has a substantial influence over gut microbiota. Human from different cultural background might have different dietary intake which might change their gut microbiota composition. For example, microbial diversity in lower taxonomic level is observed in fecal microbiota of two distinct cultural populations from the United States and Korea [71], this variation was mainly correlated with the dietary habit of this two group. Protein, carbohydrate and fat are the three major nutritional components that can dictate gut microbiota composition [67]. It has been observed that, diets enriched with fat and carbohydrates can shift the gut microbiota [72]. For example, dietary fibre (DF) is crucial for healthy diet and helps to prevent various bowel disorders and obesity [73-76]. These beneficial effect is probably gained due to incompletely fermented fibre like insoluble non-starch polysaccharides (NSP) cellulose. These

incompletely fermented fibre helps to detoxify, reduction of intracolonic pressure, accelerate colonic transition, simulate fermentation and increase biomass[77]. Fermentation of carbohydrates in eukaryotic host largely depends on gut microbiota. The organic acid produced by the microbial fermentation of carbohydrates provide energy to gut microbiota as well as peripheral eukaryotic tissue. Short-chain fatty acids [SCFAs] are one major products derived microbial degradation of carbohydrates and proteins, these are highly important for host energy. Besides providing energy the SCFAs have beneficial physiological role in the host [78]. Murine diet supplemented with SCFAs had beneficial effect like controlling adipose tissue and glucose control. These beneficial effect is exerted via a shift of the gut microbiota towards butyrate and propionate activated glucogenesis [79]. Furthermore, it has been observed that whey protein isolate (WPI) can change the microbial composition in murine as a dose dependent manner [78]. It has been observed that murine diet supplemented with WPI has higher proportion of *Lactobacillaceae* and significantly decreased levels of *Clostridiaceae* compared to murine supplemented with high fat diet [80]. This beneficial shift in gut microbiome resulted in more energy intake. Besides helping the eukaryotic host to break down food nutrients into digestible forms, gut microbiota also plays a crucial role in controlling the gut immune system. For example, SCFAs helps to lower the pH in the gut which prevents the accumulation of pathogenic bacteria.

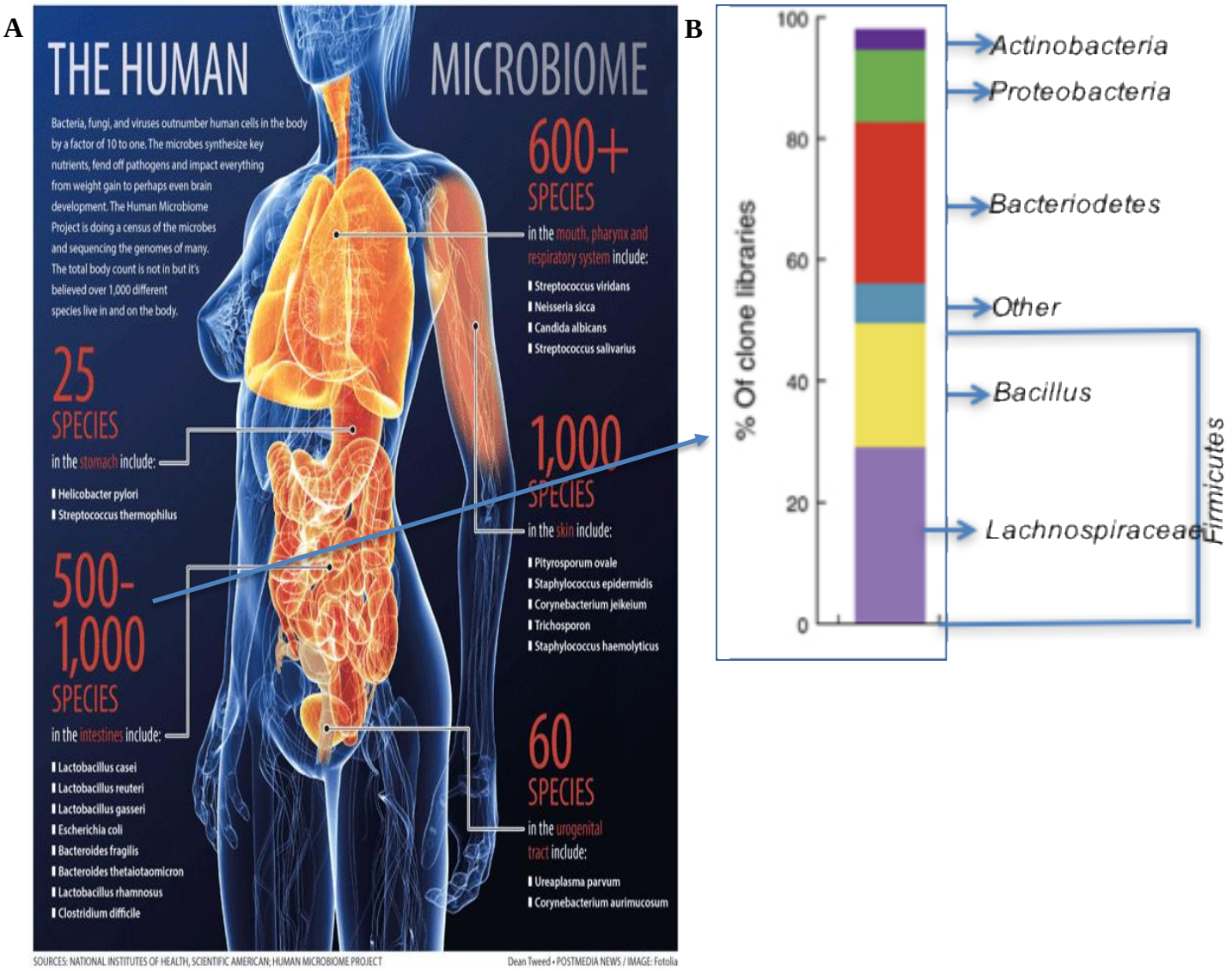

Figure 1-2. The human microbial load.
A; Schematic diagram showing microbial composition at different human body part. B; Composition of microbes at the intestine. Figures adapted from Hardin, J.R., 2015 [81] and Sartor, B., *et* al, 2012 [82] respectively.

1.4.3.1 Gut microbiota and immune system

Beside aiding the host in digestion, nutrient uptake, detoxification, the gut microbiota plays a crucial role in regulating the immune system. In the gastrointestinal tract, the immune system is regulated by the gut associated lymphoid tissue (GALT) and prevents colonization of pathogens by the commensal microbes [83]. The GALT is a crucial immune structure composed of Peyer's patches, crypt patches and isolated lymphoid follicles (ILFs) [84]. Using the GALT, antigen presenting cells (APCs) present the antigen which helps to maintain the intestinal homoeostasis. Although, the development of Peyer's patches starts in the sterile fetus using the stimulation lymphoid tissue inducer (LTi), microbial induction is required for the development of crypt patches and ILFs [84]. Excessive exposure of bacterial pathogens activates the pro-inflammatory milieu of the gut environment. It has been observed that germ-free animals have an underdeveloped GALT indicating that controlled induction by microbial antigen is crucial to maintain a healthy immune system [83, 85, 86] .The innate immune system is largely dependent on the pathogen recognition receptor (PRRs) found both on the immune cells and intestinal epithelium cells [83, 85, 86]. To date, three families of PRRs have been identified: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [83, 85, 86]. These receptors can identify the conserved microbial associated molecular patterns (MAMPs) which are found both on pathogens and commensal bacteria[87] and thus arises the question of how the gut immune system can differentiate between these two to evade unnecessary stimulation of the immune system? One proposed hypothesis is that, in a normal steady state condition, the TLR3 and TLR5 are expressed whereas TLR2 and TLR4 expressions are down-regulated[88].

TLR2 and TLR4 mainly recognize bacterial lipoprotein and lipopolysaccharide (LPS), respectively. The lower expression of TLR4 might help to maintain the intestinal homoeostasis in the presence of a large number of gram-negative commensals and bacterial LPS [67]. Another possible explanation is that the LPS structure found on the commensal and pathogenic microbes are not functionally identical which help to down-regulate the immune activation against the commensals by the host. Similarly, some bacterial flagellin can activate TLR5 whereas some cannot [67]. However, pathogens like *Helicobacter pylori* use this non-activating flagellins to evade the immune system and sustain in the host gut [89]. It has been observed that in IBD TLR signalling is altered which triggers the importance of these signalling molecules in intestinal inflammation. For example, deletion of TLR5 which recognizes bacterial flagella results in the development of spontaneous colitis [90]. Considering these, it is inevitable that controlled expression and induction of TLRs are crucial to maintain a healthy immune system. This control expression of TLRs is carried out by negative TLR regulators like TOLLIP, SIGIRR, peroxisome proliferator-activated receptor gamma (PPAR ϒ), and A20 [91]. A20, which is a zinc finger protein that inhibits the induction of TLRs, knocking out these proteins increases the susceptibility to experimental colitis [67].

The microbiota also controls the development of myeloid cells not only in the intestine but also in different organs. It has been observed that the absence of microbiota negatively impacts the development of myeloid cells in bone marrow which hampers the host to clear systemic bacterial infection [92, 93]. The complexity of gut microbiota controls myelopoiesis in the bone marrow. Similarly, SCFAs derived from microbiota

might also control myelopoiesis [94]. In fact, it has been observed that microbial influence on myelopoiesis might start before birth. For example, in a murine study it has been observed that during pregnancy antibiotic treatment resulted in fewer number of blood neutrophils in newborns [95]. The maturation of myeloid cells are also dependent on microbiota, as it has been observed that aging of neutrophils largely depends on the presence of microbiota-derived TLR ligands [96]. Besides affecting circulating myeloid cells, microbiota can also orchestrate the maturation of tissue resident myeloid. For example, SCFAs can control the gene expression profile of tissue resident macrophages in the intestine [97]. Beside the intestine, the microbiota also dictates the function of myeloid cells various other organs like the nervous system, lungs and myeloid cell trafficking. For example, the continuous replenishment of macrophages at the intestine level is largely regulated by the gut microbiota [92]. It has also been observed that the gastrointestinal motility can be controlled by the signals derived from the commensal microbiota which facilitates the interaction between enteric neurons and muscularis macrophages [98]. This regulation by commensals might be exerted via their control over the expression of a bone morphogenetic protein 2 (BMP2) and colony-stimulating factor 1 (CSF1; known as macrophage colony-stimulating factor 1) derived from muscularis macrophages and enteric neurons respectively [98]. These molecules in turn can regulate the contraction of smooth muscle cells. Beside this, innate immune arm of intestinal immune system is largely controlled by the maintenance of "Colonization resistance" (inhibition of pathogenic microbial colonization) which is largely dependent on the normal gut microbiome[99]. Colonization resistance can be maintained by the production of toxic substances by members of commensal microbiota such as

bacteriocins or short-chain fatty acids, competition with pathogens for available receptors, improvement of gut motility and/or nutrient limitation[99]. In addition to the innate immune system, gut microbiota can also control the host's adaptive immune system through T cell receptor αβ-positive intra-epithelial lymphocytes, T regulatory cells (Tregs) and T helper 17 (Th17). Further, commensal also regulate the tissue recovery after inflammation[100]. It has been observed that, dietary supplement with *Lactobacillus reuteri* enhances wound healing in mice two times faster than the control animals. *L. reuteri* induces oxytocin, a neuropeptide hormone which activates the regulatory T cells (T_{regs}) which aid to down-regulate the host inflammation and in turn collateral tissue injury[101]. Beside these, it has been also observed that mice supplemented with *Lactobacillus reuteri* have a decreased number of MPO positive neutrophils [101]. Considering these, it is now well established that the commensal microbiota largely influences the intestinal immune homoeostasis through the orchestration the myeloid cell landscape and overall their control over the maintenance of a healthy intestine.

1.4.3.2 Microbial dysbiosis and IBD

IBD is characterized by dysregulated immune responses towards the intestinal microbiome [102], however, not a single causative microbial agent has been identified which can fulfill the Koch's postulates [103]. Current technological advancements have allowed researchers to deeply analyze culture of independent microbes and these studies greatly influence the statement that alteration of gut microbial constituents rather than a single pathogen might be associated with IBD [103]. An unnatural shift or an alteration in

gut microbiota is referred as "Dysbiosis" (**Figure 1–3**) [104], and human intestinal microbiota experience temporal fluctuation influenced by food, physical environment, medication, etc [104]. In IBD, the stability of microbiota is deeply affected with the overall microbial diversity being reduced, affecting mainly Firmicutes and Bacteroides. Among Firmicutes, a decrease of *Faecalibacterium prausnitzii* has been reported in many studies [63, 105] with the bacteria being able to induce the production of IL-10 in the intestine and thus having anti-inflammatory properties and preventing intestinal inflammation in murine experimental models of colitis [106]. Besides, it has been observed that the microbial density at the mucosal epithelium is increased in active UC patients, with colonic epithelium attached bacterial being responsible to invade the epithelium barrier and induce inflammation. For example, *Fusobacterium varium* isolated from active UC patients showed cytotoxic capacity in Vero cell lines [107]. In addition, when these bacteria are transferred to mice, they developed colonic inflammation. In parallel, *Fusobacterium nucleatum* isolated from inflamed tissue specimens from active UC patients exerte invasive properties in a Caco-2 cell assay [108]. Similarly, virulence of *Escherichia coli* was found to be increased in UC patients' fecal samples [107] In CD, a decrease a microbial diversity was also observed, associated with a relative abundance of Enterobacteriaceae and *Ruminococcus gnavus*. Decrease of anti-inflammatory bacteria like *Faecalibacterium prausnitzii* has also been reported [103].

As dysbiosis being inevitable in IBD, the question whether dysbiosis is causal or a consequence of intestinal inflammation is still under debate. To answer to that last

question, studies have been done with IBD patients and their healthy relatives who share the same genetic and environmental background.

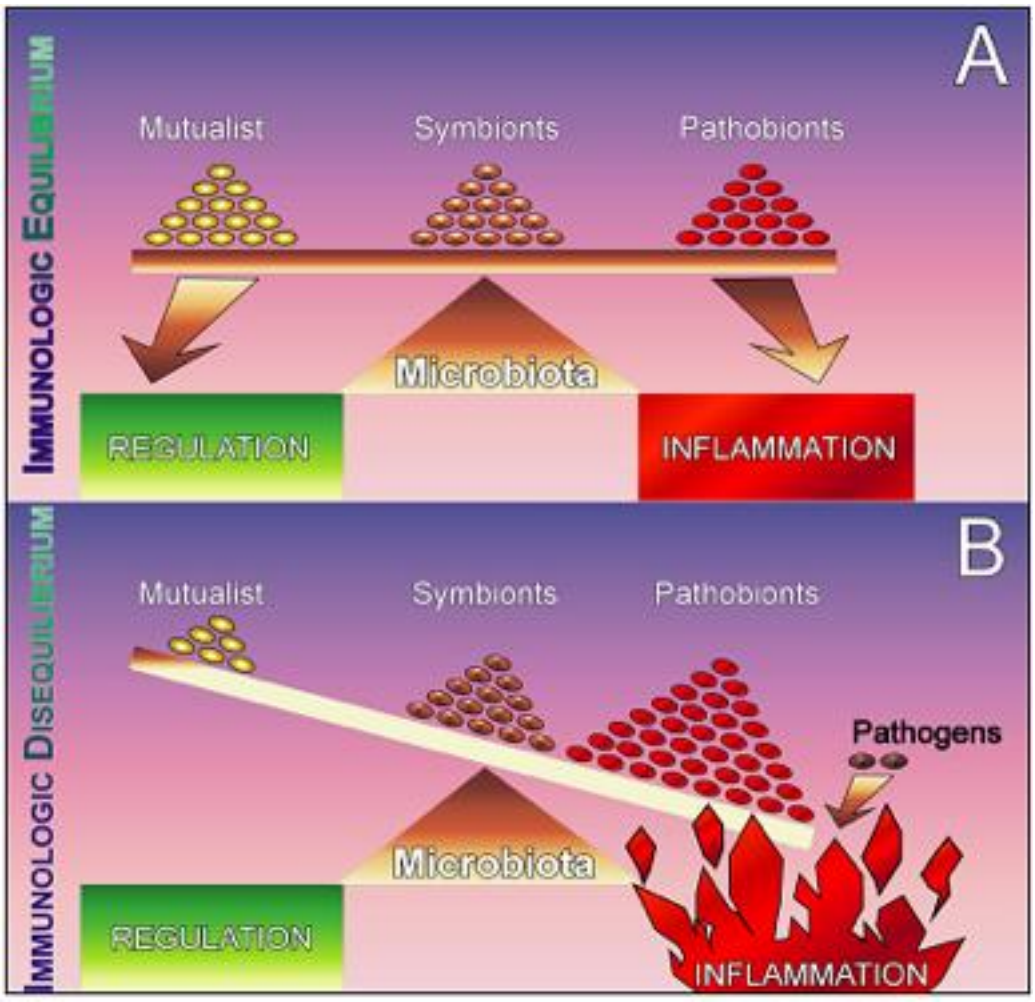

Figure 1-3. Microbial dysbiosis and immune dysfunction.
A; A healthy individual has a balanced composition of several bacterial classes. Commensals are permanent microbes of the ecosystem and might provide no benefit or harm to the host. Symbionts on the other hand benefits the host and pathobionts are detrimental to host. B; During dysbiosis, due to unnatural shift for unknown reason either pathobionts are increased and/or symbionts are decreased which might cause inflammation. Figure retrieved from Round, J.L. *et al.*2009 [104].

Twin studies revealed inconsistent microbial change between UC patients and healthy twin counterpart [109], with *F. prausnitzii* was decreased both in UC patients and their first-grade relatives [82, 110]. Similarly, CD patients' unaffected relatives also demonstrated dysbiosis, however, it was not consistent with the dysbiosis seen in CD patients [111]. Furthermore, in a twin study, monozygotic twins discordant for CD showed that healthy individuals had more diverse gut microbiota than CD counterparts. In pre-clinical stage, *NOD2* and *ATG18L* genes, which are two major CD susceptibility genes, are associated with an alteration of the gut microbiota [112]. NOD2 is expressed on intestinal epithelial cells and monocytes/macrophages and acts as a receptor for muramyl dipeptide (MDP), a cell wall component of gram-positive bacteria. It has been observed that mice devoid of NOD2 have an increased gut bacterial load, but these mice did not develop spontaneous colitis. It has also been also observed that CD patients with NOD2 mutations have reduced production of antimicrobial peptides (AMPs) from Paneth cells [113]. The production of the anti-inflammatory cytokine IL-10 also seems to be affected by NOD2 mutation in CD patients [114]. Further, replication of intracellular bacteria and bacterial antigen presentation from infected cells is heavily dependent on the NOD2 stimulation by MDP [115]. It has been reported that intracellular bacterial invasion can be determined by NOD2 which in turn brings the ATG16L to site of invasion, triggering the formation for autophagosome which then can process the intracellular pathogen [116]. These events are suggestive that the microbial dysbiosis probably occur as a consequence of genetic and environmental factor rather than inflammation.

Several studies have been done to link dysbiosis and inflammation. It has been observed that several commensals can induce colitis when presented to IL-10 deficient mice. Administration of *E. coli* or *Enterococcus faecalis* in IL-$10^{-/-}$ resulted in cecal inflammation and distal colitis respectively [116, 117], however, *Pseudomonas fluorescens* did not show any effect on colitis. In addition, *Helicobacter hepaticus*, which is known as a commensal bacterial worsen the colitic symptoms in IL-10 deficient mice [116]. The effect of gut microbiota on inflammation was further strengthened by studying mice deficient for *Tbx21/T-bet*. *Tbx21/T-bet* which are important transcription factors for Th1 cell differentiation and mice deficient of these intracellular regulators develop spontaneous colitis [118], but treatment with antibiotics lowered the colitis symptoms in these animals. Further, it has been observed that wild type mice when co-housed with *T-bet/Rag* double knock out mice developed colitis [118]. This observation indicated that dysbiosis is transferrable and can develop colitis irrespective of genetic background.

In addition to dysbiosis itself, functional changes due to dysbiosis might also lead to inflammation. It has been observed that in IBD patients gut microbial genes which are associated with carbohydrates and amino acid metabolism are decreased as opposed to genes associated with oxidative stress pathway which are increased [119]. This observation suggests that increased oxidative stress might be causal for intestinal inflammation. As discussed earlier, gut microbiota provides metabolites which are indispensable to epithelial cell function, energy uptake and development of the immune system. The SCFAs like butyrate derived from microbial degradation of non-absorbable

carbohydrates are important for energy uptake as well as production of mucin and anti-microbial peptides (AMPs) [116, 120]. *F. prausnitzii* produces both the anti-inflammatory cytokine IL-10 as well as butyrate. The bacteria decrease in IBD can reduce the amount of butyrate which might further aids in developing intestinal inflammation. Furthermore, it has been observed that sulfate-reducing bacteria (SRB) in increased in UC [121]. SRB produces hydrogen sulfide which damages the intestinal epithelial cells which in turn might cause mucosal inflammation [121]. Beside these, bacterial load on the mucus layer has been found increased in UC suggestive of a defective barrier with for example, *Ruminococcus gnavus* and *R torques* able to degrade mucin into nutrients facilitating other bacteria to reside in the mucous layer [116]. From these data, it is obvious that gut dysbiosis with a decreased microbial complexity associated with an altered immune response in genetically susceptible individual are critical for the development of IBD and experimental colitis. However, further studies are warranted to clarify the relationship between dysbiosis and inflammation.

1.4.4 Immune dysfunction

The abnormal immune system in the gut is characterized by damage to the intestinal epithelium (mucus production abnormality, anomalies in tissue repair), inflammation exacerbation and massive infiltration of immune cells to the intestine, with the immune cells producing a large amount of pro-inflammatory cytokines like TNF-α, IL-1β, IFN-γ [122, 123].

The intestinal immune system can be characterized into innate and adaptive immunity [124]. The innate immune system consists of barrier function of intestinal epithelium, anti-microbial peptides (complements, defensins, catestatin etc.), acidic pH environment in stomach to limit microbial growth, innate immune cells including dendritic cells, macrophages, neutrophil and natural killer cells [122, 123]. In line with the innate immune activation, the adaptive arm of the immune system consists of T and B cells which become activated within days due to inflammation milieu. T cells are divided in T-helper 1 (Th1), Th2, Th17 and Treg cells and compensate the innate immune system due to their much more specificity [122]. Overall, the immune system is tightly regulated and disturbance of this regulation can cause IBD.

1.4.4.1 Dysregulation of innate immune system

The 400 mm^2 intestinal epithelial cell (IEC) layer is the major physical barrier against foreign antigens and the crucial component of innate immunity [125]. Four different types of epithelial cells make the IEC, columnar cells, Paneth cells, goblet cells and neuroendocrine cells deriving from the same stem cells [126]. The columnar cells are more abundant and have finger like protrusions known as microvilli. They are covered with glycocalyx which makes them sticky in order to prevent the invasion of luminal microbes [127]. In addition, antimicrobial peptides like defensins are produced by these cells which also aid in preventing luminal invasion [128]. Defensins are divided into two categories based on their disulfide bonds: alpha defensins and beta defensins [128]. Although beta defensins are produced by columnar cell [128], alpha defensins are produced by Paneth cells [129]. Beside these, goblet cells also produce defensins and

mucin [130] which covers whole gastrointestinal tract in order to prevent microbes coming to a close proximity of epithelium layer. In addition, IEC uses antigen presenting components, pathogen recognition receptors (PRRs) in order to limit the entry of microbial and food antigens towards the mucosal immune system [131]. This is important to educate the mucosal immune system to develop a tolerance against commensal and food antigens while become activated during stress [131]. The selective permeability of the IEC allows controlled integration of nutrients, fluids and microbes [132]. This is a crucial step in maintaining intestinal homeostasis.

The epithelium integrity is compromised in IBD. It has been observed that both in CD and UC the apoptotic rate is increased [133]. This might be due to the release of pro-inflammatory cytokines like TNF-α or IFN-γ which are secreted largely during inflammation. These cytokines might increase the apoptotic rate of intestinal epithelium and thus compromising the physical barrier [133]. It has been observed that IFN-γ is associated with endocytosis of tight junction transmembrane proteins [134], that might increase the permeability of IEC, explaining probably the increased permeability of macromolecules observed in IBD patients. In addition, increased apoptotic rate compromises the barrier function [135]. In line, IEC control the electrolyte transport and anomaly in this control con induce diarrhea [136]. It has been observed that half of CD patients and almost all UC patients has diarrhea [136]. In IBD, reduction of electrolyte transport in IBD might occur due to diminished response to electrogenic anion secretion, reduction in the synthesis of epithelium sodium channels and reduced NaCl absorption [133]. Besides, several peptides including epidermal growth factor, the TGF-β family, the

insulin-like growth factor family, the fibroblast growth factor family, the colony-stimulating factor family controls the IEC integrity which seems to be compromised in IBD [137].

Another important cell of innate immune system are the dendritic cells (DC). The DCs reside in the laminal propria and Peyer's patches (PP) and are mainly responsible to sample microbial antigens and present them to T cells [138]. These DCs mainly uptake bacterial antigens through the epithelial microfold (M) cells[139]. Beside the regular M cell route, some DCs have specialized dendrites which can transverse through the gut epithelium and can uptake the bacteria [139, 140]. The DCs can rapidly recognize the microbial antigen and migrate to the mesenteric lymph node and spleen to activate T cells [140]. However, the type of activation largely depends on the type of bacteria uptake, surrounding cytokine/chemokine environment, and expression pattern of PRRs on DCs, like TLRs, C-type leptin receptors, and NOD- like receptors [140]. It also has been observed that the DCs deriving from different organs are characteristically different [140]. For example, DCs derived from PP secret large amount of IL-10 compared to spleen derived DCs when stimulated via CD40L. Blockage of IL-10 via neutralizing antibody in PP DC-T cell co-culture resulted in higher amount of IFN-γ production [117]. The IFN-γ skews naïve T cells towards T helper 1 cells which results in pro-inflammatory mille in the gut. Thus, the production of IL-10 by PP DCs are important to maintain a homeostatic condition in the intestine and to prevent exaggerate immune response against commensals. It has been observed that, both in experimental colitis and IBD patients, DCs are largely accumulated at the inflammation site [141]. The intestinal

DCs derived from CD patients demonstrated an increased expression of TLR-2 and-4 as well as CD40 which is an activation/maturation marker [142]. Furthermore, during IBD, TNF-α expressing MDC8+ monocytes are increased, precursor of mucosal DC population. Considering this, CD patients treated with anti-TNF–α antibodies portray a diminished DC activation [143, 144]. It was observed that most biopsy samples collected from CD patients have a reduced expression of thymic stromal lymphopoietin (TSLP) mRNA. TSLP is a major contributor in IL-12 secretion from DCs, therefore, due to the scarcity of TSLP, pro-inflammatory milieu cannot be controlled and large amount of IL-12 is produced by DCs which promotes Th1 response [145, 146]. Considering this, it is well evident that DCs can control the intestinal inflammation and that might take part in IBD etiology via their influence on tolerance maintenance towards commensal microbes and food antigens.

Another key important innate immune cell are neutrophils. They perform a key role in activation of immune responses and generation of gut inflammation. Neutrophils are released and migrated from the bone marrow towards site of inflammation under various chemoattractant factors stimuli [147-149]. Generally, the recruitment of neutrophils from the blood into inflamed tissue is regulated by various chemotactic factors. Different neutrophil chemotactic agents have been shown to be overproduced in mucosa affected by UC, including IL-8 [150], TNF-α [151], macrophage inflammatory protein 1α [152], and leukotriene B [153]. Under inflammatory circumstances, the production of these proteins occurs by epithelial cells and various immune cells, but neutrophils are also a major source of pro-inflammatory mediators including cytokines (IL-8, IL-6, IL-β and

TNF-α) [154], collectively, selective depletion or adhesion blockade of neutrophil by monoclonal antibody suppresses dextran sodium sulphate (DSS)-colitis [155, 156]. Quantification of granulocyte (including neutrophil) infiltration can be verified through the expression of myeloperoxidase (MPO) enzyme they express. Despite strong evidence of the pathophysiological role of neutrophils in colitis, still a little is known about the key mechanism that regulate neutrophils cell migration to the gut inflamed tissue.

The gastrointestinal tract is well known as the largest endocrine of the body [157]. The endocrine cells of the GI tract are known as enteroendocrine (EEC) cells [157]. Among various EEC cells, Enterochromaffin (EC) cells are well studied. They are the most abundant EEC cell and are characterized by secretary vesicles. These secretary vesicles are either large dense-core vesicles (LDCVs) or the smaller synaptic-like microvesicles (SLMVs). EC cells are a major source of chromogranin A (CHGA) which act as a positive immunohistochemistry marker for EC cells [157, 158]. The role of EC cells and CHGA in colitis are described later. CHGA in general colocalizes with 5-hydroxytryptamine (5-HT). It has been reported that more than 90% of 5-HT in human body is sourced in the gut[159]. It has been reported that 5-HT is responsible to increased gut motility and increased intestinal transit [160, 161]. It has been reported that $CD4^+$ T cells might interact with EC cells and increase the production of 5-HT [162]. Further, pro-inflammatory cytokine like IL-1β and/or bacterial (*Escherichia coli*) products can interact with EC cells TLR activation and increase the availability of 5-HT [163]. The altered expression of 5-HT in IBD results in diarrhea or constipation and also aids in intestinal inflammation [163].

During active inflammation, as the tight junction protein in IEC rapidly breaks down, permeability is compromised and microbial antigens migrate toward mucosal immunity [132]. Thus, the immune system become activated and a large amount of inflammatory cytokines are produced and several inflammatory cells mainly macrophages and granulocytes are migrated towards the site of inflammation [164]. All these results in excessive production of reactive oxygens, other free radicals causing collateral damage to the tissue. One of the major regulator of gut inflammation is the Signal transducer and activators of transcription 3 (STAT3). Depending on the cells type, activation of STAT3 can be both pro-inflammatory and anti-inflammatory. The impact of STAT3 activation on gut inflammation is described in detail below.

1.4.4.2 STAT3 and colitis

The altered activation of STAT3 on gut inflammation has been reported from both animal colitis models [165-167] human IBD [168, 169]. Although the special role of STAT3 is IBD etiology is not clear yet, it is known that various cytokines and growth factor can activate STAT3 via phosphorylation (pSTAT3) which leads to its dimerization and subsequent migration to the nucleus [170]. Depending on the cell types, the activated STAT3 can induce several genes regulating a variety of functions like cell growth, apoptosis, motility, cytokine production, anti-microbial activity, etc. [171-175].

Both UC and CD patients have an elevated level of intestinal pSTAT3 [169, 176]. Further, it has been reported that mononuclear cells isolated from inflamed lamina propria nor the blood mononuclear cells possess activated STAT3 [176]. It has also been

observed that the pSTAT3 level correlates with the level of inflammation in IBD patients [169]. The STAT3 activation in inflamed tissue has been observed in both innate and adaptive immune cells [170]. However, depending on cell types the outcome of STAT3 activation might be different.

Among the adoptive immune cells, CD4+ T cells play an important role in gut inflammation. It has been observed that, mice models specific for STAT3 knockout in T cells abrogate T cell proliferation [177]. This was further proven that, IL-6 dependent T cell proliferation prevents apoptosis in T cells [170]. Other reports also proved the importance of IL-6/STAT3 pathway for preventing apoptosis of pathogenic T cells [165]. Disturbance of this pathway results in amelioration of chronic inflammation. These studies suggest that STAT3 activation of adaptive immune cells specifically T cells are detrimental for IBD outcome. In fact, it has been observed that blocking of IL-6 or leptin/STAT3 pathway can effectively improve the T cell medicated experimental colitis [165-167].

During the innate immune response, the colonic epithelial cells (CECs) and phagocytic cells like macrophages play a crucial role to barrier function and immune homeostasis [170]. Various reports suggested that STAT3 activation is important among CECs to maintain barrier function and macrophage activation [173, 178, 179]. Studies using mice models specific for STAT3 knock out in innate cells but not adaptive immune cells develop terminal colitis. Surprisingly, STAT3 knockout in both CECs and macrophages are fatal [180, 181], whereas mice devoid of STAT3 in CECs or

macrophages alone can survive. This indicates the functional STAT3 is a must in either CECs and/or macrophages for animal survival.

Various molecules are activated by STAT3. In T cells, STAT 3 activation induce the anti-apoptotic genes bcl-2, bxl-2 which results in their prolonged survival [165]. In contrast, IL-10, a well-known anti-inflammatory cytokine production by macrophages and CECs are associated with STAT3 activation. Further STAT3 activation activates suppressors of cytokine signaling (SOCS) 3 which is important to inhibit colitis. It also has been observed that STAT3 activation induce trefoil factors which are important regulator of intestinal epithelial homeostasis [182]. STAT3 activation also results in increased production of mucins (MUC1, -3, -10, and -13) [183] which improved the membrane damage due to colitis. Considering this, it is assumed that STAT3 activation in innate immune cells like macrophages is beneficial for colitis outcome.

1.4.4.3. Macrophage and colitis

One of the major important innate immune cells in the gut are the macrophages [164]. Their role is highly crucial to control the barrier function in the small and large intestine. The macrophages are differentiated from monocytes after their migration from the blood. This recruitment is induced by IL-8 and TGF-β [184]. The TGF-β is expressed from the epithelium matrix and macrophages are highly populated in the intestine. In fact, they account for one fifth of total leucocytes in the intestine. These tissue resident macrophages are positioned directly beneath the intestinal epithelium and influence gut homoeostasis by eliminating invading microbes without influencing the lymphocyte

activation. They can also clear the dying cells and other cellular debris [164]. These intestinal macrophages are unique compared to macrophages of the other body counterpart. For example, they have a low expression of co-stimulatory molecules, Fc receptors of IgA and IgG, complement receptors and integrins [185], and have an increased phagocytic activity, release anti-inflammatory cytokines like IL-10 and are not induced in response to TLR stimulation[186]. These phenomena are known as inflammation anergy [187]. This inflammation anergy is thought to be mediated by TGF-β which down-regulate MyD88 and block the NFκβ pathway. Production of IL-10 by resident macrophages is crucially important to induce and maintenance of FOXP3 iTregs which are important to maintain the gut homoeostasis (**Figure 1-4A**) [164, 188]. Because of these unique properties of intestinal macrophages they are known as alternatively activated macrophages or M2 macrophages[164]. A breach in the regulation or balance between the two types of macrophages results in chronic inflammation.

Tissue infiltrating macrophages differ from their resident counterpart and are known as classically activated macrophages or M1 macrophages[189, 190]. In IBD, a large number of $CD68^{+}$ macrophages first infiltrate the intestinal mucosa. The characteristics of these infiltrating macrophages differ in CD and UC (**Figure 1-4B and 1-4C**). It has been observed that macrophages might infiltrate the muscular layer and the mesenteric fat in CD patients. Further analysis of blood monocytes from CD patients revealed that there is a reduction of classical monocytes ($CD14^{hi}CD16^{-}$) and an increase of the intermediate monocytes ($CD14^{hi}CD16^{+}$) [191, 192]. A conclusion was drawn that these peripheral monocytes are recruited to the intestinal mucosa possibly in response to C-C chemokine

ligand [192], however, these data were carried out *in vitro* and further clarification is warranted. A schematic diagram of macrophage development during homoeostasis and inflammation condition is illustrated in (**Figure 1-4D)**. In these infiltrating macrophages, the NF-κB pathway is activated and they express pro-inflammatory cytokines like TNF-α, IL-6, IL-8, IL-23, IL-1β, and IFN-γ [193]. These macrophages also secret chemokine CCL2 which further attract more monocytes [194, 195]. It is important to know that inflammation milieu of the inflamed mucosa might skew any monocyte cell lineage towards the classically activate inflammatory macrophages/M1 macrophages population. During IBD, TGF-β signaling is impaired which might also hamper the development of inflammation and anergic macrophages from newly recruited macrophages [164]. Furthermore, leaky epithelium barrier during IBD cause integration of luminal contents which induces the inflammatory response of lamina propria leukocytes [196]. The inflammatory response is largely carried out by secretion of pro-inflammatory cytokines like IL-6, TNF-α and IL-1β [197, 198].

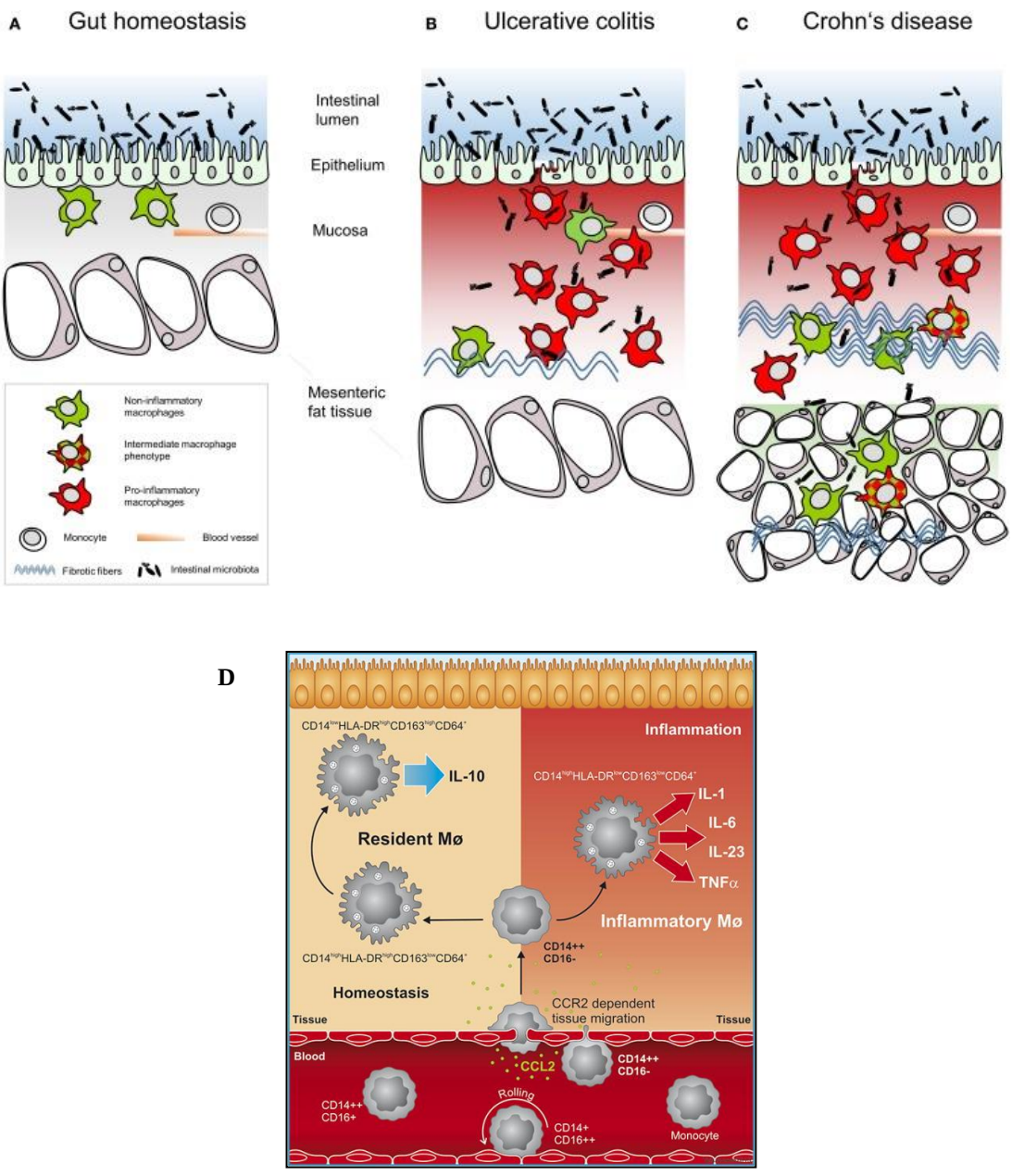

Figure 1-4. Schematic diagram of macrophages during healthy and diseased conditions.

A; Gut homeostasis, B; Ulcerative colitis, C; Crohn's disease, D; Macrophage development in intestine during homeostasis and disease condition. Under homeostatic condition the classical $CD14^{++}$ $CD16^{-}$ macrophages convert to resident macrophages which produce IL-10 and maintain homeostasis. During inflammation milieu, $CD14^{++}$ $CD16^{-}$ macrophages convert to inflammatory macrophages secreting an array of pro-inflammatory cytokines. Figure retrieved from Kuhl, A.A., *et al.*, 2015 [164]

1.4.4.4. Cytokines and their regulation of inflammation

The cytokines are small peptides which medicate cell communication, effector cell proliferation and facilitate local and systemic inflammation. Depending on functionality, cytokines can be divided in three different classes. First, the pro-inflammatory cytokines (TNF-α, IL-1, IL-6), which bind to various receptors on inflammatory cells and thus can medicate T cell proliferation, leukocyte infiltration and cell to cell communication [198]. In contrary, the second class of cytokines like IL-10, IL-5 and TGF-β are known as anti-inflammatory cytokines [198], they exert their effects by limiting lymphocyte proliferation and transforming active immune cells to their resting stage. A third category of cytokines like IFN-λ, IL-12, and IL-18 modulate the pro-inflammatory molecules [198]. The immune response in IBD is critically regulated by cytokines as they control the production inflammatory mediators like reactive oxygen metabolites, leukotrienes, platelet activating factors, etc [199, 200].

1.4.4.4.1 Pro-inflammatory cytokines TNF-α, IL-6, IL-1 and colitis

1.4.4.4.1.1 TNF-α

Activation of the NF-κβ pathways in macrophages and neutrophil results in secretion of pro-inflammatory cytokines during inflammation [193]. One of the major pro-inflammatory cytokine secreted by the activated macrophages and monocytes is the TNF-α. This pro-inflammatory cytokine exerts its effect by increasing the production of IL-1, IL-6 and facilitates the proliferation of fibroblasts and pro-coagulant factors [201, 202]. It also mediates the proliferation of cytotoxic cells and the apoptotic responses. TNF-α can modulate p38 and c-Jun N-terminal kinase pathways which facilitate the inflammatory

response [203]. TNF-α is also able to stimulate cells to secret IFN-γ which further activate several protein synthesis cascade which mediate antivirals, growth limiting and immunomodulatory effect [204]. Biopsies collected from both CD and UC showed a significant increase of TNF-like factor TL1A receptor DR3 expressing cells and IFN-γ which correlated well with the disease state [205]. The association between TNF-α and IFN-γ is further supported by down-regulation of IFN-γ producing non-lymphocyte *lamina propria* mono-nuclear cells from colon biopsies of patients who are undertaking anti-TNF-α drugs [206].

Further studies, confirming the pro-inflammatory effect of TNF-α were conducted. In mouse the lack of TNF-α induce a down-regulation of colitis [207]. In parallel, CD patients treated with infliximab which is a human-mouse chimeric anti-TNF-α antibody showed efficacy in controlling colitis [208]. Serum level of TNF-α has been found to be correlated with clinical and laboratory stage of the IBD [209].

1.4.4.4.1.2 IL-1

The IL-1 possesses pro-inflammatory role and has been found to modulate IBD outcome. Two major cytokines, IL-1α and IL-1β, are the members of the IL-1 family [210]. Both cytokines are produced by different cells via initiation of cyclooxygenase type 2, type 2 phospholipase A, and inducible nitric oxide synthetize [210]. It has been found that, epithelium cells produce IL-1 receptor antagonist (IL-1Ra) which binds to the IL-1 receptors on target cells and thus inhibiting the inflammatory action of IL-1[211].

Surprisingly, it has been observed that IL-1Ra is increased in IBD patients' plasma and tissue, this increase is thought to be a host mechanism to down-regulate the ongoing inflammation [212]. Evidence towards this is supported by a decreased ratio of IL-1Ra/IL-1 with increasing IBD flare while maintaining a balanced ration during control condition. It is assumed that, less production of IL-1Ra is associated with chronic gut inflammation [198]. Furthermore, colonic macrophages collected from IBD patients possess activated interleukin-1 converting enzyme (ICE) which in turn can release mature IL-1β [212]. In contrast, macrophages collected from normal colonic mucosa only produced ICE precursors, indicating that IL-1β is a major inflammatory regulator [212].

1.4.4.4.1.3. IL-6

Another major pro-inflammatory cytokine in the gut is IL-6. Studies suggest an increased level of IL-6 both in IBD [213, 214] and experimental colitis model [215]. IL-6 can also induce its pro-inflammatory effect via its soluble IL-6 receptor (sIL-6R) [216]. In both CD and UC, sIL-6R has been found to be increased as opposed to the remission stage [217]. It has been observed that cells lacking IL-6 receptor can receive IL-6 mediated signaling through a complex formed by IL-6 and sIL-6R [218]. IL-6 signaling might help to inhibit T cell apoptosis which might prolong T cell resistance during CD [219]. Animal studies showed that administration of anti-IL-6 receptor antibody inhibited the level of pro-inflammatory cytokine TNF-α, IFN-γ and IL-1 in experimental model of colitis [165]. Moreover, colitic animals receiving anti-IL-6 receptor antibody have significantly lower number of leucocytes and a significant increase of T cell apoptosis,

which in turn abrogated colitis symptoms [165]. All these studies confirm the pro-inflammatory effect of IL-6 in IBD.

1.4.4.4.2 Anti-inflammatory cytokines IL-10, IL-4, TGF-β and colitis.

1.4.4.4.2.1 IL-10

IL-10 is a key anti-inflammatory cytokine in gut inflammation[198]. Mucosal inflammation is attenuated by IL-10 via antigen presentation inhibition and subsequent inhibition of pro-inflammatory cytokines[220]. Using the dextran sulfate sodium (DSS) colitis model, it has been observed that the anti-inflammatory effect of IL-10 is largely exerted through a down-regulation of the nitric oxide (NO) and reactive oxygen synthesis from the macrophage population [221] and the pro-inflammatory action of classically activated macrophages is also inhibited by IL-10 [221]. Moreover, it has been observed that IFN-γ and IL-12 production by Th1 lymphocytes are also inhibited by IL-10 [220]. Furthermore, animal studies showed that IL-10 knockout increases production of pro-inflammatory cytokines which ultimately results in IBD like symptoms [222, 223], with IL-10 knockout being used as a model of IBD. In contrast, an increase of *Il10* mRNA in T lymphocytes and IL-10 positive cells are observed in UC patients [224]. Although not confirmed yet, this increase might be associated with a host mechanism that counteracts the inflammatory process [224]. Thus, proper balance of IL-10 is crucial for regulating gut inflammation.

1.4.4.4.2.2 IL-4 and TGF-β

IL-4 largely exerts it's action via inhibiting the secretion of TNF-α and IL-1 and can also inhibit macrophage colony-stimulating factor and down-regulate the synthesis of monocyte derived H_2O_2 [225-227]. IL-4 administration probably also exerts its protective effect via a down-regulation of the vascular endothelial growth factor (VEGF) in peripheral blood mononuclear cells observed in both UC and CD patients[228].

Like IL-4, TGF-β is a key immune regulator to maintain homoeostasis [198], and in several autoimmune disorders and IBD, TGF-β is decreased, suggesting a protective effect. In contrast, in UC patients an increase of TGF-β has been observed in the mononuclear cells of the lamina propria which indicate that systemic presence of this cytokine might protect host from inflammation whereas local presence of TGF-β might act as a pro-inflammatory cytokine [229]. TGF-β probably exerts its anti-inflammatory effect via epidermal growth factors, VEGF, insulin like growth factor to prevent luminal invasion and tissue repair [230, 231]. Besides, immune cells and cytokines, gut inflammation might be regulated via various epithelial endocrine cells, notably the most prominent in the gut the enterochromaffin cells.

1.5. Enterochromaffin cells (EC) and colitis

In addition to immune cells and cytokines, gut inflammation is associated with an alteration in the pro-hormone chromogranin-A (CHGA) producing EC cells [2]. EC cells are distributed throughput the gut epithelium (**Figure 1-5**) and cover more than 70% of enteroendocrine cells in the large intestine [157]. In the distal part of the colon and the

rectum more than 40% of enteroendocrine cells are EC cells. EC cells are mainly named because of their affinity to bind chromium salt [157]. After chromaffin cells, EC cells are the main source of CHGA in the gut [232] which is an important enteric mucosal signaling molecule influencing gut physiology mucosal signaling molecule influencing gut physiology [233, 234]. Changes in intestinal EC cell numbers and CHGA are observed in patients with IBD[2], however, changes have only been described regarding the number of EC cells in the context of experimental colitis [235, 236]. The association between the alteration in EC cells numbers and CHGA production in various gastrointestinal (GI) diseases highly emphasizes the potential significance of CHGA in intestinal physiopathology. Although the changes in EC cells or in CHGA amount in IBD are involved in gut physiology [237, 238], it is not clear whether the modifications play any role in immune activation of gut inflammation or not. Due to the strategic location of EC cells in gut mucosa, CHGA may play an important role in immune activation in relation to gut pathophysiology, including in IBD.

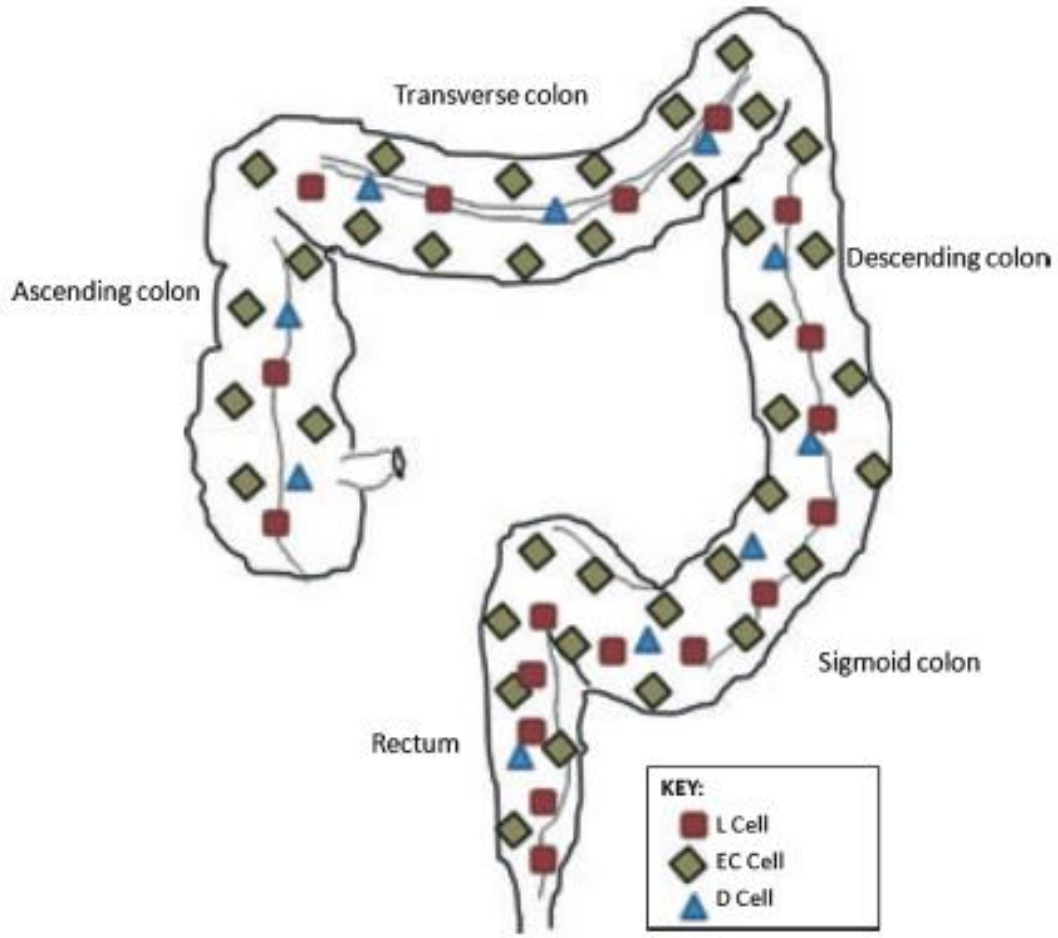

Figure 1-5. Schematic diagram representing distribution of various enteroendocrine cells.
Enterochromaffin cells are the most common enteroendocrine cells in the colon and rectum. D cells are make up around 3-4% of enteroendocrine cell population and distributed scatterly throughout the gastrointestinal tract. L cells are rarely found from proximal to terminal ileum, however their frequency increases from proximal to distal expecailly in the rectum where they account for 14% of enteroendocrine cell population. The figure was retrieved from Gunawardene, A.R., *et al.*, 2011 [157]

1.6 The Chromogranin family of proteins

The CHGA of the granin family of proteins is stored in chromaffin granules of some CNS and enteric neurons, as well as specific types of endocrine, immune and neuroendocrine cells. It is co-released along with hormones, neurotransmitters and neuropeptides [239] which are also stored in the same granules. The CHGA gene is well characterized and located at the 14q32 of human genome consisting of eight exons and seven introns. The transcript of 2 Kb is translated into the 457-residue CHGA protein. The overall homology for CHGA in different vertebrates is around 40%, but the most highly conserved regions occur at the N and C-termini, with up to 88% sequence homology. Cell and tissue-specific processing of CHGA has been described in the rat, mouse and human GI tract [240-242]. The presence of numerous pairs of basic amino acids indicate potential sites for cleavage by pro-hormone convertases (PC) 1/3 or 2, carboxypeptidase E/H [243], consistent with evidence that CHGA may serve as a pro-hormone for shorter bioactive fragments [244] **(Figure 1-6)**, as also suggested by the high sequence conservation of CgDPs.

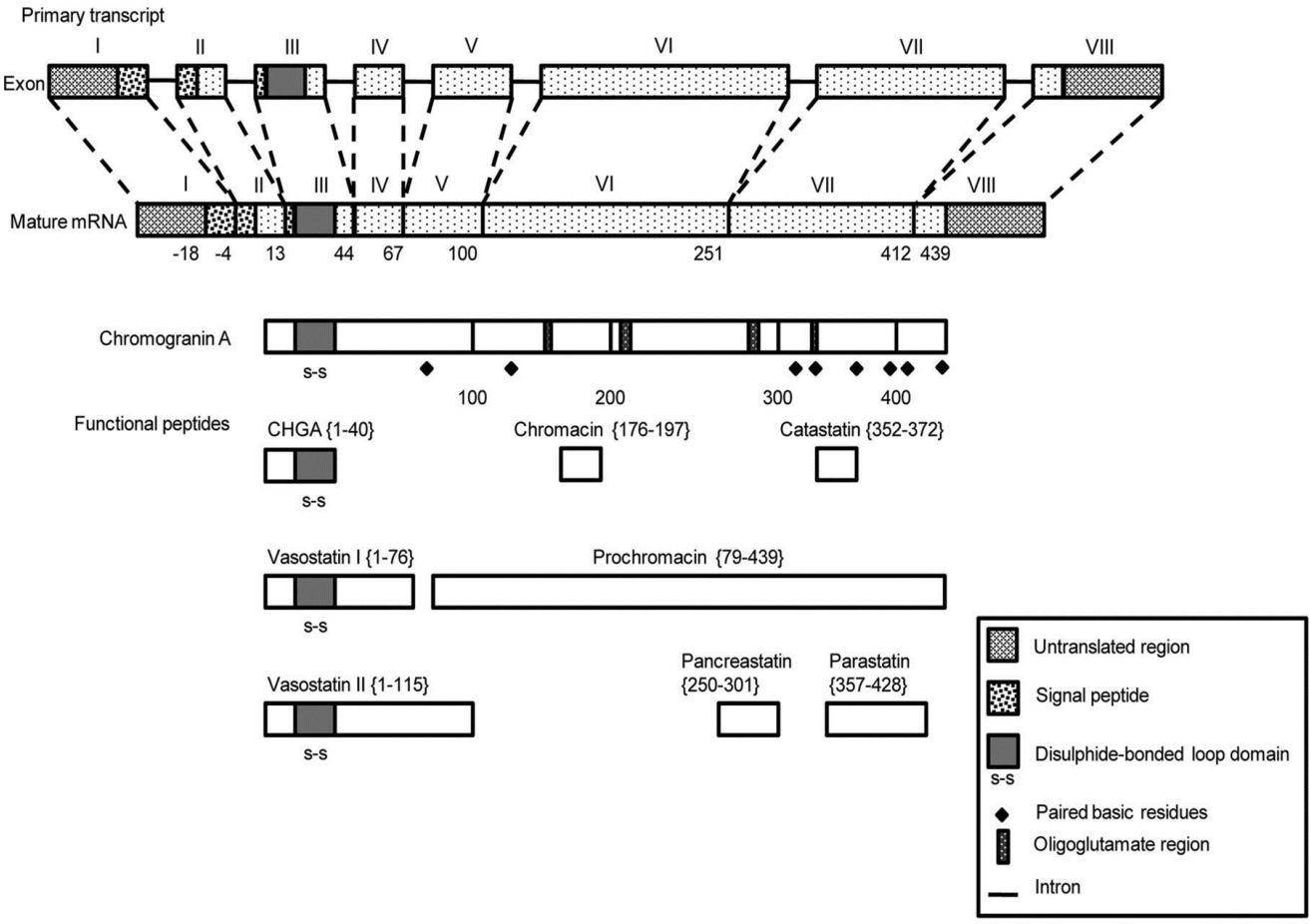

Figure 1-6. Schematic diagram representing Chromogranin A and its derived peptides.

Chromogranin A acts as a pro-hormone and several bioactive peptides are cleaved proteolytically. Figure retrieved from Valicherla, G.R., *et al.*,2013 [245]

1.6.1 CHGA-derived peptides (CgDPs)

Proteolytic fragments of CgDPs exert a broad spectrum of regulatory activities on the cardiovascular, endocrine and immune systems. Among them, pancreastatin (human $CHGA_{250–301}$) inhibits insulin release from pancreatic-islet B cells, promotes hepatic glycogenolysis, and regulates lipid metabolism; prochromacin (bovine $CHGA_{79–431}$) and chromacin (human $CHGA_{176–197}$) have antibacterial and antifungal effects; and vasostatin 2 (human $CHGA_{1–113}$) has regulatory roles in the heart and the vascular system [246]. Among its highly conserved C terminal regions, CHGA gives rise to a main peptide of biological importance: the antihypertensive peptide catestatin (human CTS; $CHGA_{352-372}$) [247-249], which have antimicrobial activity [250] and regulate smooth muscle cell proliferation [251]. CTS stimulates chemotaxis of human peripheral blood monocytes, exhibiting its maximal effect at a concentration of 1nM comparable to the established chemoattractant formylated peptide Met-Leu-Phe (fMLP) [252] **(Figure 1-7**). However, there is no study demonstrating expression, signalling, and function of CTS in gut associated diseases such as IBD.

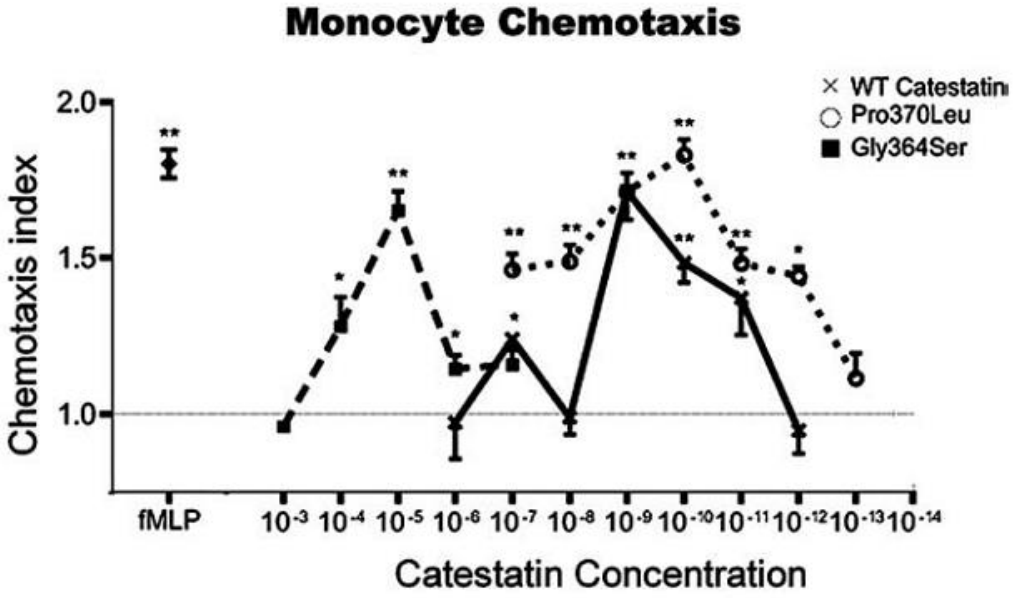

Figure 1-7. Catestatin effect on monocyte migration.
Human peripheral blood monocytes were treated with three different variants of CTS ;Wild type (10^{-12} M–10^{-6} M; crosses), Pro370Leu (10^{-13} M–10^{-7}M; circles) and Gly364Ser (10^{-7} M–10^{-3} M; squares). Pro370Leu was more potent chemoattractant than the wild type wheras it was opposite for Gly364Ser variant of catestatin. WT CTS showed it's maximum effect at 1nM concentration. fMLP used as a positive control. $^{*}P<0.05$; $^{**}P<0.01$. Figure retrived from Egger, M., *et al*, 2008 [252].

1.6.2 CgDPs in the clinical inflammatory context

In addition to the physiological context, CgDPs may play an important role in the neuroimmune interaction in relation to clinical inflammation. Circulating CHGA level, a marker for neuroendocrine tumours, has been found to be elevated in some patients with IBD [253], however, it is important to emphasis that non-specific ELISA has been used to recognize "the full CHGA", as the antibodies used, target only two specific epitopes. Therefore, being a pro-hormone, EC cells release different peptides, and the recognized epitope can be present in small or long peptides, giving us no specific indication about the type or the size of the peptide present. With this limitation, authors demonstrated that the disease activity and TNF-α level influence the "CHGA" pattern, which could reflect the neuroendocrine system activation in response to inflammation [238]. In a recent letter addressed to the aforementioned study, Sidhu and collaborators [254, 255] confirmed the observation of an elevated level of CHGA serum in both IBD and diarrhea predominant-irritable bowel syndrome patients [238]. The unifying hypothesis proposed could be that the EC cell hyperplasia produces elevated serum CHGA levels [256], but further studies of serial serum CHGA and CgDPs measurements should strengthen our understanding.

In addition to gut inflammation, the relation between TNF-α and CHGA has also been demonstrated in rheumatoid arthritis (RA), a disease that shares some common features with IBD. Correlation between CHGA and TNF-receptor (TNFR)-I and TNFR-II has been evaluated in patients before the initiation of treatment with infliximab® and were compared to the one during the treatment [257]. The authors observed a high correlation between CHGA and both receptors, moreover, they found that treatment with anti-TNF-α

mAbs abrogated the correlation between CHGA, TNFR-I and TNFR-II. Subsequently, the same group described that patients with RA had significantly higher serum levels of CHGA and TNFRs compared to controls and that the highest levels of CHGA identified the population of patients with extra-articular manifestations [257]. Taken together, these results suggest that CHGA might be involved in the pathogenesis of inflammatory autoimmune diseases through a complex interaction with TNF-α, mediated by undefined factors. In a series of papers from our collaborator, Metz-Boutigue, who has extensively published on granins, showed a link between serum concentration of CHGA and outcome in patients admitted with or without systemic inflammatory response syndrome. Not only CHGA concentrations were positively correlated with inflammatory markers like procalcitonin and C-reactive protein, but also with simplified acute physiological score (SAPS) [258, 259]. In addition, a significant association between CHGA level and periodontitis has been reported [260]. Finally, a recent study was conducted to evaluate the presence and processing of the CHGA in the vitreous of patients with diabetic retinopathy (DV), compared with nondiabetic vitreous (NDV). This study has shown differences in the presence and endogenous processing of CHGA, from DV vs. NDV. In DV, the increase of complete granins and the attenuation of their endogenous proteolytic processing (*via* PC1/2) participated in DR progression by reducing the presence of regulatory peptides, being important for the pro-/anti-angiogenic balance in the eye. All these data provide a proof of the concept that CHGA and CgDPs play an important role in the development of inflammatory pathologies.

1.6.3. CgDPs cellular mechanisms of action

Receptors for CgDPs appear not to exist. Rather, sequence similarity of CgDPs with cell penetrating peptides [261] appears to allow CgDPs to enter cells and induce calcium influx via a calmodulin (CaM)-regulated calcium-independent phospholipase (iPL) A2 pathway [262]. In addition, CgDPs regulate endothelial barrier function and protect against plasma leakage [263] by an action on the cytoskeleton. So far, our lab has examined the role of two new granin peptides on smooth muscle contraction (SMC) showing that $CHGA_{4-16}$ and $CHGA_{47-66}$ modulate spontaneous and drug-evoked SMC by in part a direct action on smooth muscle L-type calcium channels [234]. Our group and others have demonstrated that CHGA and its derived peptides play a major role in clinical and experimental pathophysiological conditions as well as inflammatory diseases [264]. Apart from these physiological studies, knowledge of pathophysiological mechanisms whereby CgDPs exert their action remains rudimentary, especially in the context of gut inflammation. Consequently, the link between CgDPs and colitis remains to be established.

1.7. Model of experimental colitis

Much of the recent progress understanding about immunity has been achieved by the study of experimental animal models of intestinal inflammation [265]. Although these models do not represent the complexity of human disease and do not replace studies with patient material, they are valuable tools for studying many important disease aspects which are difficult to address in humans. A number of animal models are available to

study IBD. Among them chemically induced animal models are widely used due to their induction simplicity, immediate onset of inflammation and better control [265]

1.7.1. Dextran sulphate sodium (DSS)

The most widely used and characterized experimental model of UC is the dextran sodium sulphate (DSS)-induced colitis, which was developed by administration of DSS in the drinking water. DSS mouse model was first described by Japanese scientist Okayasu in 1990 [266]. Acute, chronic model of colitis can be induced by changing the concentration and duration of DSS exposure. The severity of colitis also depends on the molecular weight of DSS [267]. The exact etiology of DSS induced colitis is still unknown. However, there are reports of increased rates of apoptosis and decreased proliferation of epithelium in acute DSS colitis leading to defective epithelium integrity. Epithelium integrity is crucial for preventing luminal microbiota invasion through intestinal mucosa. Due to the loss of epithelium integrity altered activation of immune system can take place which was evident through a significant increased expression of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-ß IFN-g and IL-12) in the colon of DSS colitic animals [268, 269]. Due to the acute effect of this model, this model is crucial to understand the innate immune mechanism in gut inflammation. Further, the impact of microbial flora in the pathogenesis of DSS colitis [270] make it a suitable model to study the role of microbial dysbiosis in the etiology of the disease.

1.7.2. Dinitrobenzene sulfonic acid (DNBS)

Another model for colitis is dinitrobenzene sulfonic acid (DNBS)-induced colitis model. In this model, DNBS is administered intrarectally (*i.r.*) in varying concentration of ethanol [271]. The administration of ethanol is crucial to damage the epithelium barrier which allows penetration of DNBS. The DNBS works as an hapten agent to make the luminal contents more immunogenic, which then disrupts the intestinal immune homoeostasis. The characteristic features of DNBS induce colitis is bloody diarrhea, intestinal wall thickening, weight loss, *etc*. The symptoms vary deepening on the type of animal, dose and exposure timing. The benefit of using DNBS over DSS is its low cost, rapid colitis induction, however, DNBS handling require more expertise than DSS.

1.7.3. Other chemically induced colitis models

Other than DSS and DNBS, one of the most widely used chemical agents to induce colitis is trinitrobenzene sulfonic acid (TNBS). TNBS is a chemical agent and can be used to induce both acute and chronic inflammation in rodents [272] as well as pigs [273], rabbits [274], guinea pigs and event non-human primates. TNBS like DNBS need to be administered i.r. with ethanol [275]. It is important to consider the genetic background of rodents while using TNBS as a chemical agent to induce colitis. For example, it has been observed C57BL/6 mice are generally resistant to TNBS induced tissue injury whereas other BALB/C mice are prone to TNBS induced gut inflammation [275, 276]. The classical characteristics of TNBS induced colitis is weight loss, bloody diarrhea and transmural intestinal inflammation. Because of the instability of the tri-nitrile residue, TNBS is less and less used and it is being replaced by DNBS.

Another chemical agent used to induce colitis is oxazolone. This chemical agent acts like a hapten and used to induce Th2 type immune response [277]. It has been observed that, in murine models oxalozone administration results in body weight loss, diarrhea, ulcers [277]. Oxalozone administration results in active tissue injury which makes it an excellent choice to study acute intestinal inflammation [277, 278].

As colorectal cancer is common in IBD patients, a model to study colorectal tumours is needed. It has been observed that, administration of DSS along with Azoxymethane (AOM) results in chronic intestinal inflammation which ultimately progressed to colorectal cancer [279]. It is also noteworthy that, treatment with concurrent administration of DSS with AOM results in increased expression of IL-4 and IFN-γ which are characteristic features of UC [280]. Thus, this chemically induced model allows researchers to study the inflammatory stare of IBD and to further link it with colorectal tumours.

Acetic acid is also used to induce colitis which produces tissue necrosis and edema confined to the colon [281]. Due to the low cost and ease handling of this agent, some researchers use this model to induce UC like symptoms. Although activation of NF-κB and other inflammatory mediators are observed in acetic acid induced model [282], it is assumed that the tissue injury observed immediately after acetic acid administration is not due to immune response [281].

Beside these chemical induced models, research have carried out to establish other forms of colitis model like genetically engineered model where specific genes are knocked out [278]. Through genetic manipulation spontaneous colitis models were developed where colitis mimicking to IBD can be studied without external manipulation. For example, C3H/HeJBir mouse model develop chronic inflammation and develop characteristic features like ulcers, crypt abscesses, etc [283]. This model is an excellent choice to study the genetic etiology of IBD. Mice with STAT3 deficiency in their myeloid cell lines also develop colitis spontaneously [173]. STAT3 in macrophages and neutrophils are crucial to induce IL-10 production [278] and the disruption of this pathway will interfere with the development of the inflammatory process. Similar to this, IL-10 knock out mice also develop spontaneous colitis.

Although, there is a large amount of research performed to identify the perfect animal model to capture human IBD characteristics, none of them can recapitulate all the disease, but chemical induce models are still the best choice to study colitis due to their ease of administration, reproducible results and low cost.

1.8. Current IBD therapies

A number of approaches were undertaken during the last decade to develop new drugs against this complex disease [284]. The primary aim of these therapeutic approaches is to improve the quality of life of the patients via inducing and maintaining a remission stage [284]. Treatment options include various conservative approaches as well as surgical operations to the patients when resistant to the drugs. The drugs currently in

the market for IBD can be classified in 5 categories: anti-inflammatory drugs, immunosuppressants, biological agents, antibiotics, and drugs for symptomatic relief [284]. Some examples of these drugs are summarized in Table 1-1.

Table 1-1. Various drug categories currently used to treat IBD.

The table retrieved from John K Triantafillidis J K et.al. [284]

Drug Group	Drugs
Biologics	• Infiximab, adalimumab
Anti-inflammatory	• Mesalazine • Corticosteroids (prednisolone, methylprednisolone, butesonide)
Immunosuppressives	• Azathioprine, 6-mercaptopurine, methotrexate, cyclosporin, tacrolimus
Antibiotics	• Metronidazole, ornidazole, clarithromycin, rifaximin ciprooxacin, anti-Tuberculosis
Probiotics	

1.8.1. Biological agents

The TNF-α is one of the major pro-inflammatory cytokines that worsen the IBD outcome [284]. Various biological agents against this pro-inflammatory cytokine were developed and thought to have a significant treatment effect in IBD. Two popular anti-TNF-α drug approved are infliximab and adalimumab [284].

1.8.1.1. Infliximab (IFX)

This is an immunoglobulin G1 (IgG1) type chimeric antibody against TNF-α. The monoclonal antibody works by binding to circulating and membrane bound TNF-α. This induced the cytotoxicity of activated T cells and in turn cell clearance [284]. IFX has been found to be effective in mucosal healing in both UC and CD. In a randomized study using UC patients, more than 60% of patients receiving IFX responded to the drug [285]. It has also been observed that patients who are resistant to iv steroids also responded to this drug. It is also documented that patients receiving a single dose (5mg/Kg of IFX) are less likely to undergo for colectomy [286]. Using a clinical cohort of fistulizing CD, it has been observed that 55% of patients receiving IFX at 5 mg/kg achieved a complete closure of fistulas. However, in the same cohort 38% of patients receiving IFX at 10 mg/kg achieved a complete closure of fistulas while only 13% of patients receiving placebo improved to the point of complete closure to fistulas [208].

1.8.1.2. Adalimumab (ADA)

The ADA is also a humanized monoclonal antibody against TNF-α and is administered subcutaneously [284]. In a clinical trial composing of 299 CD patients,

ADA showed an efficacy rate of 36% compared to 12% in the placebo group [287]. The optimal dose for this antibody is 160mg/kg on week 0 followed by 80 mg/kg on week 2 [287]. ADA is found to be effective in IFX non-responders [284, 288]. It has also been observed that moderate to severe CD patients receiving ADA maintain remission stage and requires less hospitalization compared to placebo patients [289]. ADA is also found to be effective in UC patients who became resistant against corticosteroids [284]. Currently, it has been observed that 27% of UC patients resistant to IFX can achieve clinical remission while receiving ADA within week 12 [284].

1.8.1.3. Limitations of anti TNF-α therapy

The most fearing factor for these anti TNF-α biologics are their side effects which include opportunistic infections, development of malignancy and sometimes autoimmunity in individuals receiving anti TNF-α drugs [290]. Because anti TNF-α are strong down-regulator of immune response, it is highly recommended to perform a long-term follow-up of these patients [290]. Despite this, a meta-study analyzed the safety of anti-TNF-α involving 21 studies composed of a total 5256 patients where 3341 individuals receive the drug. The authors did not show any significant change in the mortality rate or frequency of malignancy between groups [291]. These anti-TNF-α drugs have been approved and are safe during pregnancy [284, 292], but, before administration biological agents, patients should be tested for tuberculosis (Tb) [293], as these immunosuppressive agents might activate latent TB.

1.8.2. Anti-inflammatory drugs

Anti-inflammatory drugs are in generally the most commonly used drugs to treat IBD. The drugs that fall under this category are mesalazine and corticosteroids (prednisolone, methylprednisolone, butesonide) [284].

1.8.2.1. Mesalazine

Mesalazine is believed to be the active moiety of sulfasalazine. Sulfasazine is a conjugate of 5-aminosalicylic acid and sulfapyridine [294]. The mode of action of mesalazine is not well described yet. The therapeutic activity of mesalazine is not really known. However it is believed to modulate chemical mediators of inflammation, especially prostaglandins and leukotrienes [295]. Mesalazine is also believed to down-regulate the production of inflammatory prostaglandins probably via inhibiting the cyclooxygenase enzyme which is a major component fort the arachidonic acid cascade [295]. This drug has been found to be effective in maintaining remission stage in active UC patients [284]. In a clinical study, where moderately active UC patients were included the drug showed a successive rate by week 6 in 70% and 66% of patients who received the drug 4.8mg/day (d) and 2.4 mg/d respectively [296]. Although current data supports the use of mesalazine to maintain remission in UC patients, the use of this drug against CD is not supportive yet [284].

1.8.2.2. Corticosteroids (CSs)

CSs effectively block the early manifestation of inflammation like inhibiting vascular permeability, vasodilation, and preventing infiltration by neutrophils. These drugs are

also effective to inhibit fibroblast activation and collage deposition. CSs can also inhibit the production of inflammatory cytokines, and further inhibit the production of nuclear factor-κβ (NF-κβ). The NF-κβ is crucial to initiate immune response and interference with their production down-regulate the inflammation. CSs can be used either orally or systematically in combination with another drug or alone. Currently prednisolone, methylprednisolone, and budesonide are most widely used CSs. In moderate to active UC patients, CSs are administered at 20 to 60 mg/dwhich then is gradually reduced to 10mg/d. In case of CD patients, CSs were found to be effective to improve patients towards remission. However, mucosal healing was not observed in these patients.

1.8.2.3. Limitation of anti-inflammatory drugs

Mesalazine at a lower dose (1–2 g/d) was not effective in CD patients when it was compared to placebo. At a higher dose (3–4.5g/d) also, mesalazine failed to improve the remission in CD patients when compared to placebo. Mesalazine when compared to Azathioprine (AZ), a well-known immunosuppressive drug did not show any benefit regarding relapse in CD patients undergone for surgery. The data supports the inefficacy of mesalazine to use as a drug of choice for CD patients.

On the other hand, CSs although showed some efficacy in UC patients to induce remission, they could maintain remission. Further, many patients do not respond to CSs. Further, budesonide, a popular locally acting CSs did show any efficacy for UC patients. In case of CD patients, although CSs shed light to induce faster remission rates, patients suffered from adverse side effects. Furthermore, development of resistance against

steroids is common in CD patients. CSs are not effective to maintain remission in CD patients too. All the data supports that, further studies are warranted to find the optimal dose, safer alternative CSs to treat IBD. It is also assumed that CSs might be beneficial for a particular phenotype in IBD which also need to be further investigated.

1.8.3. Antibiotics

Various broad-spectrum antibiotics are recently tested out to treat CD patients [297]. Antibiotics that are currently used are metronidazole, ornidazole, ciprofloxacin, tobramycin, clarithromycin, cotrimoxazole, and anti-TB treatment. Some of these antibiotics are discussed below.

Since 1970, the efficacy of metronidazole is tested out. Although in a clinical study using CD patients, metronidazole failed to show a difference between treatment and placebo group [298]. Again, in another clinical study, the effect of metronidazole compared to sulfasalazine was tested to treat CD patients. No difference was found between two groups. However, patients resistant to sulfasalazine responded to metronidazole [299]. However, the efficacy of this antibiotic was found in combination with ciprofloxacin. It was observed that remission rate in CD patients receiving 250 mg of metronidazole four times daily in addition to ciprofloxacin 500 mg twice daily was comparable to regular steroid treatment [300]. However due to severe side effects of metronidazole like nausea, anorexia, dysgeusia, dyspepsia, and peripheral neuropathy, its use is limited [297]. Another antibiotic named Ornidazole, has chemical property and antimicrobial structure resembling with metronidazole. Ornidazole at 500 mg/d was

effective to down-regulate the Crohn's Disease Activity Index (CDAI) within 4 weeks of treatment in a group of 25 CD patients [301]. The overall health was improved including reduction in abdominal pain, reduced serum C level and reduction in the recurrence rate [301]. Beside these two antibiotics, Ciprofloxaxcin is widely used in patients with active CD. Ciprofloxacin showed efficacy to reduce Crohn's Disease Activity Index (CDAI) when administered at 500mg twice daily compared placebo. In a clinical study composed of 72 CD patients, Ciprofloxacin were administered at 500 mg twice daily and metronidazole 250 mg three times daily. Within 10 weeks of treatment, 68% of patients achieved clinical remission while 76% of patients showed clinical response [302]. In another comparison study with mesalazine, 56% of CD patients who received ciprofloxacin received clinical remission compared to 55% of patients who received mesalazine [303] It is assumed that ciprofloxacin is effective in active CD patients who have inflammation in the colon [297]. Another antibiotic tested to treat IBD is Clarithromycin which easily penetrate in macrophages and is a well-known broad-spectrum antibiotic. In a clinical study, Clarithromycin were administered for 3 months in active CD patients. Although in the first month the drug showed efficacy, the benefit was abolished in the subsequent months. This suggests that bacterial resistance might occur to this antibiotic which attenuate the effect of the antibiotic [304].

1.8.3.1. Anti-TB treatment

Mycobacterium avium subsp. *paratuberculosis* (MAP) was found to be associated with CD. However, in a double blinded study with 213 active CD patients were randomized to a clarithromycin 750 mg/d, rifabutin 450 mg/d, clofazimine 50 mg/d or

placebo. Within 16 weeks 66% patients receiving antibiotics were in remission which was higher than patients in the placebo group (50%). However, in the following year there was no observable difference between two groups. This nullifies the fact MAP as a possible cause of CD [305].

1.8.3.2. Limitation of antibiotics

Although antibiotics shed some light in IBD treatment, still a lot of research is required. It is not well understood why only a limited number of antibiotics shows efficacy in IBD treatment. The mechanism of action for are not well understood yet. Development of bacteria resistance against antibiotics is also a major problem. CD patients treated with ciprofloxacin concurrently develop a infection with *Yersinia enterocolitica*. It is important to know that ciprofloxacin is effective against *Y. enterocolitica* [297]. So the development of secondary infection in these CD patient is not well understood yet. All these data are suggestive for more effective research to develop a therapeutic antibiotic against IBD treatment.

1.8.4. Probiotics

Microbial dysbiosis is evident in IBD and various live non-pathogenic microbes can be used to counteract the dysbiosis and are known as Probiotics [284]. Currently, probiotics used for IBD treatment are consisting of *Saccharomyces boulardii* yeast or lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* spp. [284, 306].

Probiotics can improve the overall gastrointestinal health through various ways like reducing intestinal pH, preventing colonization of pathogenic microbes and/or via manipulating the overall immune response. For example, it has been observed that in UC patients *Lactobacillus paracasei* significantly down-regulate the plasma and lymphocyte content of pro-inflammatory cytokines [306]. *S. boulardii* can interfere the production of NF-kβ which down-regulate the production of pro-inflammatory cytokines by interfering with the global mediator of inflammation NF- κβ. The proteins secreted from *S. boulardii* can also modulate the mitogen-activated protein kinases ERK1/2 and p38. Further, it can also inhibit bacterial overgrowth and cleaves *Clostridioides difficile* toxin A via releasing a protease [307].

Probiotics have shown promising effect when administered in UC patients. A product marketed by Sigma-Tau Pharmaceutics, Inc, Gaithersburg, MD name as VSL#3 were effective to achieve clinical remission in mild to moderately active UC patients [308]. In a clinical study composed of UC patients, VSL3 was effective to down-regulate the UC activity index score (UCDAI) by 50% in 63.1% of patients compared to 40.8% patients in the placebo group. Remission was also higher in VSL3 group (47.7%) compared to a placebo (32.4%). It can be used as a safe supplement in UC patients who are under mesalazine treatment. In addition to this, efficacy of non-pathogenic *E. coli* to treat UC compared to traditional therapy mesalazine was checked. It was observed that 75% of UC patients received mesalazine achieved remission compared to 68% of UC patients who

received non-pathogenic *E. coli* as a treatment. This non-significant difference indicated the non-pathogenic *E. coli* has a comparable effect rate towards mesalazine in treating moderate to active UC patients [309]. Compared to this, *L. rhamnosus* GG strain was also found to be effective UC patients to decrease the relapse rate when administered alone or as a supplement with mesalazine. Further, UC patients receiving Bifidobacteria-fermented milk supplement had significantly fewer exacerbations compared to non-supplemented group [310],

1.8.4.1 Limitation of Probiotics

Although beneficial effects are observed with probiotic treatment, there are several limitations yet to be addressed. It is important to know that beneficiary effect for a particular probiotic strain might not be truthful for another individual. In addition, the exact dose for probiotics are not determined yet, although it is believed that several billions are required to develop a gut colony [311]. Further, no beneficial effect has been yet recorded for treating CD patients with probiotics. Although considered safe, patients can suffer from bloating and flatulence while taking probiotics. Besides, extreme caution should be taken while treating critically ill or immunocompromised patients with probiotics. Probiotic treatment and antibiotic treatment should have period 2 hours in between. Finally, there is a scarcity of data in regard to effective probiotic strain, the dosage or particular patient group [284]. Thus, more research are required to introduce probiotics as a sole treatment option for IBD.

Chapter 02. Part 02. Rationale

2.1 Study Rationale

The apparent therapeutic effect of biological therapy [5] and corticosteroids underscores the importance of the dysregulated immune response. However, some patients are resistant to these drugs, and all of these therapeutic agents have adverse side effects [6]. For example, monoclonal antibodies to TNF-α are used to treat severely ill patients which are expensive; require indefinite use, and make the patients more vulnerable to secondary infections and potentially even malignant complications [312]. These drawbacks limit the enthusiasm for using these agents earlier in the treatment paradigm, so we are in need of new therapeutic agents.

The CHGA-derived peptide (CgDPs) modulate calcium-independent phospholipase A2 ($iPLA_2$) activity through calmodulin (CaM) binding. Several studies have demonstrated that local inflammation and cytokine release from human macrophages can be intracellular calcium dependent [313, 314]. Oxidase activation, cell degranulation and priming response to a wide variety of pro-inflammatory molecules in polymorphonuclear neutrophils (PMNs) are also mediated through calcium signaling [262]. Moreover, $iPLA_2$ signalling and the nuclear factor-κβ (NF-κβ)-mediated overproduction influx of key pro-inflammatory mediators (IL-1ß, IL-6, TNF-α and IL-12) is attributed to the initiation and progression of colonic inflammation [315]. Microbial dysbiosis might also be a major cause of IBD [63]. The peptide catestatin (CTS) derived from C terminal portion of CHGA is cationic in nature and exerts some antimicrobial effect [250, 316]. As mentioned earlier, this peptide also possesses

immnoregulatory activity like as a chemoattractant [252]. Considering the location of EC cells, it is likely that CgDPs can influence regulation of gut inflammation by modulating production of inflammatory cytokines from innate immune cells or through their possible effect on immune cells or gut microbiome. **I investigated the role of CTS in the production of cytokines in the context of gut inflammation and determine its role on macrophage activation and gut microbiota. As macrophages are the major source of pro-inflammatory mediators, and as CTS can penetrate in macrophages [262], it is reasonable to hypothesize that CTS can modulate these innate immune cells and ultimately affect colitis.**

2.2 Research hypothesis and aims

Although altered CHGA expression has been described in IBD [238], there is currently no information regarding its role or the role of its derived peptides in the development of colitis. Consequently, in-depth studies characterizing the presence and the effect of CTS are critical to determine its impact on colitis. Therefore, this project aimed to determine the role of this bioactive fragment in the pathophysiology of colitis using an experimental murine model. For that purpose, below hypotheses were developed:

1. CHGA or CgDPs expression is increased in experimental mouse models mimicking colitis in humans.
2. CTS plays regulate gut inflammation via macrophage activation.
3. CTS/CTL modulates the gut microbiota.

To accept or reject these hypothesis, following aims were developed:

Aim 1: To determine the expression of CHGA and CTS in both human IBD and experimental murine model of colitis.

Aim 2: To determine the effect of CTS on gut inflammation using acute murine model of colitis.

Aim 3: To determine the effect of CTS on gut microbiota composition and function.

Aim 4: To determine the effect of CTS on reactivation of gut inflammation using murine quiescence model of colitis mimicking more to the natural history of IBD.

The results summarizing Aim 1 and 2 were published in Biochemical Pharmacology in 2014 [317]. Aim 3 summarizing the effect of CTS on naïve gut microbiota and has been published in Frontiers in Microbiology in 2017 [318]. Further the effect of DSS of murine gut microbiota was observed and is published in Journal of Basic Microbiology in 2016 [319]. Finally, all results summarizing Aim 4 has been published in Frontiers in Immunology in 2017 [320].

Chapter 03. Part 03. Aim 1 and 2

3. Catestatin decreases macrophage function in two mouse models of experimental colitis

This collaborative work was published in:

Biochemcial Pharmacology, 2014 [317]

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Contribution

MFR, JEG, carried out the majority of the experiments. MHB provided some of the peptides and C.N.B. provided the serum. MFR and JE.G. analyzed the data. MFR. wrote the first draft of the manuscript. JE.G., reviewed the draft and provided comments. All authors approved the final submission and declare that no potential competing interests exist.

Competing of interest

The authors have no competing of interest.

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3.i. Abstract

Mucosal inflammation in patients with inflammatory bowel disease (IBD) is characterized by an alteration of prohormone Chromogranin A (CHGA) production. The recent demonstration of an implication of CHGA in collagenous colitis and immune regulation provides a potential link between CHGA-derived peptides (catestatin, CTS) and gut inflammation. Colitis was induced by administration of dextran sulfate sodium or 2, 4 dinitrobenzenesulfonic acid to C57BL/6 mice. Treatment with human (h)CTS or its proximal or distal part was started one day before colitis induction and colonic inflammatory markers were determined. Pro-inflammatory cytokines were evaluated in peritoneal isolated and bone marrow derived macrophages (BMDMs); p-STAT3 level was studied. Serum levels of CHGA and CTS were assessed in experimental colitis and in a separate study in IBD patients and healthy controls. We show that sera from IBD patients and that in experimental colitis conditions the colonic level of mouse (m)CHGA and mCTS are significantly increased. Moreover, *in vivo* treatment with human (h)CTS reduced the disease onset and suppressed exacerbated inflammatory responses in preclinical settings of colitis associated with an increase of p-STAT3. *In vitro*, *h*CTS treatment decreased proinflammatory cytokine release by peritoneal macrophages and BMDMs and increased p-STAT3 levels. These results support the hypothesis that CTS is increased during colitis and that hCTS modulates intestinal inflammation *via* the macrophage population and through a STAT-3 dependent pathway in a murine model of colitis. Identification of the molecular mechanism underlying the protective role of this peptide may lead to a novel therapeutic option in IBD.

Key words: Catestatin, chromogranin A, experimental colitis, macrophages, STAT3.

3.1. Introduction

Inflammatory bowel diseases (IBDs), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a chronic relapsing and remitting course that results from intestinal inflammation [9]. The apparent therapeutic benefit of biological therapy (tumor necrosis factor- alpha (TNF-α)–neutralizing antibody) [5], corticosteroids and thiopurines underscores the importance of the dysregulated immune response. However, some patients are resistant to these drugs, and all of these therapeutic agents have adverse side effects [6, 321]. For the most ill patients, monoclonal antibodies to TNF-α are used, however, these agents are expensive, require indefinite use, and concern for infectious and potentially even malignant complications [312] limit physicians' decisions to introduce these agents earlier in the treatment paradigm. Therefore, new cost effective agents need to be developed.

Mucosal changes in IBD are reflected by mucosal and transmural inflammation accompanied by a prominent infiltrate of activated cells from both the innate and adaptive immune systems. The release of inflammatory mediators, including pro-inflammatory cytokines from immune cells [322, 323], mediates tissue injury and exacerbation of IBD. In addition to immune cells, inflammation in the gut is associated with an alteration in enterochromaffin (EC) cells releasing mainly serotonin and chromogranin-A (CHGA) [2].

The human CHGA gene consists of eight exons separated by seven introns and has been mapped to chromosome 14q32.16. It translates to a 457 amino acids protein

containing a signal peptide of 18 amino acids associated with a mature human CHGA protein of 439 amino acids. After chromaffin cells, EC cells are the main source of CHGA in the gut [232] which is an important enteric mucosal signalling molecule influencing gut physiology [233, 234]. The overall homology for CHGA in different vertebrates is around 40%, but the most highly conserved regions occur at the N and C-termini, which show up to 88% sequence homology. Cell- and tissue-specific processing of CHGA has been described in the rat, mouse and human GI tract [240]. The presence of numerous pairs of basic amino acids indicate potential sites for cleavage by prohormone convertases 1/3 or 2, carboxypeptidase E/H [243], consistent with evidence that CHGA may serve as a prohormone for shorter bioactive fragments [244]. Proteolytic fragments of CHGA exert a broad spectrum of regulatory activities on the cardiovascular, endocrine and immune systems [324]. Among its highly conserved C-terminal regions, CHGA gives rise to main peptides of biological importance: the antihypertensive peptide catestatin (human CTS; $hCHGA_{352-372}$) [247-249] and its short version cateslytin (human CTL; $hCHGA_{352-366}$), which have immune regulation properties [262] and regulate smooth muscle cell proliferation [251]. Besides the physiological context, CHGA or CHGA-derived peptides (CgDPs) may play an important role in the immune interaction in relation to inflammation.

Recently, links between the serum CHGA concentration and outcome in patients admitted with systemic inflammatory response syndrome, periodontitis, diabetic retinopathy [260] and rheumatoid arthritis [257] have been reported. Moreover, elevated levels of CHGA have been found in patients with IBD [238], lymphocytic colitis [325],

and collagenous colitis [326], where at long term the disease can lead to the development of colon cancer.

Patient with IBD have been reported to have an increased risk of colorectal cancer [327, 328], and risk factors include the severity of inflammation, a family history of colon cancer, and disease duration [329]. It is suspected that chronic inflammation, immune cell activation, and specific release of molecules promoting cell migration are implicated in the long-term development of dysplasia and colon carcinoma. For decades, CHGA has been used as a marker for colorectal carcinoma and transitional mucosa [324]. Thus, a close relation between chronic inflammation, immune cell activation and release of cell migration-promoting molecules should exist. A recent work from Rumio et al. demonstrated that the conserved N-terminal fragment of CHGA (vasostatin-1) has a protective effect in mice developing acute and chronic colitis [330].

Macrophages play a key role in host defense against bacterial pathogens that stimulate them *via* the activation of Toll-like receptors. Macrophage activation results in the secretion of proinflammatory cytokines such as TNF-α, interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) and in the induction of a Th-1 cytokine response, with the production of interferon-gamma (INF-γ). In this context, macrophages are considered to be classical proinflammatory effector cells [331]. Human studies revealed that there is a significant increase in the numbers of macrophages within the inflamed tissue and the peripheral blood of patients with CD or UC [141, 332]. In addition, macrophage depletion in the dextran sulfate sodium (DSS) colitis model almost completely inhibited experimental

colitis [333]. Recent data has demonstrated that CTS can modulate monocyte migration [262].

Therefore, to evaluate the potential of hCTS and to decipher the sequence of the peptides implicated, herein we evaluated the effect of hCTS and its proximal and distal sequence in two murine models of colitis and evaluated their effects on macrophages. We report that treatments with hCTS, or its derived sequences, significantly ameliorate disease severity and inhibit inflammation in the context of DSS- and 2, 4 dinitrobenzene sulfonic acid (DNBS)-induced experimental colitis. This therapeutic efficacy is mediated through the regulation of peritoneal macrophages cytokine production *via* an intracellular mechanism that implicates the signal transducer and activator of transcription 3 (STAT-3) protein.

3.2. Material and methods

3.2.1 Animals

Male C57BL/6 (7–9 weeks old) mice were purchased from Charles Rivers (Canada) and maintained in the animal care facility at the University of Manitoba under specific pathogen-free conditions. All experiments were approved by the University of Manitoba Animal Ethics Committee (10-073) and conducted under the Canadian guidelines for animal research.

3.2.2 Peptides used

hCTS ($hCHGA_{352-372}$: SSMKLSFRARAYGFRGPGPQL [248]); modified hCTS ($shCHGA_{352-372}$: SLPRRQLPSSAGMRGGKFAYF); hCTS, proximal sequence ($hCHGA_{352-366}$: SSMKLSFRARAYGFR), and hCTS, distal sequence ($hCHGA_{360-372}$: ARAYGFRGPGPQL) were used (gift from Dr. Metz-Boutigue or purchased from Biopeptide Co., Inc, San Diego, CA). The peptides were used at different doses ranging from 0.5 to 1.5mg/kg/d as reflected by previous published data related to the use of peptide for intra-rectal injection [334].

3.2.3 DSS and 2, 4 DNBS induced colitis

DSS (molecular weight [MW], 40 kilodaltons: MP Biomedicals, Soho, OH) was added to the drinking water at a final concentration of 5% (wt/vol) for 5 days [266, 333]. Controls were time-matched and consisted of mice that received normal drinking water only. Mean DSS consumption was noted per cage each day. For the DNBS study, mice

were anaesthetized using Isoflurane® (Abbott, Toronto, Canada). PE-90 tubing (10 cm long; ClayAdam, Parisppany, NJ) that was attached to a tuberculin syringe (BD, Mississauga, Canada) was inserted 3.5 cm into the colon. Colitis was induced by administration of 100µl of 4mg of DNBS solution (ICN Biomedical Inc. Aurora, OH) in 30% ethanol (Sigma, Mississauga, Canada) and left for 3 days [335]. For the DNBS study mice were supplied with 6% sucrose in their drinking water to prevent dehydration.

3.2.4 Assessment of colitis severity - disease activity index (DAI)

DAI scores have historically correlated well with the pathological findings in a DSS-induced model of IBD [336]. DAI is the combined score of weight loss, stool consistency, and bleeding. Scores were defined as follows: weight: 0, no loss; 1, 5%–10%; 2, 10%–15%; 3, 15%–20%; and 4, 20% weight loss; 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, Canada). DAI was scored from day 0 to day 5 during DSS treatment.

3.2.5 Macrophage isolation

Five or 3 days after the beginning of the DSS or the DNBS induction respectively, resident peritoneal cells were collected as described [337] with a slight modification. Sterile PBS (Gibco BRL Life Technologies, Grand Island, NY) (10ml) was injected into the caudal half of the peritoneal cavity using a 25-gauge needle (BD, Mississauga, Canada) and the body was shaken for 15sec. By inserting a 19-gauge needle (BD, Mississauga, Canada) resident peritoneal cells were slowly withdrawn and resident

peritoneal macrophages were purified by adhesion. Resident peritoneal cells were plated in macrophage culture medium (RPMI 1640 supplemented with 10% HI FCS containing 50 IU of penicillin, 50 µg streptomycin, and 2 mM glutamine per milliliter (Gibco BRL Life Technologies, Grand Island, NY)) for 60 mins at 37°C. Non-adherent cells were removed by washing five times with 500 µl of warm PBS. The overall cell viability of the adherent cell before and after treatment was greater than 97%, and more than 94% of the cells were macrophages using DiffQuick (Jorgensen Laboratory, Loveland, CO) staining. Primary bone marrow-derived macrophages (BMDMs) from naive C57BL/6 mice were generated as previously described [338] and were cultured in complete RPMI-10 medium. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Lipopolysaccharide (LPS, Sigma, Mississauga, Canada) was added to the cultures at a final concentration of 100ng/ml. In a separated set CgDPs were added to the medium at a final concentration of 10^{-7} or 10^{-5}M one hour before the LPS was added in the presence or absence of STAT3 V blocker (10^{-5}M, STATTIC; Sigma, Mississauga, Canada). Supernatants were collected 24hrs after LPS was either added or not to assess IL-1β, IL-6 and TNF-α levels in supernatants using ELISA.

3.2.6 Macroscopic scores

After macrophage isolation, mice were sacrificed and the abdominal cavity was opened, the colon was located, and observations on distension, fluid content, hyperaemia, and erythema were recorded. The colon was removed and opened longitudinally, and macroscopic damage was immediately assessed on the full section of the colon.

Macroscopic scores were performed using a previously described scoring system for DSS colitis [336] and for DNBS colitis [339].

3.2.7 C-reactive protein (CRP) assay in serum

At sacrifice date, blood was collected by intracardiac puncture in anaesthetized (Isoflurane®, Abbott, Toronto, Canada) mice. CRP levels were determined using an ELISA commercial kit (Immuno-Consultants, Portland, OR).

3.2.8 Colonic histology and myeloperoxidase (MPO) activity

Formalin (Sigma, Mississauga, Canada) -fixed colon segments coming from the splenic flexure were paraffin (Sigma, Mississauga, Canada) -embedded and 3-µm sections were stained using hematoxylin-eosin (H&E) (Sigma, Mississauga, Canada). Colonic damage was scored based on a published scoring system that considers architectural derangements, goblet cell depletion, oedema/ulceration, and degree of inflammatory cell infiltrate [336]. MPO activity was determined following an established protocol [340]. Briefly, MPO activity, used as a marker of granulocyte infiltration, was extracted and the activity was measured using a modified version of the method described by Bradley [341]. Tissue samples were homogenized (50mg/ml) in ice-cold 50mM potassium phosphate buffer (pH 6.0) (Gibco BRL Life Technologies, Grand Island, NY) containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma, Mississauga, Canada). The homogenate was freeze-thawed three times, briefly sonicated, and then centrifuged at 12000 rpm for 12min at 4°C. The supernatant was then added to a solution of O-dianisidine (Sigma, Mississauga, Canada) and hydrogen peroxide (Sigma,

Mississauga, Canada). The absorbance of the colorimetric reaction was measured by a spectrophotometer (Biotek, Winoosk, WT). MPO is expressed in units per milligram of wet tissue, 1 unit being the quantity of enzyme able to convert 10^{-6}M of hydrogen peroxide to water in 1 minute at room temperature.

3.2.9 CHGA, CTS, cytokines and p-STAT3 levels

Colonic samples were homogenized in 700µl of Tris· HCl buffer containing protease inhibitors (Sigma, Mississauga, Canada). Samples were centrifuged for 30 min, and the supernatant was frozen at -80°C until assay. Commercial ELISA were used to determine cytokine levels (IL-1β, IL-6 and TNF-α), (R&D Systems, Minneapolis, MN), human and mouse CHGA and CTS level (CUSABIO, Cedarlane, Burlington, Canada). Cell lysates from colonic samples and macrophages were used to detect p-STAT3 (STAT3 [pY705] ELISA, LifeScience, Burlington, Canada)

3.2.10 Human sera

Sera were collected from persons with Crohn's disease (n=15), ulcerative colitis (n=15) and from healthy controls who did not have any known chronic immune disease or first-degree relatives with known chronic immune diseases (n=15).

3.2.11 Statistical analysis

Results are presented as the mean ± SEM. Statistical analysis was performed using one or two-way ANOVA followed by the Tukey-Kramer multiple comparisons *post hoc*

analysis, and a P value of <0.05 considered significant with n=8 to 12 depending on the groups tested (Prism 5, GraphPad, La Jolla, CA).

3.3. Results

3.3.1 mCHGA and mCTS in the context of DSS-induced colitis and IBD

First, we examined the relation between mCHGA and mCTS during the development of experimental colitis using the DSS and DNBS models. In this context, our data demonstrate a significant increase of mCHGA during the development of colitis (**Figure 3-1A, C**) confirming previously published clinical data [238, 325, 326]. However, for the first time this was correlated with an increase of colonic mCTS when compared to the control group (**Figure 3-1B, D**). To determine the translational applicability of our study, we determined the level of hCHGA and hCTS in IBD patients. hCHGA and hCTS levels were significantly higher in persons with IBD than in healthy subjects. In the IBD group there were no significant differences in mean hCHGA and hCTS levels among those with UC and CD **(Figure 3-1E, F).** These data demonstrate that in the context of colitis m and hCTS are up regulated and may act as a pro- or anti-inflammatory regulators.

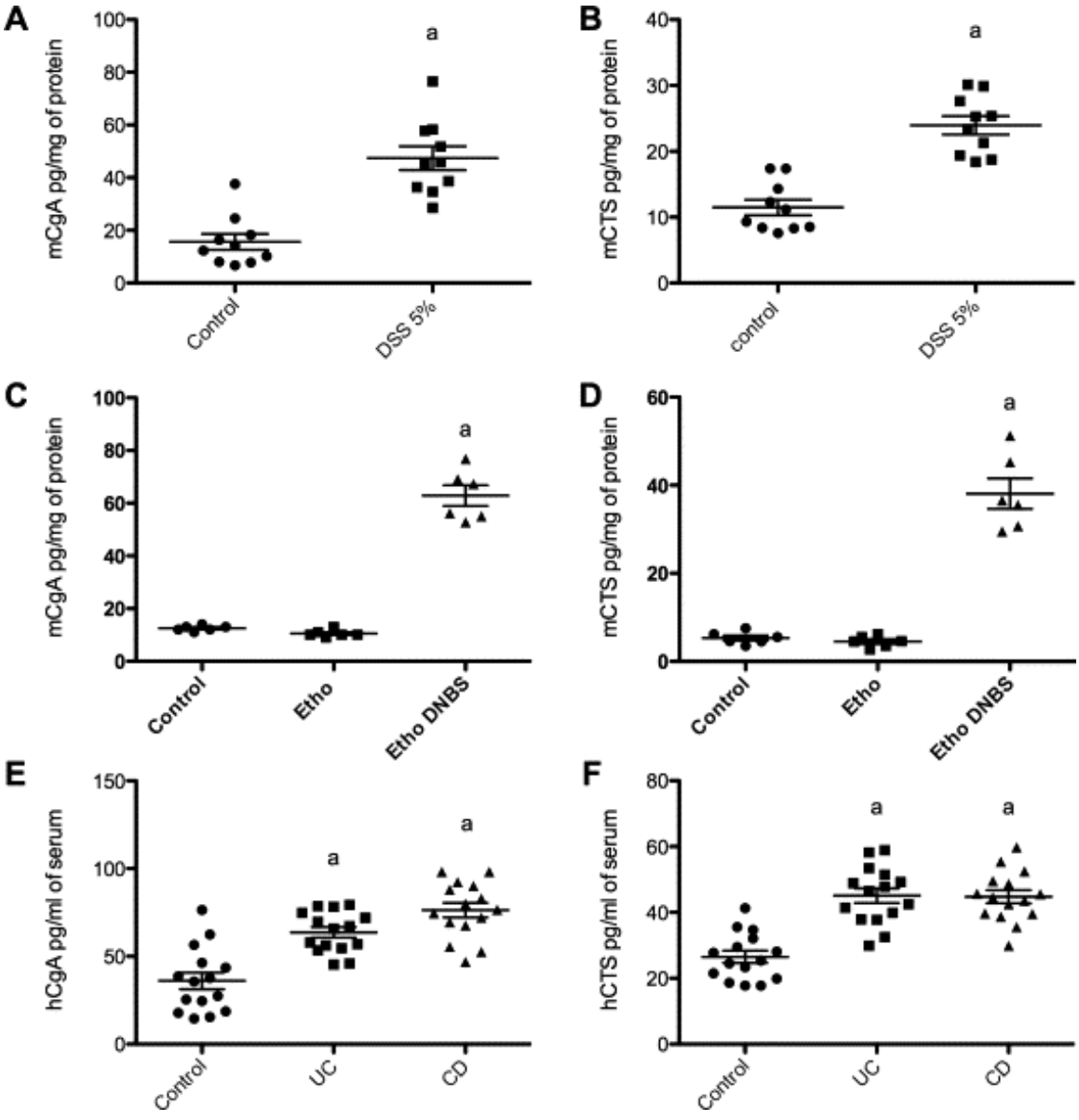

Figure 3-1. Differential expression of mouse (m) and human (h) CHGA and CHGA$_{352-372}$ (catestatin, CTS) in colonic inflammatory conditions.
A, B*:* The presence of the mCHGA and mCTS was detected at the distal part of the colon in dextran sulfate sodium (DSS)-induced colitis. ***C, D****:* The presence of the mCHGA and mCTS was detected at the distal part of the colon in 2, 4 dinitrobenzene sulfonic acid (DNBS)-induced colitis. Values are shown as the mean ± SEM. Samples were collected on day 3 or 5 post- DNBS or DSS induction; mice per group n≥ 6. ***E, F****:* The presence of hCHGA and hCTS was detected in the serum from persons with non-inflammed colon (control, n=15), with ulcerative colitis (UC, n=15) or Crohn's disease (CD, n=15). $^{a}p<0.05$ compared to control groups, ANOVA followed by the Dunnett multiple comparisons *post hoc* analysis. m or hCHGA or CTS were measured using commercially available ELISA kits. CgA represents Chromogranin A,.

3.3.2 The effect of $hCHGA_{352-372}$, $hCHGA_{360-372}$ and $hCHGA_{352-366}$ in the absence of colitis

To determine if the three peptides have some direct effect in the absence of colitis, we injected the peptides for six days (daily i.r. injection). $hCHGA_{352-372}$, $hCHGA_{360-372}$ or $hCHGA_{352-366}$ caused no changes in weight gain, colonic appearance or histology, CRP, MPO, or cytokine levels in C57BL/6 without colitis **(Figure 3-3 to 3-6 and 3- 8).**

3.3.3 The effect of $hCHGA_{352-372}$, $hCHGA_{360-372}$ and $hCHGA_{352-366}$ on DAI

To determine the potential regulatory effect related to hCTS on colonic inflammation, we studied the effect of the full sequence of hCTS ($hCHGA_{352-372}$), its proximal ($hCHGA_{352-366}$) and distal ($hCHGA_{360-372}$) part using the DSS-induced experimental model of colitis. Colitis was characterized by weight loss and frequent stools; this was evident by day 4 in the saline treated group of mice **(Figure 3-2)**. In $hCHGA_{352-372}$-treated (1.5mg/kg/d, 6 days, i.r.) mice, the onset of colitis was delayed as injury reflected in the DAI was seen within 5 days (**Figure 3-2A, B**) and the differences between groups reached statistical significance on day 4 and 5 of DSS regime. This effect was dose-dependent (0.5, 1 and 1.5mg/kg/d, i.r.) and the highest reduction was achieved with a dose of 1.5mg/kg/d; the modified peptides ($shCHGA_{352-372}$, 1.5mg/kg/d, 6 days, i.r.) did not show any significant effects **(Figure 3-2A)**. Daily administration of $hCHGA_{360-372}$ or $hCHGA_{352-366}$ (1.5 mg/kg/d, i.r.) showed a significantly lower DAI for the two last days when compared to saline-DSS group **(Figure 3-2B),** but no differences were seen between the peptides.

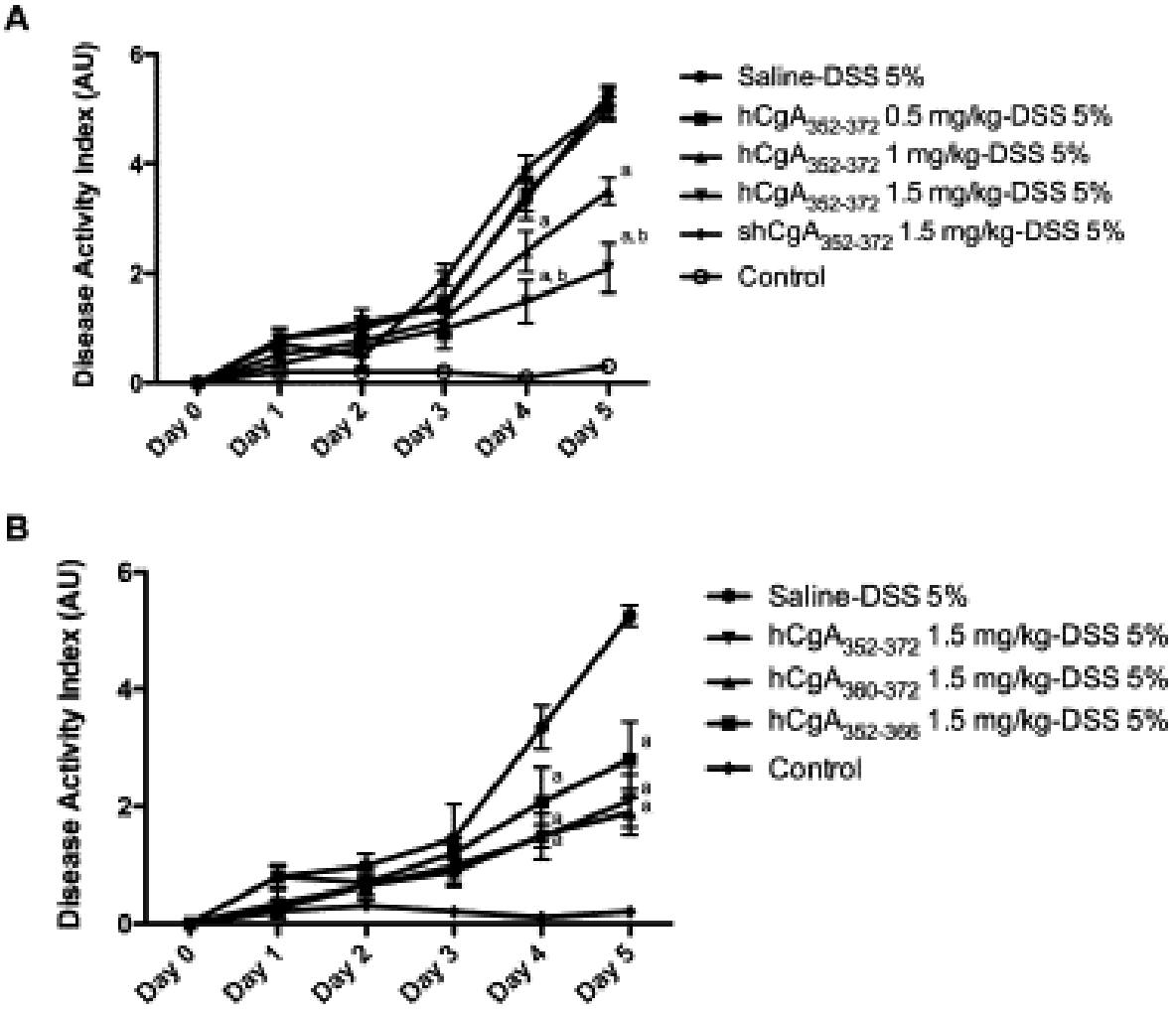

Figure 3-2. Human (h)CHGA352-372, hCHGA360-372 and hCHGA352-366 treatment alleviate the disease activity index in dextran sulphate sodium (DSS)-induced colitis. Treatments (6 days, intra-rectal) were started one day prior to colitis induction. ***A:*** Disease activity index; dose-dependent effect of hCHGA352-372 peptide (0.5, 1, 1.5 mg/kg/d, 6 days) ***B:*** Disease activity index; hCHGA352-372 hCHGA360-372, hCHGA352-366 peptide (1.5 mg/kg/d, 6 days). Values are shown as the mean ± SEM, n ≥8 mice per group. [a]$p<0.05$ compared to saline DSS-treated group, [b]$p<0.05$ compared to hCHGA352-372 peptide (1 mg/kg/d, 6 days) DSS-treated group. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis. Control represents data obtained in non-colitic non-treated mice, because no significant differences were determined between this group and any other non-colitic treated group of animals. CgA represents Chromogranin A; sh represents the modified hCHGA352-372 peptide.

3.3.4 The effect of hCHGA$_{352-372,}$ hCHGA$_{360-372}$ and hCHGA$_{352-366}$ on macroscopic score

At sacrifice day, feces consistency, hyperaemia and presence of blood were assessed. As shown in **(Figure 3-3A, B)**, hCHGA$_{352-372}$ treatments (0.5, 1 and 1.5mg/kg/d, 6 days, i.r.) significantly decreased the macroscopic scores and increased the length of colon. The modified peptides (shCHGA$_{352-372,}$ 1.5mg/kg/d, 6 days, i.r.) did not show any significant effects. The decreased severity of colitis in hCHGA$_{360-372}$- or hCHGA$_{352-366}$ treated mice (1.5 mg/kg/d, 6 days, i.r.) compared to saline–DSS group was further demonstrated by the 1.72 and 1.81-fold decrease in the macroscopic damage score respectively (**Figure 3-3A**).

3.3.5 The effect of hCHGA$_{352-372}$, hCHGA$_{360-372}$ and hCHGA$_{352-366}$ on the colonic MPO activity

MPO a marker of granulocyte infiltration was studied. DSS treatment increased the MPO activity from 0.6±0.1 U/mg in control mice to 2.23±0.2 U/mg **(Figure 3-3C)**, but treatment with hCHGA$_{352-372}$ (0.5, 1 and 1.5mg/kg/d, 6 days, i.r.) resulted in a significantly lower MPO activity when compared to saline–DSS group. The modified peptides (shCHGA$_{352-372,}$ 1.5mg/kg/d, 6 days, i.r.) did not show any significant effects. Treatments with hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (1.5 mg/kg/d, 6 days, i.r.) resulted in a significantly lower MPO activity of 1.33±0.4 and 1.37±0.3 U/mg respectively compared to saline–DSS group **(Figure 3-3C).**

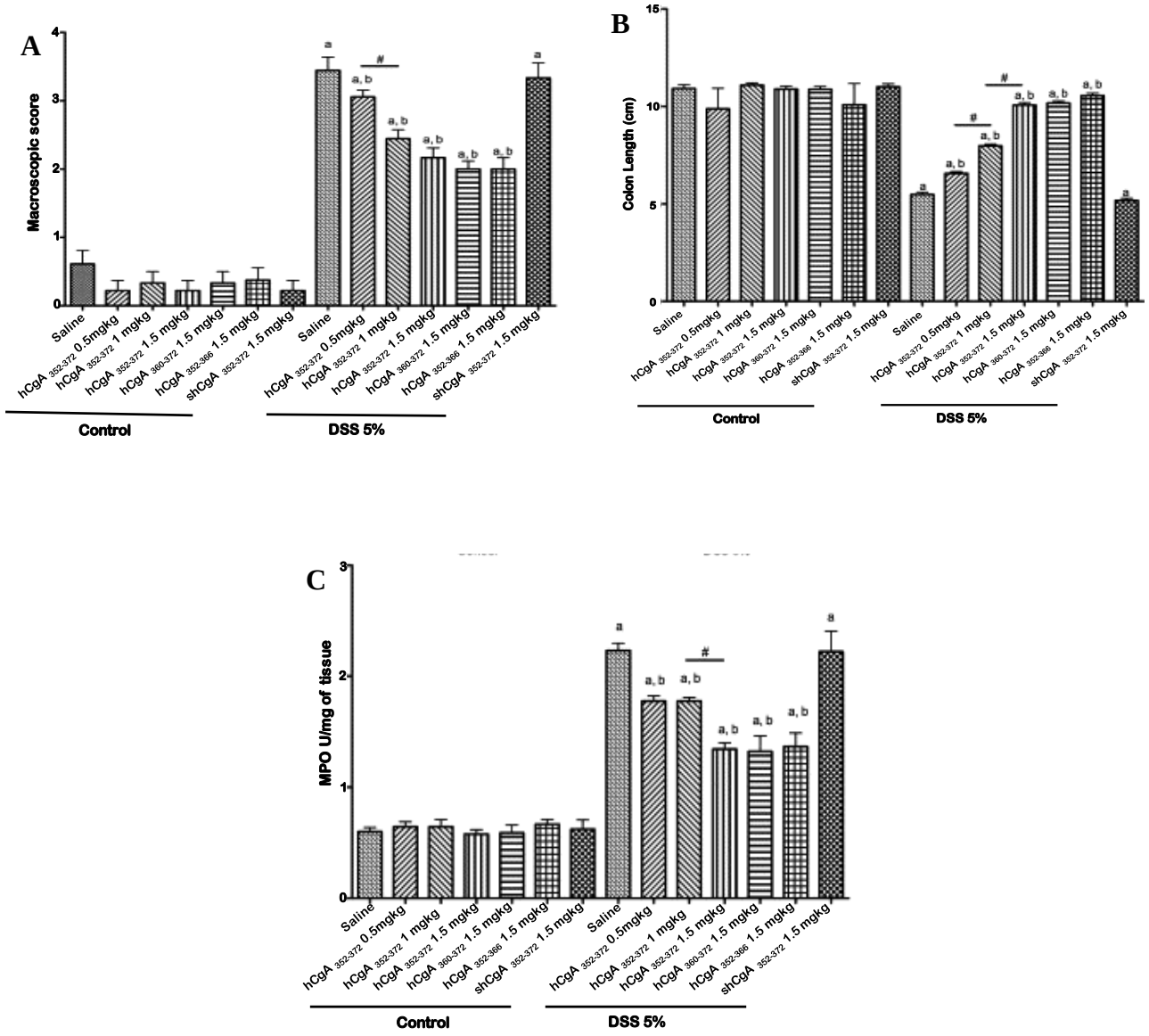

Figure 3-3. Human (h)CHGA$_{352-372}$, hCHGA$_{360-372}$ and hCHGA$_{352-366}$ treatment alleviate disease activity, colon length and colonic myeloperoxidase (MPO) activity in dextran sulfate sodium (DSS)-induced colitis.

Treatments (6 days, intra-rectal 0.5, 1, 1.5 mg/kg/d, 6 days) were started one day prior to colitis induction. ***A:*** Macroscopic score; ***B:*** Colon length; ***C:*** MPO activity. Values are shown as the mean ± SEM. Samples were collected on day 5 post-DSS; n ≥8 mice per group. ^{a}P<0.05 compared to control group, ^{b}P<0.05 compared to saline DSS-treated group. $^{\#}$P<0.05. ANOVA followed by Tukey multiple comparisons *post hoc* analysis. sh represents the modified hCHGA$_{352-372}$ peptide.

3.3.6 The effect of $hCHGA_{352-372}$, $hCHGA_{360-372}$ and $hCHGA_{352-366}$ on the histological score

Mucosal inflammation and infiltration was assessed through histological scoring. DSS colitis induces a significant increase of cell infiltration **(Figure 3-4A(a))**. As shown in **Figure 3-4A, (b) $hCHGA_{352-372}$, (c) $hCHGA_{360-372}$ or (d) $hCHGA_{352-366}$** treatments (1.5mg/kg/d, 6 days, i.r.) significantly decreased the severity of colitis. This was associated with a reduction in the loss of tissue architecture and edema, and a decrease in the mixed immune cell infiltrate (mononuclear cells, neutrophils, and eosinophils). The modified peptides ($shCHGA_{352-372}$, 1.5mg/kg/d, 6 days, i.r.) did not show any significant effects **(Figure 3-4A(e)).** Treatments with $hCHGA_{352-372}$, $hCHGA_{360-372}$ or $hCHGA_{352-366}$ (1.5 mg/kg/d, 6 days, i.r.) decreased the histological score from 2.7±0.1 to 1.3±0.1, 1.2±0.2 and 1.3±0.1 respectively (Figure 4B).

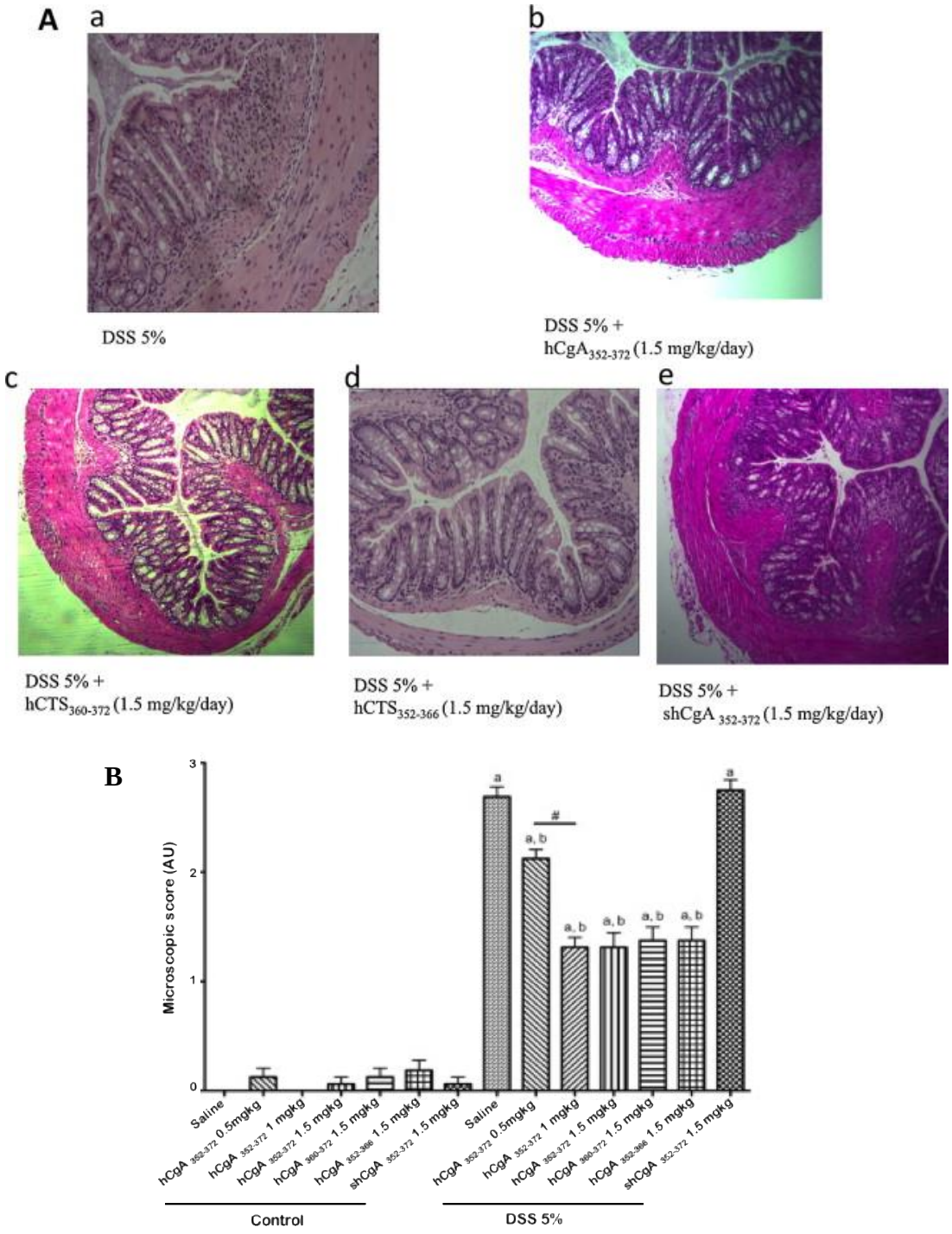

Figure 3-4. Human (h)CHGA$_{352-372}$, hCHGA$_{360-372}$ and hCHGA$_{352-366}$ treatment alleviate histological score in dextran sulfate sodium (DSS)-induced colitis.

A: Appearance of the colon ***a:*** in mice with DSS-induced colitis; ***b:*** *in* hCHGA$_{352-372}$, ***c:*** in hCHGA$_{360-372}$ ***d:*** in hCHGA$_{352-366}$, and ***e:*** in shCHGA$_{352-372}$ (1.5 mg/kg/d, intra-rectal, 6 days)-treated mice with DSS-induced colitis ***B:*** Histological score; values are shown as the mean± SEM. Samples were collected on day 5 post-DSS; n≥8 mice per group. [a]P<0.05 compared to control group, [b]P<0.05 compared to saline DSS-treated group. Hematoxylin and eosin staining, 100X magnifications. sh represents the modified hCHGA$_{352-372}$ peptide.

3.3.7 The effect of $hCHGA_{352-372}$, $hCHGA_{360-372}$ and $hCHGA_{352-366}$ on serum CRP level

To confirm any potential effect on systemic inflammation, serum CRP levels were studied. CRP decreased from 35.63±0.7 in colitic mice to 25.14±0.22µg/ml in colitic mice treated with $hCHGA_{352-372}$ (1.5mg/kg/d, 6 days, i.r.) and this effect was dose-dependent (**Figure 3-5A)**. The modified peptides ($shCHGA_{352-372,}$ 1.5mg/kg/d, 6 days, i.r.) did not show any significant effects (Figure 5A). The decreased severity of colitis in $hCHGA_{360-372}$- or $hCHGA_{352-366}$- treated mice (1.5 mg/kg/d, 6 days, i.r.) compared to saline–DSS group was further demonstrated by a 1.5 and 1.6-fold decrease in serum CRP level respectively **(Figure 3-5A)**.

3.3.8 The effect of $hCHGA_{352-372}$, $hCHGA_{360-372}$ and $hCHGA_{352-366}$ on colonic cytokine levels

To determine more precisely the mucosal inflammatory states, we studied the colonic pro-inflammatory cytokine levels. We found significant decrease in the levels of IL-1β (2-fold), IL-6 (1.8-fold) and TNF-α (1.9-fold) in the colon of colitic mice treated with $hCHGA_{352-372}$ (1.5mg/kg/d, 6 days, i.r.) when compared to the saline-DSS group, this effect was dose-dependent **(Figure 3-5 B-D)**. The modified peptides ($shCHGA_{352-372,}$ 1.5mg/kg/d, 6 days, i.r.) did not show any significant anti-inflammatory effects on the development of colitis **(Figure 3-4)**. IL-1β levels in $hCHGA_{360-372}$ and $hCHGA_{352-366}$-treated colitic mice (1.5mg/kg/d, 6 days, i.r.) were 2.35 and 2.46-fold lower respectively compared to the saline-DSS group **(Figure 3-5B)** and IL-6 and TNF-α levels were 1.79-17.4 and 2 1.68-fold lower, respectively **(Figure 3-5C, D)**.

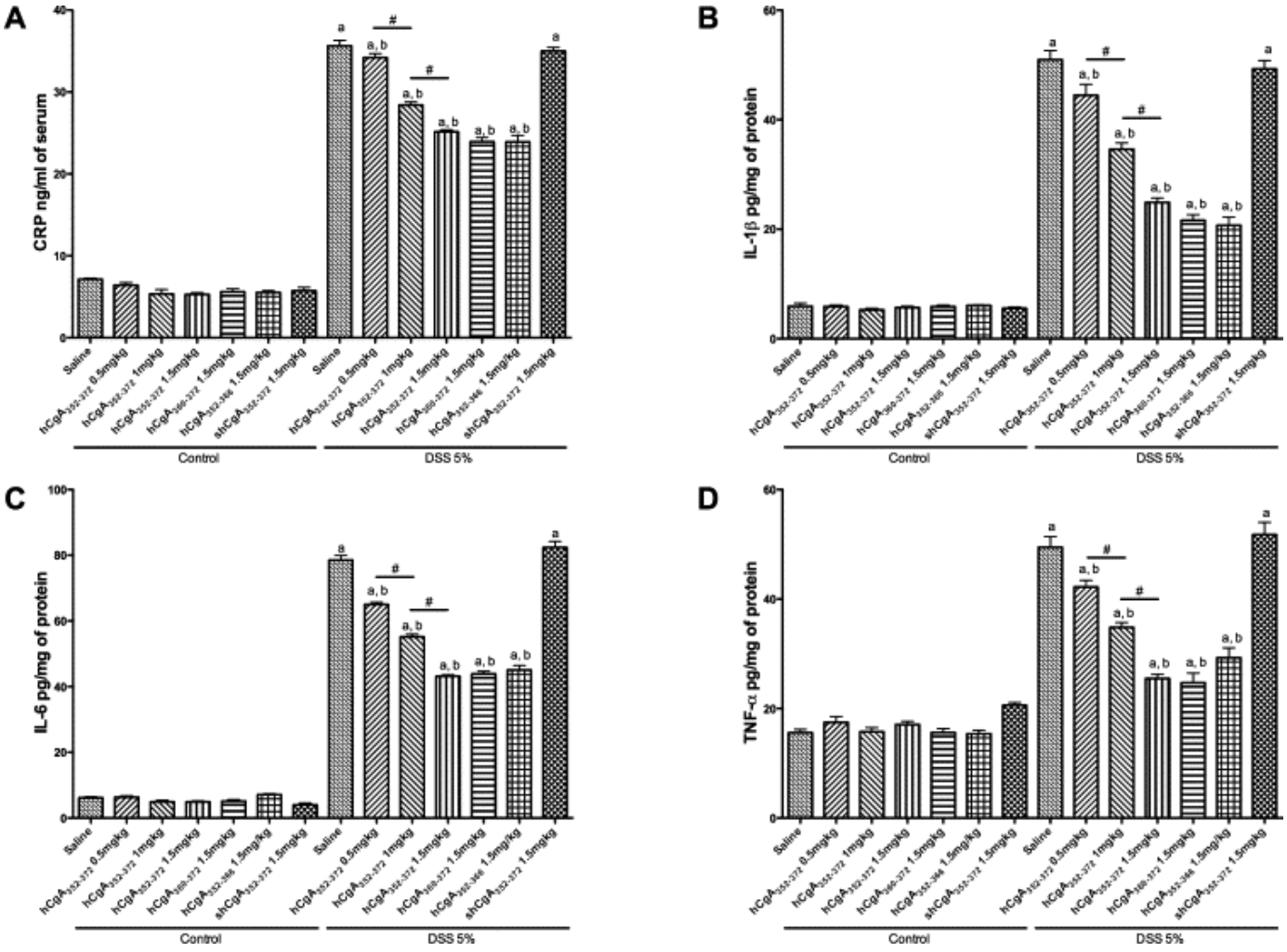

Figure 3-5. Human (h)CHGA$_{352-372}$, hCHGA$_{360-372}$ and hCHGA$_{352-366}$ decrease serum C-reactive protein level and proinflammatory colonic cytokines in dextran sulfate sodium (DSS)-induced colitis.

Treatments (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/d, 6 days) were started one day prior to colitis induction *A:* Serum C-reactive protein (CRP); *B:* Colonic interleukin (IL)-1β amount; *C:* Colonic IL-6 amount; *D:* Colonic tumor necrosis factor (TNF)-α amount. Values are shown as the mean ± SEM. Samples were collected on day 5 post-DSS; n≥8 mice per group. ^{a}P<0.05 compared to control group, ^{b}P<0.05 compared to saline DSS-treated group, $^{\#}$P<0.05. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis. CRP, IL-1β, IL-6 and TNF-α were measured using commercially available ELISA kits. sh represents the modified hCHGA$_{352-372}$ peptide.

3.3.9 $hCHGA_{352-372}$, $hCHGA_{360-372}$ or $hCHGA_{352-366}$ regulate macrophage pro-inflammatory cytokines release

Monocytes and macrophages are an important component in the development of colitis, and recent data demonstrated that those cells are the main producer of IL-1β, IL-6 and TNF-α. To elucidate the mechanism by which the hCTS is influencing the development of colitis, we next investigated the role of the three peptides in macrophage function in relation to gut inflammation. This role was further studied by examining the ability of hCTS to inhibit macrophages to produce pro-inflammatory cytokines by isolating macrophages from the peritoneal cavity of non-colitic or colitic mice treated or not *in vivo* and *in vitro* with the different peptides. Alternatively, BMDMs were isolated from naïve mice and IL-1β, IL-6 and TNF-α production was assessed in the presence or absence of the three peptides. Peritoneal macrophages isolated from the saline-DSS group revealed an increased release of IL-1β, IL-6 and TNF-α when compared to the saline-DSS group **(Figure 3-6)**; however, this was not evident in peritoneal macrophages isolated from the DSS group treated *ex vivo* with $hCHGA_{352-372}$, $hCHGA_{360-372}$ or $hCHGA_{352-366}$ (1.5 mg/kg/d, i.r.) **(Figure 3-6)**.

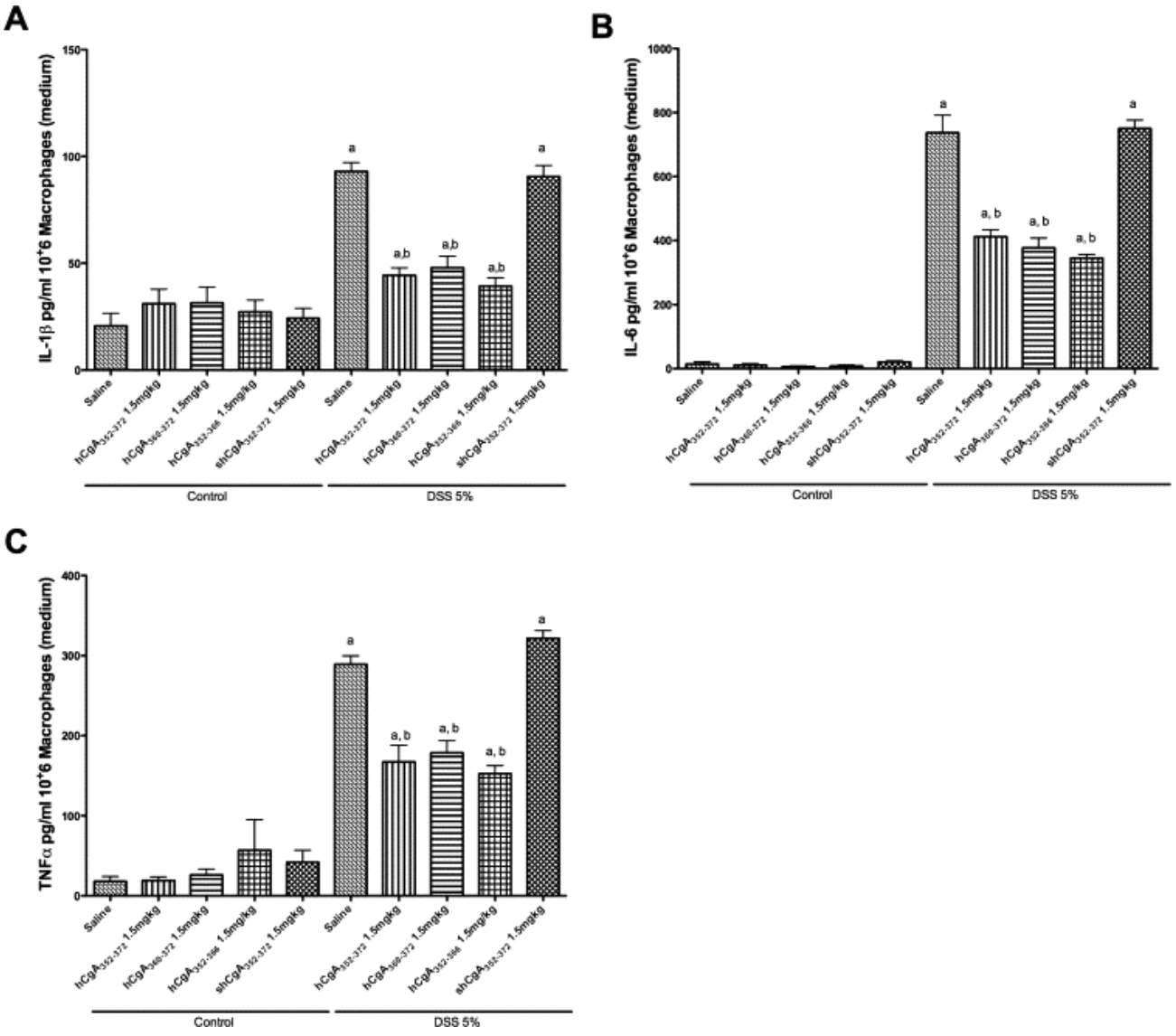

Figure 3-6. Functional role of peritoneal macrophages in the amelioration of colitis induced by hCHGA352-372, hCHGA360-372 or hCHGA352-366 (1.5 mg/kg/d, intra-rectal, 6 days) treatments.

Peritoneal macrophages were isolated from *in vivo* colitic $hCHGA_{352-372}$, $hCHGA_{360-372}$ or $hCHGA_{352-366}$-treated mice with dextran sulfate sodium (DSS)-induced colitis. Interleukin (IL)-1β **(*A*)**, IL-6 **(*B*)** and tumor necrosis factor (TNF)-α **(*C*)** were measured in conditioned media (24h) using commercially available ELISA kits. $^{a}P<0.05$ compared to control group respectively, $^{b}P<0.05$ compared to saline DSS-treated group, ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, $n \geq 8$. Values are shown as the mean ± SEM of four separate experiments. sh represents the modified $hCHGA_{352-372}$ peptide.

We next examined whether direct cell culture treatments with the peptides decreased the release of cytokine from peritoneal macrophages and BMDMs isolated from naïve control mouse. Peritoneal macrophages cultured with LPS (100 ng/ml^{-1}) and treated with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (10^{-7} or 10^{-5}M) demonstrated a significant decreased release in the three pro-inflammatory cytokines **(Figure 3-7A, C, E)**; this effect was confirmed using BMDMs **(Figure 3-7B, D, F).**

In both experiments, the modified peptides (shCHGA$_{352-372}$) (10^{-5}M) did not show any significant effects on the cytokine release **(Figure 3-6 & 3-7).**

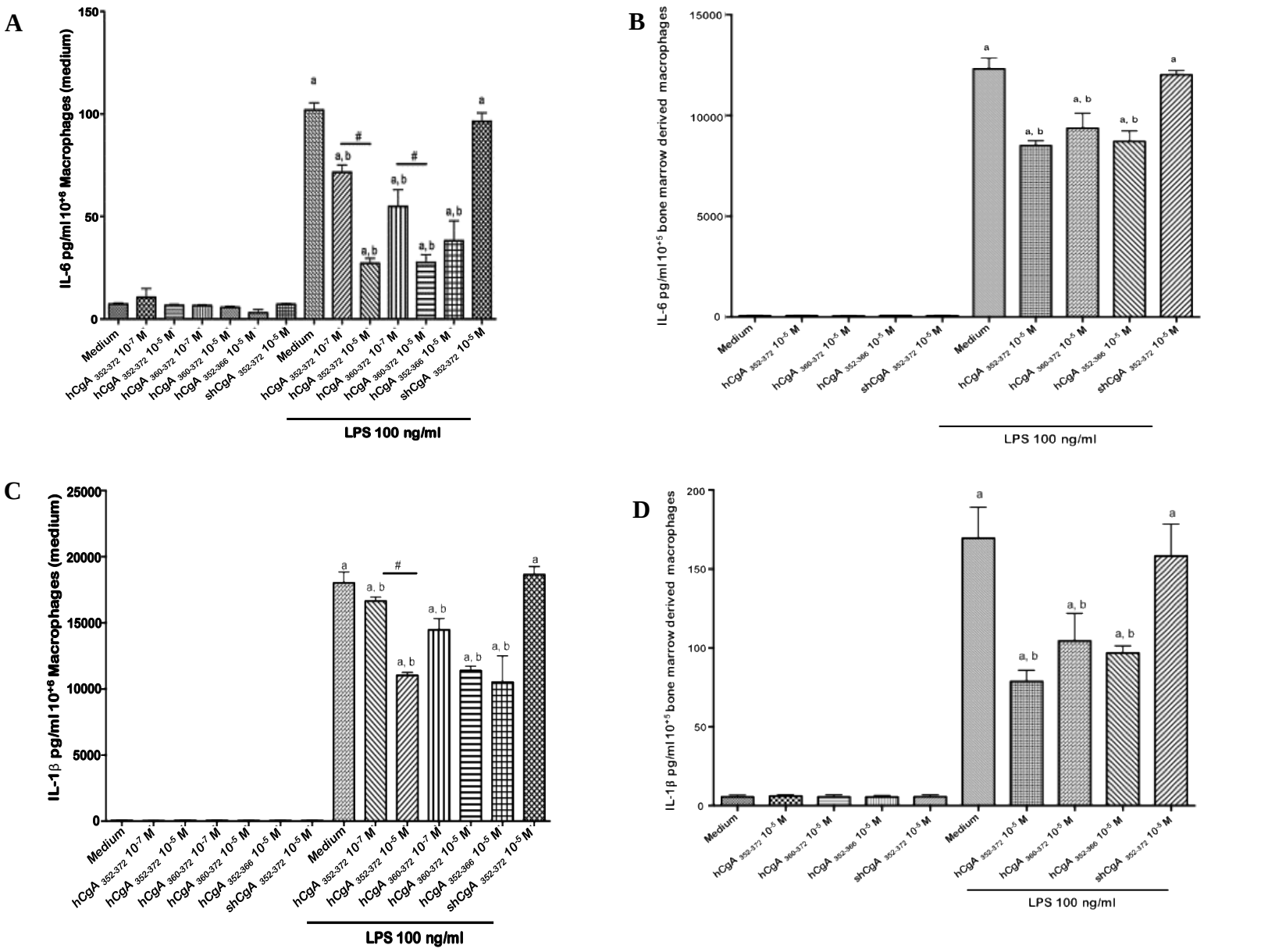

Figure 3-7 continued

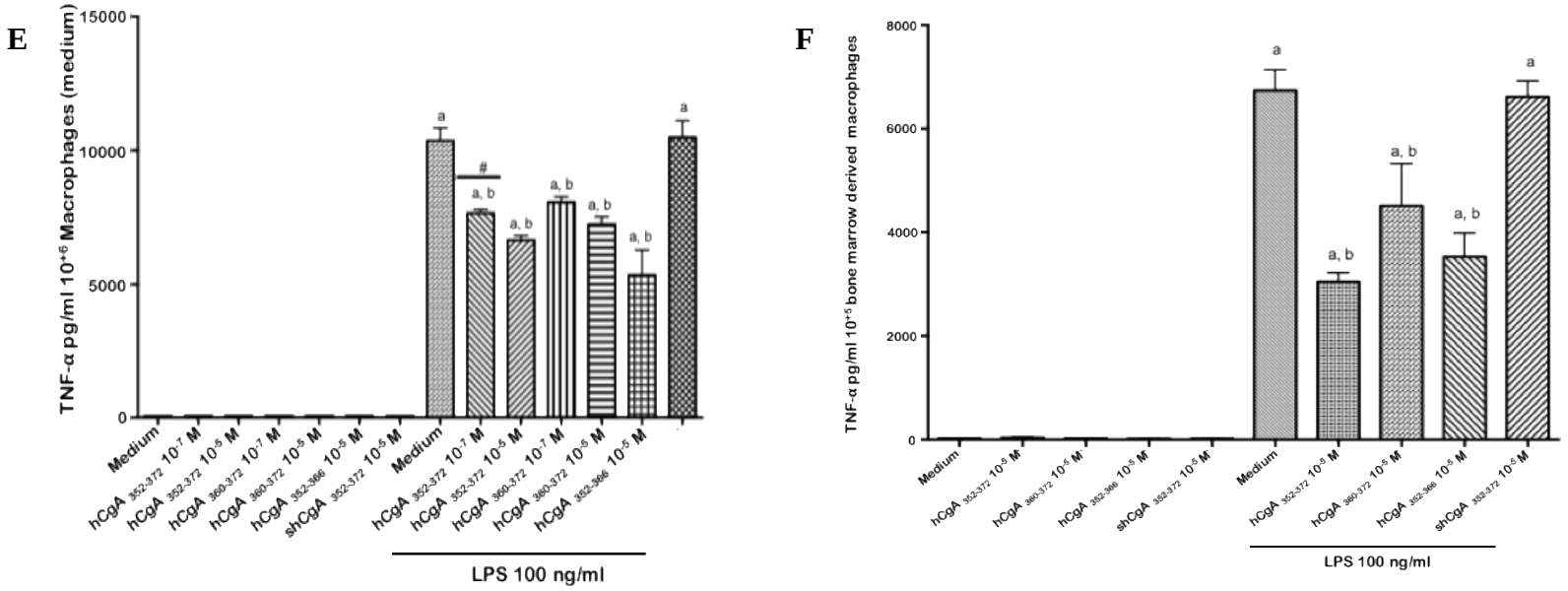

Figure 3-7. Functional role of naive peritoneal and bone marrow-derived macrophages (BMDMs).
Lipopolysaccharide (LPS)-stimulated (100 ng/ml^{-1}) peritoneal mouse macrophages and BMDMs cultures from naive control mouse treated *in vitro* with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (10^{-7}M or 10^{-5}M). Interleukin (IL)-6 ***(A, B)***, IL-1β ***(C, D)*** and TNF-α ***(E, F)*** were measured in conditioned medium (24h) using commercially available ELISA kits. BMDMs and naive peritoneal macrophages isolated from naive mouse are less potent producers of proinflammatory cytokines when treated with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (10^{-5}M). The modified peptides (sh) did not have any effects. $^{a}P<0.05$ compared to control non-LPS treated group, $^{b}P<0.05$ compared to medium LPS-treated group, ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, n≥5. $^{\#}P<0.05$. The values are shown as the mean ± SEM of four separate experiments.

3.3.10 hCHGA$_{352-372}$, hCHGA-$_{360-372}$ or hCHGA$_{352-366}$ increase p-STAT3 level

In physiological and pathological conditions, CTS has been described to increase the level of phosphorylated STAT-3 (p-STAT3) [342]. Therefore, to determine the possible mechanism by which hCTS can affect DSS-induced colitis, we obtained cytosolic and nuclear fractions from macrophages and colon to study the level of p-STAT3. Colitis was associated with an increase of colonic p-STAT3 level. We found a significantly higher level of p-STAT3 in the colon of colitic mice treated with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (1.5mg/kg/d, 6 days, i.r.) when compared to saline-DSS group **(Figure 3-8A)**. Low concentration of hCHGA$_{352-372}$ (0.5, 1 mg/kg/d, 6 days, i.r.) or the modified shCHGA$_{352-372}$ (1.5 mg/kg/d, 6 days, i.r.) peptides did not show any significant effects.

In parallel, a significant increase of p-STAT3 was observed in peritoneal macrophages isolated from mice treated *in vivo* with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (1.5 mg/kg/d, 6 days, i.r.) when compared to saline-DSS group **(Figure 3-8B)**. Low concentration of hCHGA$_{352-372}$ (0.5, 1 mg/kg/d, 6 days, i.r.) or the modified shCHGA$_{352-372}$ (1.5 mg/kg/d, 6 days, i.r.) peptides did not show any significant effects.

We described the same effect when naïve peritoneal macrophages were isolated and stimulated with LPS (100ng/ml) in the presence of absence of the peptides (10^{-5}M) **(Figure 3-8C)**. Low concentration of hCHGA$_{352-372}$ or hCHGA$_{360-372}$ (10^{-7}M) or the modified shCHGA$_{352-372}$ (10^{-5}M) peptides did not show any significant effects.

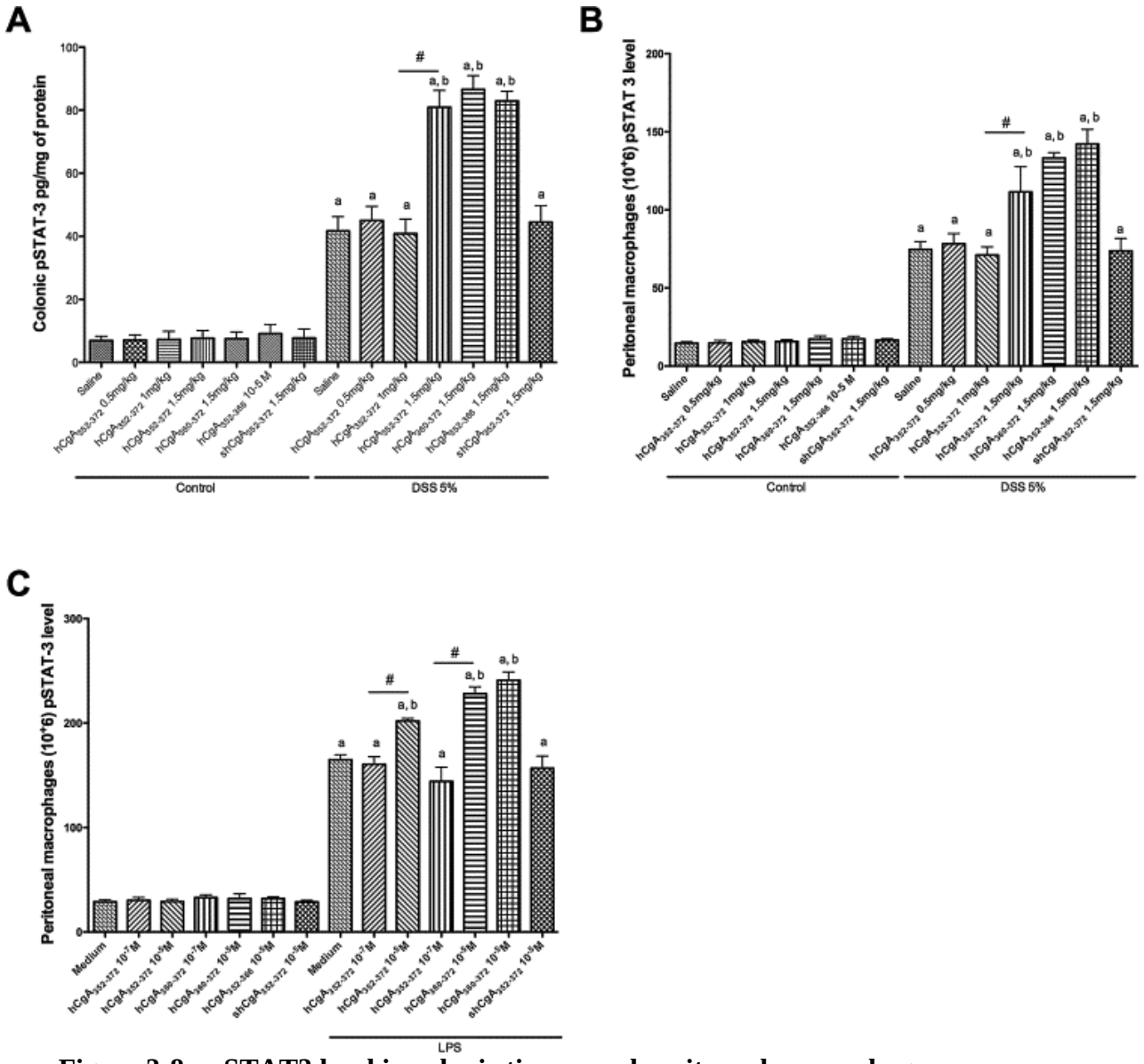

Figure 3-8. p-STAT3 level in colonic tissues and peritoneal macrophages.

A: Colonic tissue isolated from *in vivo* colitic and non-colitic hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$-treated mice (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/d, 6 days); ***B:*** Peritoneal macrophages isolated from *in vivo* colitic and non-colitic hCHGA352-372, hCHGA$_{360-372}$ or hCHGA$_{352-366}$-treated mice (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/d, 6 days); ***C:*** Naive peritoneal macrophages isolated from non-colitic mice and treated *in vitro* with hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ (10^{-7}M or 10^{-5}M) and stimulated with lipopolysaccharide (LPS) (100 ng/ml). p-STAT3 was measured using commercially available ELISA kits. ^{a}P<0.05 compared to non-colitic or in the absence of LPS, ^{b}P<0.05 compared to saline DSS-treated or medium LPS-treated groups, $^{\#}$P<0.05. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, n≥5. The values are shown as the mean ± SEM of four separate experiments. sh represents the modified hCHGA352-372 peptide.

In the presence of the STATTIC (STAT3 blocker; 10^{-5}M), the beneficial effect of the treatment on peritoneal macrophages cytokine release was abolished **(Table 3-1)**.

3.3.11 The level of mCHGA and mCTS and the effect of hCHGA$_{352-372}$, hCHGA-$_{360-372}$ and hCHGA$_{352-366}$ on DNBS-induced colitis

To determine whether the above-described changes were restricted to the DSS-based model, we performed studies using the DNBS-based model of experimental colitis. As shown in **table 3-2**, hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ treatments significantly decreased the macroscopic scores at day 3 after DNBS induction. DNBS increased MPO activity from 1.3±0.1 U/mg in control mice to 4.3±0.2 U/mg and hCHGA$_{352-372}$, hCHGA$_{360-372}$ or hCHGA$_{352-366}$ treatment (1.5mg/kg/d) resulted in significantly lower MPO activity compared to DNBS-treated controls. In addition, we found significantly decreases in the IL-1β, IL-6 and TNF-α levels in the colon of DNBS treated mice with hCHGA$_{352-372}$, hCHGA$_{352-366}$ and hCHGA$_{360-372}$ when compared to DNBS-treated controls **(Table 3-2).**

Table 3-1. Functional role of *STAT3* in the release of pro-inflammatory cytokines.
(A) Peritoneal macrophages (10^{+6} cells) isolated from colitic in vivo $hCHGA_{352–372}$, $hCHGA_{360–372}$ or $hCHGA_{352–366}$ -treated mice (1.5 mg/kg/d, i.r., 6 days) and treated in vitro with STAT3 inhibitor V (STATTIC; 10^{-5} M); (B) Naive peritoneal macrophages (10^{+6} cells) isolated from non-colitic mice stimulated with LPS (100 ng/ml) and treated in vitro with $hCHGA_{352–372}$, $hCHGA_{352–366}$ and $hCHGA_{360–372}$ (10^{-5} M) in the presence or absence of STATTIC (10^{-5} M). Interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α levels were measured in conditioned media (24 h) using commercially available ELISA kits. [a]P < 0.05 compared to colitic medium non-treated group. [b]P < 0.05 compared to colitic medium treated group. ANOVA followed by the Dunnett multiple comparisons post hoc analysis, n ≥ 6. The values are shown as the mean ± SEM of four separate experiments.

A

			$hCHGA_{352-372}$ 1.5mgkg		$hCHGA_{360-372}$ 1.5mgkg		$hCHGA_{352-366}$ 1.5mgkg		
	Medium	STATTIC	Medium	STATTIC	Medium	STATTIC	Medium	STATTIC	
IL-6 (pg/ml)	93 ± 3.4	104 ± 12	44.3 ± 3[a]	89.4 ± 9.7[b]	47.9 ± 6.3[a]	78.9 ± 8.8[b]	39.2 ± 3.9[a]	97.4 ± 7.3[b]	A
IL-1ß (pg/ml)	736 ± 55	889 ± 42	412 ± 22[a]	823 ± 67[b]	377 ± 30[a]	745 ± 65[b]	345 ± 11[a]	798 ± 43[b]	
TNF-α (pg/ml)	289 ± 10	334 ± 25	167 ± 20[a]	245 ± 48[b]	178 ± 15[a]	274 ± 34[b]	152 ± 10[a]	235 ± 46[b]	

B

			$hCHGA_{352-372}$ $10^{-5}M$		$hCHGA_{360-372}$ $10^{-5}M$		$hCHGA_{352-366}$ $10^{-5}M$	
	Medium	STATTIC	Medium	STATTIC	Medium	STATTIC	Medium	STATTIC
IL-6								
(pg/ml)	111 ± 13	137 ± 12	27.1 ± 5[a]	99.7 ± 19[b]	29.5 ± 4[a]	84.3 ± 12.7[b]	38.2 ± 9[a]	77.2 ± 14[b]
IL-1ß								
(pg/ml)	18,014 ± 818	19,342 ± 921	11,024 ± 93[a]	19,354 ± 756[b]	11,387 ± 339[a]	18,649 ± 382[b]	10,509 ± 692[a]	17,484 ± 789[b]
TNF-α								
(pg/ml)	10359±478	10942±921	6644±177[a]	9892±534[b]	6239±285[a]	10374±437[b]	5343±939[a]	10100±453[b]

Table 3-2. $hCHGA_{352–372}$, $hCHGA_{360–372}$ or $hCHGA_{352–366}$ reduce the severity of 2,4-dinitrobenzene sulfonic acid (DNBS)—induced colitis.

$hCHGA_{352–372}$, $hCHGA_{360–372}$ or $hCHGA_{352–366}$ (1.5 mg/kg/d, 4 days, i.p.) treatment started one day before disease induction. Macroscopic score, myeloperoxidase (MPO) activity and cytokine profile in colonic tissue were determined 3 days post-DNBS induction. Values are shown as the mean ± SEM, $n \geq 8$. *BLS (below the lowest standard). sh represents the modified $hCHGA_{352–372}$ peptide. Dunnett multiple comparisons post hoc analysis.[a]$P < 0.05$ vs saline. [b]$P < 0.05$ vs DNBS 4 mg + ethanol 30%-saline treated group

	Saline	Ethanol 30%	DNBS 4 mg +Etho 30% +saline	DNBS 4 mg +Etho 30% +$hCHGA_{352-372}$ 1.5 mg/kg/d	DNBS 4 mg +Etho 30% +$hCHGA_{360–372}$ 1.5 mg/kg/d	DNBS 4 mg +Etho 30% +$hCHGA_{352–366}$ 1.5 mg/kg/d	DNBS 4 mg +Etho 30% +$shCHGA_{352–372}$ 1.5 mg/kg/d
Macroscopic (Score)	0.1 ± 0.4	0.6 ± 0.5	4.3 ± 0.8[a]	1.6 ± 0.8[b]	1.8 ± 0.7[b]	2.3 ± 0.6[b]	4.7 ± 0.9
MPO(U/mg/tissue)	0.5 ± 0.1	1.3 ± 0.1	4.3 ± 0.2[a]	1.5 ± 0.18[b]	2.1 ± 0.15[b]	1.9 ± 0.2[b]	4.8 ± 0.7
IL-6 (pg/ml)	BLS	BLS	79.5 ± 8.1[a]	35.5 ± 5.5[b]	31.3 ± 4.8[b]	37.2 ± 6[b]	83.2 ± 8.9
IL-1ß (pg/ml)	BLS	BLS	166 ± 10[a]	101 ± 7[b]	109 ± 6[b]	95 ± 18[b]	177.2 ± 11
TNF-α (pg/ml)	BLS	6 ± 2.7	185 ± 9[a]	66 ± 12[b]	57 ± 13[b]	54 ± 14[b]	181 ± 18

3.4. Discussion

It was recently demonstrated that there are significant increases in CHGA in several subtypes of colitis [238, 254, 326] and that hCTS regulates immune cells and the STAT-3 pathway [342]. The present study shows that h and mCTS are significantly increased during the development of colitis. Moreover, we showed that hCTS attenuates the inflammatory response during experimental colitis. Colitis induced by DSS and DNBS were less severe in mice treated with hCTS and could be mimicked by the proximal and distal part of the peptide. Conversely, administration of the scramble peptide did not modify the course of colonic inflammation. A protective role for hCTS in macrophage and BMDM cytokine release *via* the STAT-3 pathway implicates a role for macrophages in this anti-inflammatory effect. Taken together, these findings extend the influence of hCTS to intestinal inflammation and immune regulation.

The most widely used and characterized experimental model of UC is the DSS-induced colitis, which was developed by administration of DSS in the drinking water. DSS induces a very reproducible acute colitis characterized by mucosal inflammation with ulcerations, body weight loss, and bloody diarrhoea infiltrations [343], polymorphonuclear cells, macrophages, lymphocytes infiltration and changes regarding the number of EC cells [235, 236]

After chromaffin cells, EC cells are the main source of CHGA and its derived peptides in the gut [232], which are an important enteric mucosal signalling molecules influencing gut physiology [233, 234]. Changes in intestinal EC cell numbers and hCHGA levels are

observed in patients with IBD [253-255, 344] and in diarrhea predominant-irritable bowel syndrome patients [238]. The unifying hypothesis proposed relies on EC cell hyperplasia and the potential neuroendocrine system activation in response to inflammation [238] that produces elevated serum CHGA levels [256]. In this study, we have shown that induction of DSS or DNBS colitis was correlated with a significant increase in mCHGA and mCTS expression. In parallel, using serum from persons with IBD we have demonstrated that the levels of hCHGA and hCTS were significantly higher in IBD than controls, confirming previously published data[326]. Since subjects with IBD were drawn from the general population and not a clinic we did not have access to their clinical data; hence it would be important to determine how hCHGA and hCTS levels vary by disease activity status. Considering there were similar levels in CD and UC we think it is less likely that levels would vary by phenotype but this remains to be proven. This can be explained by the fact the CHGA or its derived peptides may have some anti-inflammatory proprieties that need to be expressed not only during the acute phase but also during the remission phase to keep the inflammation under control *via* some anti-inflammatory mechanisms. Our data demonstrate for the first time an increased in h and mCTS in colitis and suggest that its role should be examined during the development of inflammatory conditions.

To determine the role of hCTS in colitis, we have shown that preventive administration of hCTS not only attenuated the severity of inflammation associated with DSS-induced colitis but also reduced the production of pro-inflammatory mediators in the gut. The attenuation of DSS-induced inflammation in treated mice was observed in all the

parameters examined, including disease activity, macroscopic and histologic scores, and MPO and CRP activity.

MPO is an enzyme contained in azurophilic granules of neutrophils and in other myeloid cells, and as such, it is commonly used as an index of inflammation [345]. Previous studies reported an extensive accumulation of neutrophils and a significant increase in the serum CRP level and colonic MPO activity in DSS-colitis [335]. In this study, we observed significantly lower levels of serum CRP and colonic MPO activity in mice treated with hCTS after induction of colitis. Our data confirm the potential relation between CgDPs and CRP level as described in the context of systemic inflammatory response syndrome [258, 259]. It is noteworthy that the modified peptide did not show any effect on colitis, confirming the role of wild-type sequence. These findings, along with the reduction in colonic IL-1β, IL-6 and TNF-α suggest that hCTS has an important role in the pathogenesis of colitis by regulating the infiltration of inflammatory cells and production of pro-inflammatory mediators in the colon.

To define the role of the proximal and distal part of hCTS, we studied two specific sequences. Preventive treatment with $hCHGA_{352-366}$ and $hCHGA_{360-372}$ decreased colonic inflammation. There was associated with a significant decrease in disease activity, macroscopic and histological scores in colitic mice treated with the two peptides. Moreover, we demonstrated a significant down-regulation of MPO activity, serum CRP and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). These observations further provide evidence in favour of a crucial role of hCTS in regulation of gut inflammation

and might define the importance of studying the common sequence shared by the three peptides.

To gain mechanistic insight, it is necessary to identify the cell types involved in the regulation of gut inflammation by hCTS. Gut inflammation is characterized by mucosal recruitment of macrophages which play a critical role in intestinal inflammatory responses through the secretion of chemokines and cytokines, and through antigen presentation to T lymphocytes [346]. Macrophages also play a key role in the host defense against bacterial pathogens, which stimulate macrophages *via* the activation of toll-like receptors [347]. Macrophage activation by pathogens results in the secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α and in the induction of a Th-1 cytokine response. In this context, macrophages are considered to be classical pro-inflammatory effector cells [331]. Due to the strategic location of EC and macrophages, it is likely that CgDPs may play an important role in regulation of macrophages in colitis. In the present study, we observed a lower amount of IL-1β, IL-6 and TNF-α released in the culture supernatant of macrophages isolated from the peritoneal cavity of colitic mice treated *in vivo* with the three peptides.

Receptors for CgDPs appear not to exist, but the sequence similarity of CgDPs with cell penetrating peptides [261] appears to allow the peptides to enter cells [262] and could explain the intracellular effect. We confirmed the proinflammatory cytokine regulation on naïve macrophages and BMDMs treated *in vitro* with the peptides. Using an ELISA method of detection, this down-regulation was associated with an increase of p-STAT3

level, corroborating the study of Bandyopadhyay et al. where, using a western blot analysis, they described that *in vitro* hCTS treatment of an adipose explant from diet-induced obese mice stimulates STAT3 phosphorylation[342]. In our study, the increase of p-STAT3 and the decrease in pro-inflammatory cytokines confirm the data demonstrating that mice lacking STAT3-deficient macrophages are characterized by excessive cytokine release and develop colitis [173, 180]. Conversely, STAT3 activation in Socs3-deficient macrophages triggers a strong anti-inflammatory response [348]. Taken together, these observations suggest that hCTS plays an important role in gut inflammation by influencing the macrophage production of cytokines *via* the STAT3 protein and a yet unknown mechanism. This effect is in contrast to the data that demonstrated that hyperactivation of STAT3 results in severe colitis [166] and that STAT3 is constitutively activated in T cells from a Crohn's patient [168]. Therefore, the effect presented in our study might be restricted to the macrophages population.

Finally, we investigated the role of hCTS using the DNBS-based model of experimental colitis to see whether the observed role of hCTS using the DSS model was specific to this model. DNBS-colitis considered as a model of CD, is characterized by transmural inflammation [349, 350]. Similarly, we observed significant attenuation in colonic inflammation in DNBS-treated mice.

It is conceivable that other factors contribute to the protective effect of the treatment in our study. For example, it is known that CgDPs can influence monocyte trafficking [262], and thus, it is possible that hCTS may target the macrophages and polymorphonuclear neutrophils in a quantitave manners, correlating with the significant decrease of MPO

activity described in our study. Conversely, the bovine CTS sequence stimulated rat mast cell histamine release [351]; however, we consider it is unlikely that these mechanisms made a significant contribution because mast cell activation portrays a pro-inflammatory effect not visible in our study. One potentially confounding factor is the antibacterial role of CTS [352]. Over the last 5 years, much attention has been given to the gut microbiota in relation to IBD development. It has been shown that a change in gut microbiota could affect the development of experimental colitis [353]; therefore intrarectal infusion of the peptides might induce a beneficial gut microbiota dysbiosis, which subsequently can affect the development of colitis.

The direct correlate of our findings and the exact role of the significant hCTS increase in IBD patients are not clear. However, due to its major implication in the development of in colorectal carcinoma and transitional mucosa[354], it is possible to speculate that hCTS may be released to prevent, the long-term progression of chronic intestinal inflammation to cancer [328]. Conversely, it needs to be highlighted, that p-STAT3 activation also plays an important role in the development of colitis-associated cancer [355], therefore, additional data using this experimental model are needed. Furthermore, studies of serial serum CHGA and CgDPs measurements, or targeting different immune cells (e.g. DC, CD4+ T cell, T-reg cells) will strengthen our understanding.

This study, taken together with others, offers potential new therapeutic approaches to the management of IBD. The present study reveals a novel function of hCTS in regulation of gut inflammation in relation to the activation of pro-inflammatory cytokine

production. In addition, this study demonstrated a p-STAT3 dependent molecular mechanism of hCTS-mediated activation of immune cells in the context of inflammation. Up-regulation of mCTS signalling in response to chemical stimuli like DSS or DNBS can take part in lowering gut inflammation by influencing immune cell activation, and by decreasing production of inflammatory mediators. In addition to enhancing our understanding of the pathogenesis of experimental colitis, this study provides novel data on CTS in the context of immunoendocrine interactions in the gut and in intestinal homeostasis.

Part 04 Chapter 04. Aim 3

4.0 Bridge to Chapter 4

The study in the previous chapter demonstrated an anti-inflammatory effect of CTS in the context of acute gut inflammation [317]. However, CTS is a well-known antimicrobial peptide, with various *in vitro* studies indicating that CTS is effective against gram-positive bacteria and gram-negative bacteria as well as against yeast and filamentous fungi [356, 357]. In addition, CHGA, the precursor of CTS has been reported to be a strong modulator of gut microbiota [358]. Despite this, no report until recently on the impact of CTS on gut microbiota *in vivo.*

DSS colitis was the choice of experimental colitis in this project as it is the most widely used colitis model [266]. Several reports suggested that gut microbial dysbiosis is indispensable during the development of gut inflammation [359, 360], however, the mechanism of action of DSS is not well understood yet and its impact on gut microbiota has not been studied. Furthermore, like the environmental impact on IBD outcome [361], the housing condition and surrounding confounding factors like diet, stress level, lab personnel can impact the colitis induced by DSS [362] by affecting the gut microbiota.

In the first part of this Chapter, we investigated the impact of CTS on gut microbiota of naive mice [318]. In the second part of this Chapter 04, we investigated the impact of DSS on gut microbiota which allowed us to address fair comparison with the previously published work [319].

4.1 Human Catestatin Alters Gut Microbiota Composition in Mice

This collaborative work was published in:

Frontiers in Microbiology, 2017 [318]

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***Contributorship*:**

MFR, PM, NE conducted the experiment and performed the analyses. The study was designed by JEG and MM. MM provided the peptide. MFR, EK, PM, NE, and JEG interpreted the data and wrote the manuscript.

***Conflict of interest*:**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

***Source of support*:**

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4.1.i. Abstract

The mammalian intestinal tract is heavily colonized with a dense, complex and diversified microbial populations. In healthy individuals, an array of epithelial antimicrobial agents is secreted in the gut to aid intestinal homeostasis. Enterochromaffin cells (EC) in the intestinal epithelium are a major source of chromograninA (CHGA), which is a pro-hormone and can be cleaved into many bioactive peptides that include catestatin (CTS). This study was carried out to evaluate the possible impact of CTS on gut microbiota *in vivo* using a mouse model. The CTS (Human $CHGA_{352–372}$) or normal saline was intrarectally administered in C57BL/6 male mice for 6 days and then sacrificed. Feces and colonic mucosa tissue samples were collected, DNA was extracted, the V4 region of bacterial 16S rRNA gene was amplified and subjected to MiSeq Illumina sequencing. The α-diversity was calculated using Chao 1 and β-diversity was determined using QIIME. Differences at the genus level were determined using partial least square discriminant analysis (PLS-DA). Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was used to predict functional capacity of bacterial community. CTS treatment did not modify bacterial richness in fecal and colonic mucosa-associated microbiota; however, treatment significantly modified bacterial community composition between the groups. Also, CTS-treated mice had a significantly lower relative abundance of Firmicutes and higher abundance of Bacteroidetes, observed only in fecal samples. However, at lower phylogenetic levels, PLS-DA analysis revealed that some bacterial taxa were significantly associated with the CTS-treated mice in both fecal and colonic mucosa-associated samples. In addition, differences in predicted microbial functional pathways in both fecal and colonic mucosa-

associated samples were detected. The results support the hypothesis that CTS treatment modulates gut microbiota composition under non-pathophysiological conditions. The data may open new avenues for the development of a potential new line of antimicrobial peptides and their use as therapeutic agents to treat several inflammatory conditions of the gastrointestinal tract, such as inflammatory bowel disease (IBD), inflammatory bowel syndrome (IBS) or other health conditions.

Keyword: gut microbiota, intestinal homeostasis, chromogranin A (CHGA), catestatin (CTS), antimicrobial peptides, microbial dysbiosis

4.1.1. Introduction

Over the last 15 years, bacterial multi-drug resistance (MDR) has emerged as a result of several socio-economical reasons, such as the use of surface antibacterial agents that are now available in many household products [363], antibiotic over-prescription, or failing to complete a course of antibiotics [363]. Although due to MDR new line of antibiotics are required, development of new antibiotics has been reduced by pharmaceutical companies because of the cost and complexity of clinical trials [364]. Currently, there are relatively few new antimicrobials in development.

The gastrointestinal tract is heavily colonized with an average of 10^{14} microbes that represent thousands of species, which is 10 times more than the total number of cells in the human body [63]. More than 90% of this bacterial populations belong to two major phyla: gram-negative Bacteriodetes and gram-positive Firmicutes [69, 70], with the remaining belong to low-abundance phyla such as Proteobacteria and Actinobacteria. Viruses, protists and fungi are also other members of gut microbiome [69, 70]. In healthy individuals and during certain age categories, microbial diversity in the intestine is stable over time and demonstrates a symbiotic relationship with the host [63], but a shift in microbial composition, referred to as dysbiosis, has been described in several of pathologies [359, 365, 366]. Gut microbiota helps to digest food items. Various metabolites produced by the resident microbiota play a significant role in host physiology, metabolism and immune function. For example, gut microbiota can activate toll-like receptors (TLRs) in the gut epithelium, which in turn can affect the expression of antimicrobial peptides such as angiogenins [367, 368]. In addition to the innate immune

system, gut microbiota can also control the host's adaptive immune system through T cell receptor αβ-positive intraepithelial lymphocytes, regulatory T cells and T helper 17 cells [69]. Overall, gut homeostasis is largely dependent on the proper balance and composition of gut microbiome [369].

At the mucosal level the epithelium plays a major role in limiting the passage/penetration of bacteria to the sub-mucosa from the gut lumen. Antimicrobial peptides (AMPs) secreted by epithelial cells have a broad spectrum effect against bacteria and are a part of an ancient defense mechanism that is present in virtually all mammals [370]. In the gastrointestinal (GI) tract, specialized intestinal epithelial cells or circulating inflammatory cells are a major source of these AMPs [370]. Within the epithelium, Paneth cells are the main producer of AMPs but new data indicate that enterochromaffin (EC) cells can hypothetically also produce certain types of AMPs [371].

EC cells are the major source of chromogranin A (CHGA) [372], a family of highly acidic proteins. The CHGA gene is localized at 14q32 in the human genome, consisting of 8 exons and 7 introns, and its 2-Kb transcript is translated into the 457-residue CHGA protein. The overall homology for CHGA in different vertebrates is ~ 40%, but the most highly conserved regions occur at the N- and C-termini, which show up to 88% sequence homology. Cell- and tissue-specific CHGA processing has been described in the rat, mouse and human GI tract [240-242]. The CHGA primary structure from its cDNA sequence shows the presence of numerous pairs of basic amino acids. These are potential sites for cleavage by prohormone convertases (PC) 1/3 or 2, and carboxypeptidase E/H

[243], which is consistent with evidence that CHGA may serve as a prohormone for shorter bioactive fragments [244]; this is also suggested by the high sequence conservation of CHGA-derived peptides. But in the gut, peptides can be highly sensitive to enzymes present in the luminal environment. Proteolytic fragments of CHGA-derived peptides exert a broad spectrum of regulatory activities on the cardiovascular, endocrine and immune systems. Among its highly conserved C-terminal regions, CHGA gives rise to a peptide of biological importance: the antihypertensive peptide catestatin (human CTS; $CHGA_{352-372}$) [247-249], which has restricted antimicrobial activity against *Staphylococcus aureus in vitro* [250]. Similar to other AMPs, CTS can interact with anionic components of fungi and bacteria. As a result, the microbial membrane is permeabilized, leading to cell lysis [356]. *In vitro* studies have demonstrated that CTS is effective against gram-positive bacteria such as *S. aureus* and group A *Streptococcus*, gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, yeasts such as *Candida albicans* and filamentous fungi such as *Aspergillus niger*, *Aspergillus fumigatus* and *Trichophyton rubrum* [356, 357]. However, to date, there has been no indication that the *in vitro* data can be reproduced using an *in vivo* model and there is no indication about the type of microbiota affected, as the colonic mucosa-associated population differs completely for the population present in the feces [373].

Despite the effects between the CTS and *S. aureus*, *E. coli* and *P. aeruginosa in vitro*, the effects of *in vivo* CTS treatment on the different type of gut microbiota are unknown. Our aim was to assess the composition of fecal and colonic mucosa-associated microbiota and functional alterations in mice that were exposed to CTS for 6 days.

4.1.2 Materials and Methods

4.1.2.1. Animals

Male C57BL/6 mice (7–9 weeks old) were purchased from Charles River (Canada) and maintained in the animal care facility at the University of Manitoba. The experimental protocol was approved by the University of Manitoba Animal Ethics Committee (15-010) and the research was conducted according to the Canadian Guidelines for Animal Research [374, 375]. Two groups of four and eight mice were studied, one receiving the vehicle solution and one receiving intra-rectal (i.r.) injection of CTS for 6 days. By using mice from the same sex, source, age, and keeping them in co-housed conditions while receiving the same food, the environmental effects on gut microbiota were minimized.

4.1.2.2. Peptide

The CTS (Human $CHGA_{352–372}$: SSMKLSFRARAYGFRGPGPQL) [248] was used (Biopeptide Co., Inc, San Diego, CA, USA), and the peptide was injected (i.r.) at 1.5 mg/per kg body weight per day for 6 days. Saline (0.9%) was injected in the control group. Mice were anaesthetized using Isoflurane (Abbott, Toronto, ON, Canada). PE-90 tubing (10 cm long; ClayAdam, Parisppany, NJ, USA), which was attached to a tuberculin syringe (BD, Mississauga, ON, Canada), was inserted 3.5 cm into the colon. The dose was determined according to our previous published study [317].

4.1.2.3. *Assessment of physiological condition*

Weight loss, stool consistency and bleeding were assessed daily to determine any possible physical changes in the mice as a result of CTS treatment [336] Scores were defined as follows: weight: 0, no loss; 1, 5–10%; 2, 10–15%; 3, 15–20%; and 4, 20% weight loss; stool: 0, normal; 2, loose stool; and 4, diarrhea; 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).

4.1.2.4. Fecal and tissue sample collection

Samples were collected 6 days post-treatment induction, after euthanasia under isoflurane (Abbot) anaesthesia. The macroscopic score was determined on the sacrifice day based on stool consistency, rectal prolapse and rectal and colonic bleeding. On the day of sacrifice, the colon was opened and approximately a 250 mg fecal sample was collected near the rectal opening. In addition, a portion of the colon tissue was collected within 5 cm from rectal opening. Approximately 50 mg of mucosa scrapings were collected from these colon tissue. All samples were collected in individual collector tubes from each animal and snap frozen in liquid nitrogen and preserved at -80°C until use.

4.1.2.5. DNA extraction and quality check

Samples were homogenized at room temperature, and genomic DNA was extracted using a ZR Tissue and Insect DNA extraction Kit (Zymo Research Corp., Orange, CA, USA). Fecal DNA extraction was performed using a ZR fecal DNA extraction kit (Zymo Research Corp., Orange, CA). Both DNA extraction kits have a bead-beating step to mechanically lyse microbial cells. DNA was quantified using a Nanodrop 2000

spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA samples were normalized to achieve a concentration of 20 ng/µl, and quality-checked by PCR amplification of 16S rRNA gene using primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') [376, 377]. Amplicons were verified by agarose gel electrophoresis.

4.1.2.6. Library construction and Illumina sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. [378] Briefly, the V4 region of the 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers [379, 380]. A reverse PCR primer was indexed with 12-base Golay barcodes to allow for sample multiplexing. The PCR reaction for each sample was performed in duplicate and contained 1.0 µl of pre-normalized DNA, 1.0 µl each of 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 followed by an extension step at 72°C for 10 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). PCR products were then purified using a ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample, and quantified using Picogreen dsDNA (Invitrogen, Carlsbad, CA, 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, and the concentration was measured through optical density using a 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 (5'-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3'), read2 (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3') and index read (5'-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3') were synthesized and purified using polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, 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, 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.

4.1.2.7. Bioinformatics analyses

Bioinformatics analyses were performed as described previously [378]. Briefly, the PANDAseq assembler [381] 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 using downstream computational pipelines in the open source software package QIIME [382]. Chimeric reads were filtered using UCHIME [383] and sequences were assigned to operational taxonomic units

(OTU) using the QIIME implementation of UCLUST[384] at the 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using an RDP classifier [385] and aligned with the Greengenes (v. 13.5) core reference database [386] using PyNAST algorithms [387]. The phylogenetic tree was built with FastTree 2.1.3 [388]for additional comparisons between microbial communities.

4.1.2.8 Alpha (α)- and beta (β)-diversity analyses

Within-community diversity (α-diversity) was calculated using QIIME and differences between control and CTS groups were determined using SAS (SAS 9.3). An α rarefaction curve was generated using a Chao 1 estimator of species richness [389] with 10 sampling repetitions at each sampling depth. An even depth of ~ 15,700 sequences per sample was used for calculation of richness and diversity indices. To compare microbial composition between samples, β-diversity was measured by calculating the weighted and unweighted UniFrac distances [390] using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots using PRIMER v6 software [391]. Permutational multivariate analysis of variance of Bray-Curtis distance (PERMANOVA) [392] was used to calculate *P*-values and test for significant differences in β-diversity among treatment groups.

4.1.2.9. *Partial least square discriminant analysis*

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on the genus data to identify the effects of treatments [378, 393]. The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing categories of variables on X. In this case, X

variables were the bacterial genera and the Y variables were observations of different treatment groups compared together. To avoid over-parameterization of the model, the variable influence on the projection (VIP) value was estimated for each genus, and genera with VIP < 0.50 were removed from the final model [394, 395]. R^2 estimate then was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristic of each treatment group and the results were visualized by PLS-DA loading scatter plots.

4.1.2.10 Metagenomic functional prediction

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 [396]. 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 [396]. The KEGG data were analyzed using STAMP (STatistical Analysis of Metage- nomic Profiles) [397].

4.1.2.11 Other statistical analysis

The SAS UNIVARIATE procedure (SAS 9.3) was used to test the normality of residuals for α biodiversity data. Non-normally distributed data were log transformed and then used to assess the effect of sampling using the SAS MIXED procedure. The SAS MIXED procedure was used, as described above, to test for significant changes in the proportions of different phyla among the groups of interest. Differences between groups were considered significant at $P< 0.05$ while trends were observed at $P< 0.1$.

4.1.3. Results

4.1.3.1. Descriptive analysis

Over the 6 days of treatment, daily intrarectal infusion of the peptide did not show any effect on the weight, stool consistency and presence of blood in the feces. The data are consistent with our previous findings [317] (data not shown).

4.13.2. Sample assessment by Illumina sequencing

After sacrifice, we collected fecal and colon samples from a total of 12 mice. Among these, the control group received normal saline intrarectally and the others received CTS i.r. (1.5 mg per kg body weight for 6 days). During the DNA extraction process, one colonic mucosa sample from the saline-treated group was discarded because of poor quality or purity, resulting in a total of 12 useable fecal samples and 11 useable colonic mucosa-associated microbiota samples for Illumina sequencing. For fecal samples, a total of 328,085 sequences were generated. After quality-filtering steps, an average of 27,340 high quality sequences per sample was obtained. For colonic mucosa samples, a total of 207,123 sequences were generated. After quality-filtering steps, an average of 18,829 high-quality sequences per sample was obtained.

4.1.3.3. CTS exposure did not significantly influence α-diversity in fecal and colonic mucosa samples in mice

Bacterial richness and diversity from both fecal and colonic mucosa samples between control and CTS-treated groups were calculated. However, no significant differences were observed in both fecal and colon samples (data not shown).

4.1.3.4. CTS treatment significantly influenced β-diversity in fecal samples but not in colonic mucosa samples in mice

Bacterial communities from fecal samples of CTS-treated mice clustered separately ($P < 0.05$) from controls suggesting that the treatment modified the fecal bacterial profile (**Figure 4.1-1)**. However, there was no significant change in the bacterial community composition in colonic samples in CTS-treated mice compared to controls (**Figure 4.1-2).**

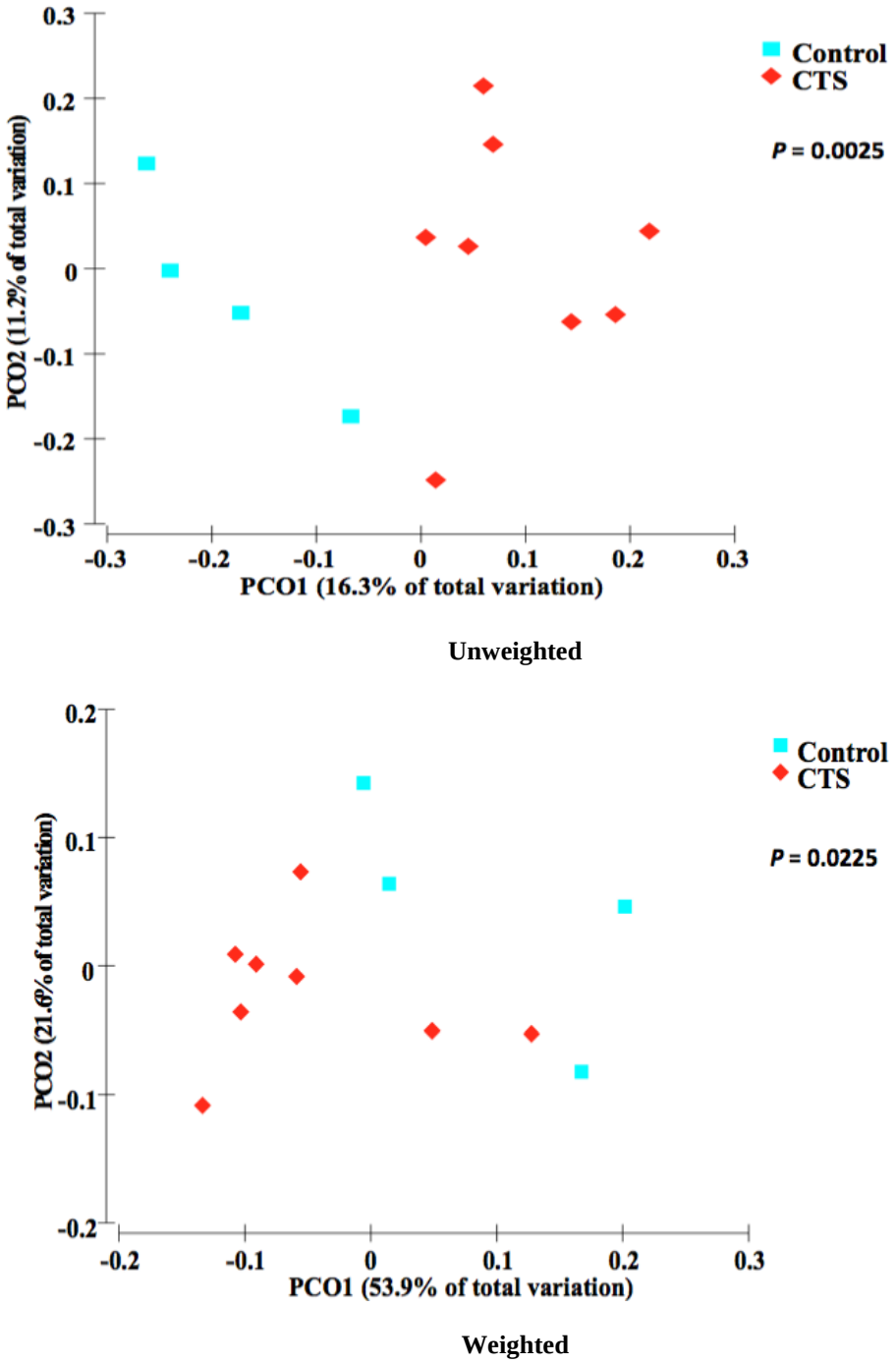


Figure 4.1-1. PCoA based on the unweighted and weighted UniFrac distance metric. Each colored point represents a fecal sample obtained from one mice and it is colored according to different treatment (CTS or Control). P-values were calculated using PERMANOVA. Samples clustered according to treatment status of the mice (P values < 0.05).

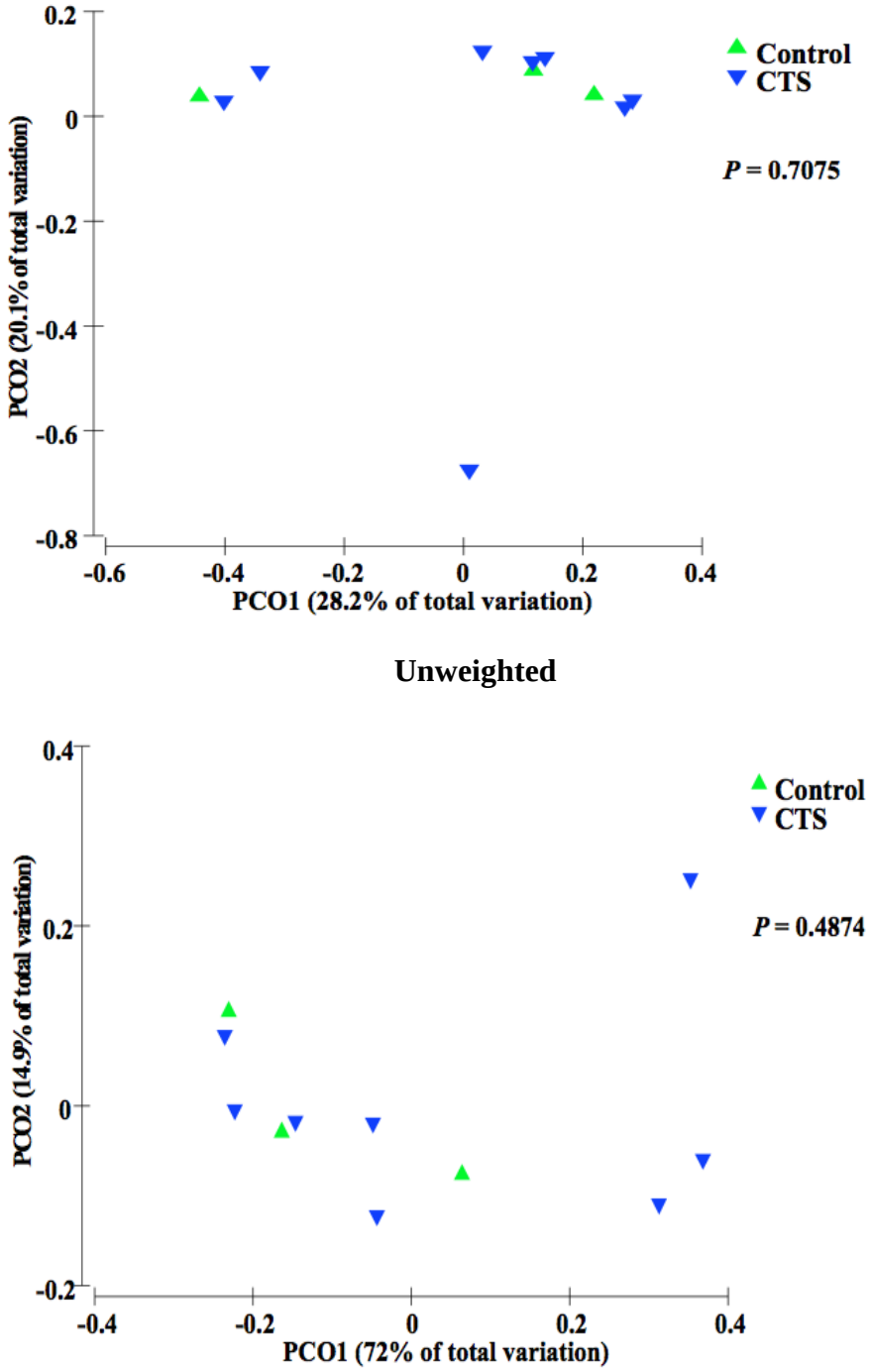


Figure 4.1-2. PCoA based on the unweighted and weighted UniFrac distance metric. Each colored point represents a colonic mucosa-associated sample obtained from one mice and it is colored according to different treatment (CTS or Control). P values were calculated using PERMANOVA. Samples did not cluster according to treatment status of the mice (P values > 0.05)

4.1.3.5. CTS treatment influenced fecal but not colonic mucos- associated bacterial community composition at the phylum level in mice

In the fecal samples, a total of 10 phyla were identified, of which 4 phyla were considered to be abundant within the community (≥ 1%); these included Firmicutes, Bacteroidetes, Proteobacteria and Deferribacteres. The other six phyla were in low abundance within the community (< 1%) and included Actinobacteria, Cyanobacteria, Fibrobacteres, TM7, Tenericutes and Verrucomicrobia (**Table 4.1-1**). Among the four abundant phyla, CTS treatment increased the relative abundance of Bacteroidetes ($P < 0.05$) and decreased the Firmicutes population ($P < 0.001$) in the feces (**Figure 4.1-3**).

In the colonic mucosa samples, a total of 19 phyla were identified, of which 4 phyla were considered to be abundant within the community; these included Firmicutes, Bacteroidetes, Proteobacteria and Deferribacteres. The other 15 phyla were in low abundance within the community, and included Acidobacteria, Actinobacteria, Armatimonadetes, Chlamydiae, Chlorobi, Cyanobacteria, Fibrobacteres, Lentisphaerae, OD1, OP3, Planctomycetes, Spirochaetes, TM7, Tenericutes and Verrucomicrobia (**Table 4.1-2**). CTS treatment had no significant impact on the relative abundance of bacterial phyla (**Figure 4.1-4**).

Table 4.1-1. Relative abundance of bacterial phyla in fecal samples.

Phylum	Groups	Mean percentage of sequence in total bacterial community	SEM
Unclassified	Control	0.496921	0.092571
	CTS	0.414249	0.04264
Actinobacteria	Control	0.511464	0.202226
	CTS	0.187706	0.09371
Bacteroidetes	Control	59.59139	4.253899
	CTS	73.99889	2.680188
Cyanobacteria	Control	0.017379	0.006038
	CTS	0.152001	0.041196
Deferribacteres	Control	1.709693	1.021632
	CTS	1.143747	0.782717
Fibrobacteres	Control	0.001941	0.00115
	CTS	0.000541	0.000541
Firmicutes	Control	33.80289	3.56062
	CTS	20.55645	1.827242
Proteobacteria	Control	2.218421	0.409325
	CTS	2.872719	0.488761
TM7	Control	0.003883	0.002301
	CTS	0.003098	0.001035
Tenericutes	Control	0.314479	0.089961
	CTS	0.404554	0.187125
Verrucomicrobia	Control	1.331539	0.730169
	CTS	0.266048	0.129101

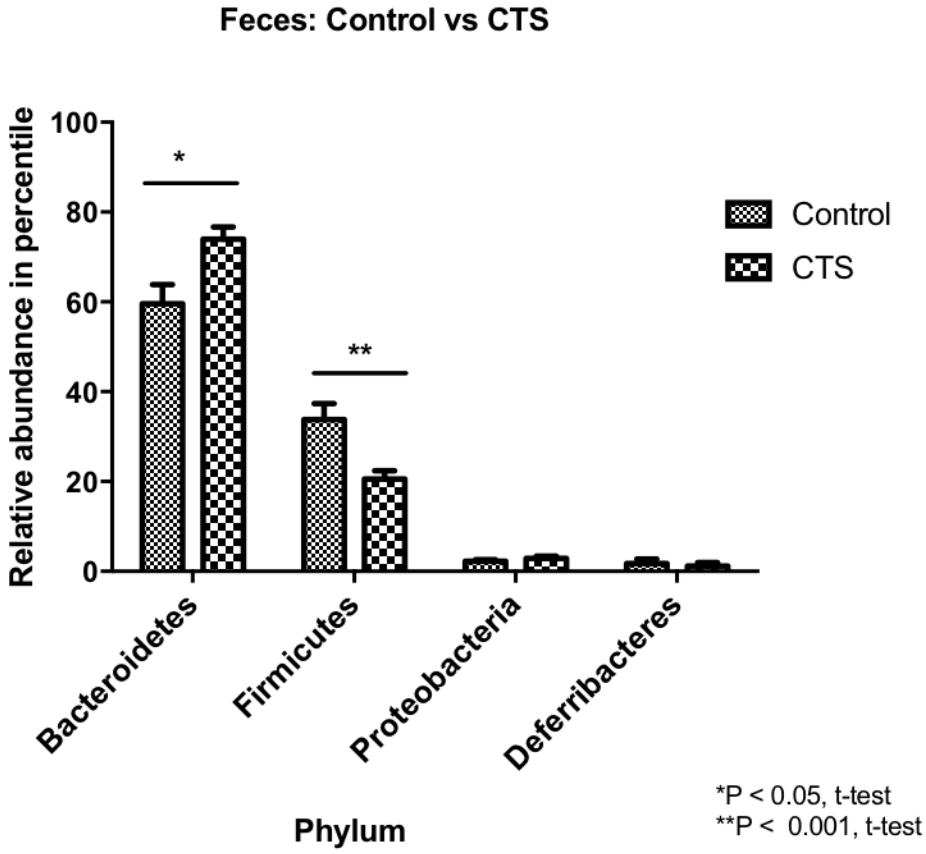


Figure 4.1- 3. Effect of CTS treatment on the abundant phyla (≥1%) present in the fecal samples.

After quality filtering steps, 10 bacterial phyla were identified in fecal samples. Among these, four phyla were consideredabundant within the community (≥1%), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. CTS treated mice had higher proportion ($P < 0.05$, t-test) of Bacteroidetes and lower abundance ($P < 0.01$, t-test) of Firmicutes in the feces compared to control animals.

Table 4.1-2. Relative abundance of bacterial phyla in colonic mucosa samples.

Phylum	Groups	Mean percentage of sequence in total bacterial population	SEM
Unclassified	Control	0.122989	0.050167
	CTS	0.291527	0.19307
Acidobacteria	Control	0.025735	0.010402
	CTS	0.033388	0.00685
Actinobacteria	Control	0.095085	0.095085
	CTS	0.072405	0.031928
Armatimonadetes	Control	0	0
	CTS	0.000879	0.000879
Bacteroidetes	Control	8.06612	5.42359
	CTS	21.0435	6.556034
Chlamydiae	Control	0	0
	CTS	0.00306	0.002007
Chlorobi	Control	0	0
	CTS	0.002098	0.002098
Cyanobacteria	Control	0.039247	0.022309
	CTS	0.175241	0.063441
Deferribacteres	Control	6.923494	3.656936
	CTS	5.949701	2.168418
Fibrobacteres	Control	0	0
	CTS	0.013779	0.012973
Firmicutes	Control	10.13424	6.855156

	CTS	12.51866	5.161134
Lentisphaerae	Control	0	0
	CTS	0.00457	0.00457
OD1	Control	0	0
	CTS	0.004988	0.00332
OP3	Control	0	0
	CTS	0.001604	0.001604
Planctomycetes	Control	0.002067	0.002067
	CTS	0.004664	0.001662
Proteobacteria	Control	72.02106	16.32041
	CTS	59.16764	11.01085
Spirochaetes	Control	0	0
	CTS	0.082258	0.082258
TM7	Control	0	0
	CTS	0.007409	0.00482
Tenericutes	Control	0.037096	0.019253
	CTS	0.371448	0.170574
Verrucomicrobia	Control	2.532872	2.52412
	CTS	0.142801	0.117295

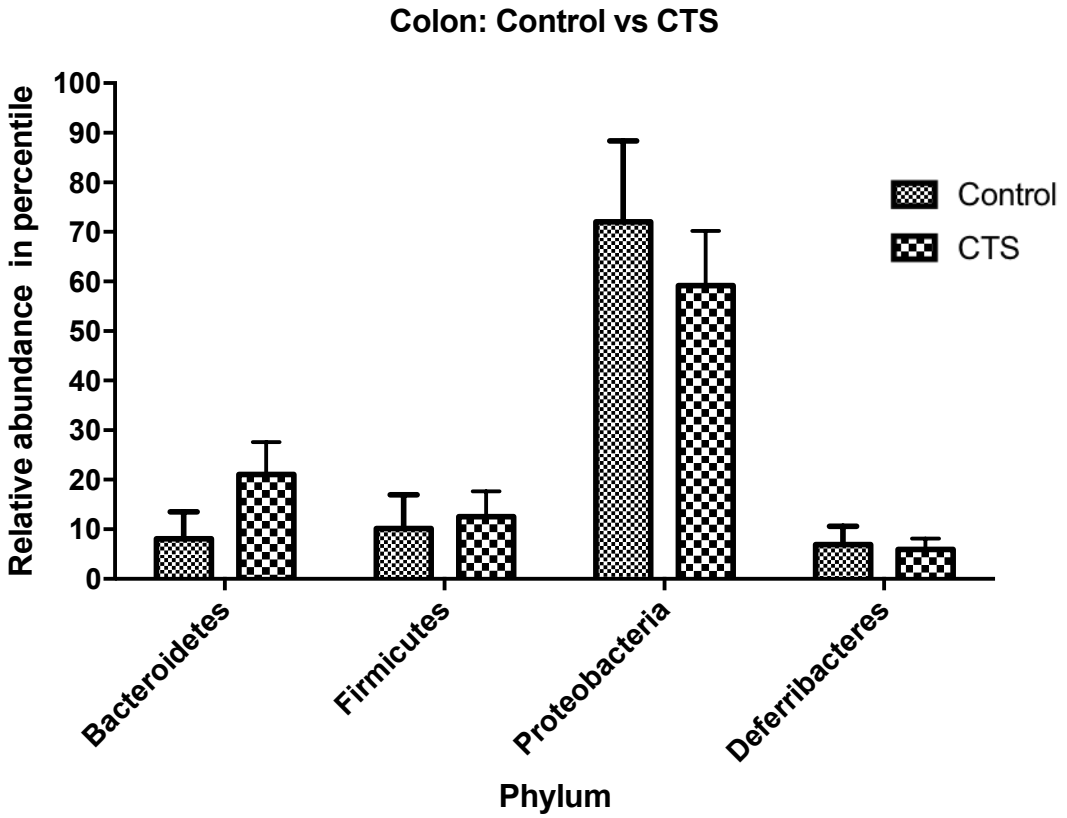
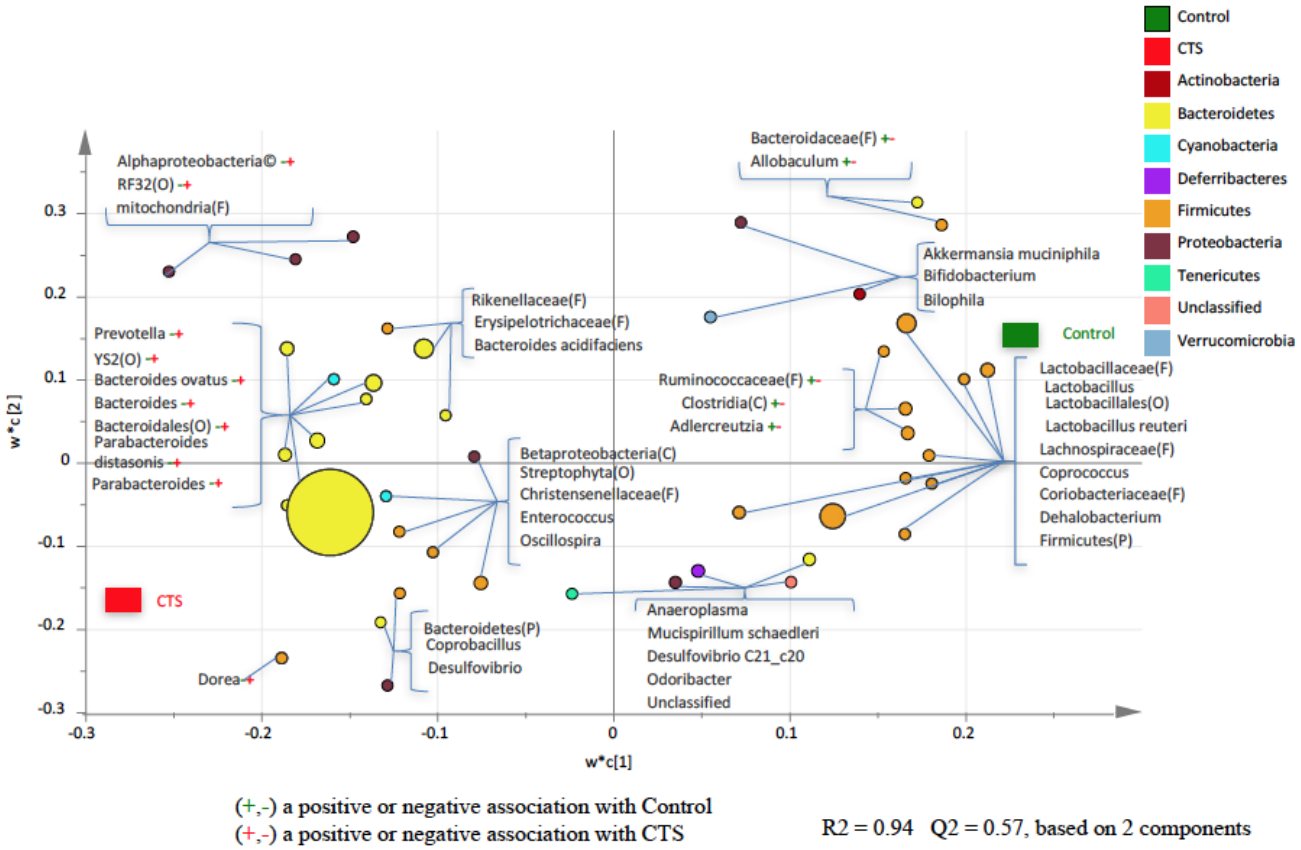


Figure 4.1-4. Effect of CTS treatment on the abundant phyla (≥1%) present in the colonic mucosa-associated samples.
After quality filtering steps, 19 bacterial phyla were identified in colonic mucosa samples. Among these, four phyla were considered abundant within the community (≥1%), including Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres. CTS treatment did not change the abundance of these phyla in colonic mucosa samples.

4.1.3.6. CTS treatment influenced fecal bacterial community composition at lower phylogenetic levels in mice

A total of 86 bacterial taxa were identified. While majority of taxa were classified at the genus or species levels, some were only classified at the phylum (P), class (C), rrder (O), or family (F) levels. Of the 86 taxa, 54 taxa were considered abundant within the community, while 32 were in low abundance. Results of the relative abundance of various genera with percentages of sequences ≥ 0.01% of community were analyzed using PLS-DA to identify bacteria that were most characteristic of CTS or Control treatments. The PLS-DA analysis showed that the genera *Prevotella, Bacteroides, Ovatus, Parabacteroidesdistarosis, Parabacteroides* and *Dorea* were positively associated with the CTS treatment in the fecal samples (R^2 = 0.94, Q^2 = 0.57) (**Figure 4.1-5)**. In addition, members of Alpharoteobacteria (Class), Bacteroidales (Order), RF32 (Order), and YS2 (Order) also showed a positive association with CTS treatment in the fecal samples (R^2 = 0.94, Q^2 = 0.57). A negative association with the members of *Adlercreutzia*, *Allobaculum*, Bacteroidaceae(Family), Clostridia (Class) and Ruminococcaceae (Family) were evident in the fecal samples collected from CTS-treated mice (R^2 = 0.94, Q^2 = 0.57).

Feces: Control vs CTS



(+,-) a positive or negative association with Control
(+,-) a positive or negative association with CTS

R2 = 0.94 Q2 = 0.57, based on 2 components

Figure 4.1-5. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the Control or CTS treatments in the mice fecal samples.

All taxa are colored based on the phyla to which they belong. Some sequences could only be affiliated to phylum (P), order (O), family (F), or class (C) levels. Specific taxa were significantly associated with each treatment group, which may be an indicator of an alteration in the physiological or metabolic processes that the taxa may influence.

4.13.7. CTS treatment influenced colonic mucosa-associated bacterial community composition at lower taxonomical levels in mice

A total of 179 taxa were identified. Of these 84 taxa were considered abundant within the community, while 95 taxa were in low abundance. The relative abundance of various genera/taxa with sequence percentages ≥ 0.01% of community were analyzed using PLS-DA to identify bacteria that were most characteristic of the CTS or control treatments. The PLS-DA analysis of the colonic mucosa samples showed that genera *Bifidobacterium* and *Stenotrophomonas* had a positive association with the CTS treatment (R^2 = 0.32, Q^2 = 0.242) (**Figure 4.1-6**). Members of Bacteroidales (Order), Chitinophagaceae (Family), Clostridiaceae (Family), Clostridiales (Order), Coriobacteriaceae (Family), Pseudomonadaceae (Family), Rikenellaceae (Family), Ruminococcaceae (Family) and YS2 (Order) also showed a positive association with the CTS treatment in the colonic mucosa samples (R^2 = 0.32, Q^2 = 0.242) (**Figure 4.1-6**).

Colon: Control vs CTS

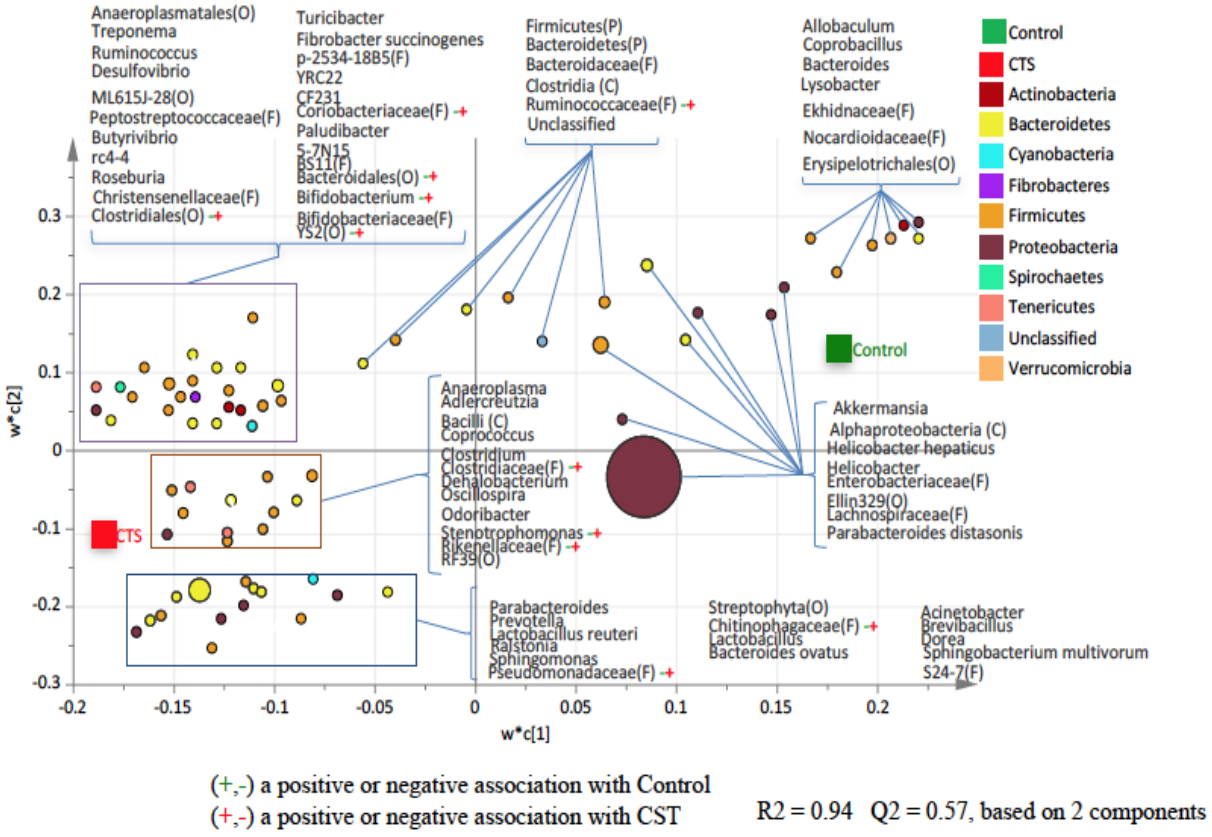


Figure 4.1-6. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the Control or CTS treatments in the mice colonic mucosa-associated samples.
All taxa are colored based on the phyla to which they belong. Some sequences could only be affiliated to phylum (P), order (O), family (F), or class (C) levels. Specific taxa were significantly associated with each treatment group, which may be an indicator of an alteration in the physiological or metabolic processes that the taxa may influence.

4.1.3.8. CTS treatment significantly influenced the predicted functional and metabolic pathways of fecal and colonic mucosa-associated microbiota in mice

To determine the functional KEGG pathways that could be associated with the observed microbial changes, we compared the functional pathways for the microbiota in fecal and colonic mucosa samples from the CTS-treated group with those of the control mice. Several metabolic pathways were determined. Subsystems or pathways that have a significant positive or negative correlation with CTS treatment are shown in **Figures 4.1-7** and **8**. In the fecal samples from CTS-treated mice, chlorocyclohexane and chlorobenzene degradation were underrepresented (P=0.015) **(Figure 4.1-7)**. However, nitrogen metabolism was enriched in the fecal samples from CTS-treated mice (P=0.033; **Figure 4.1-7**). In the colonic mucosa samples from CTS treated mice, nicotinate and nicotinamide metabolism, cell division and ribosome biogenesis were enriched compared to the controls (P< 0.05; **Figure 4.1-8**).

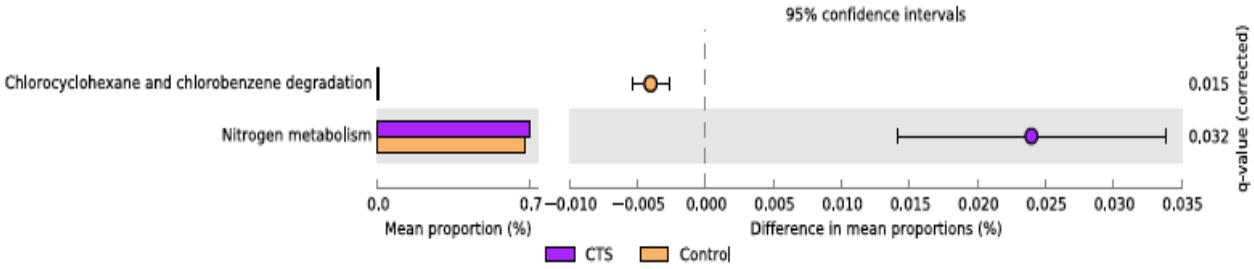


Figure 4.1-7. Subsystems and pathways enriched or decreased within the Catestatin (CTS) or (Control) mice fecal samples.
Corrected *P*-values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented in the CTS or Control mice fecal samples have a positive or (negative) difference between mean proportions and are indicated by purple or (orange) coloring, respectively.

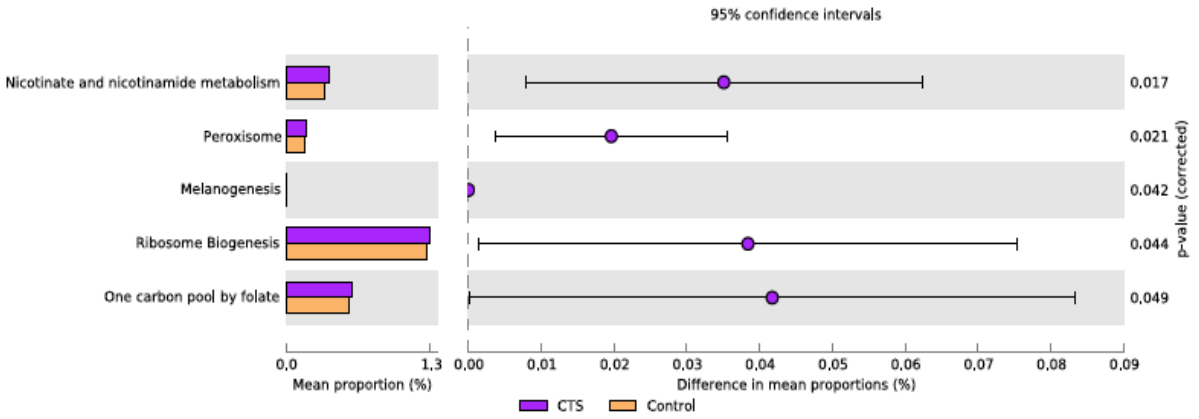


Figure 4.1-8. Subsystems and pathways enriched or decreased within the Catestatin (CTS) or (Control) mice colonic mucosa-associated samples.
Subsystems or pathways overrepresented in the CTS or Control mice colon samples have a positive or (negative) difference between mean proportions and are indicated by Purple or (orange) coloring, respectively.

4.1.4. Discussion

The mammalian intestine continuously encounters more microorganisms than any other tissue, and survival of the mammals largely depends on their unique adaption in the world of microorganisms. Specific intestinal epithelial cells release several antimicrobial peptides, which are critical for maintaining a stable ecological environment that favors commensal and targeting pathological microorganisms [398]. Moreover, these are also important for inhibiting ongoing inflammatory responses. The CTS, a highly conserved CHGA peptide that is present in intestinal EC cells, has been described as a peptide with some immunomodulatory activities during acute experimental colitis [317] and restricted *in vitro* antibacterial activities [352], but also antifungal and antiviral activity [356, 357]. Moreover, in a recent report, it has been observed that the CHGA, the precursor of CTS strongly regulates human gut microbiome [358]. However, there are no documented studies demonstrating the effect of CTS on gut microbiota using *in vivo* models. Here, we show that i.r. infusion of CTS modulates gut microbiota composition under physiological conditions.

Based on the α-diversity, we observed that bacterial richness and diversity in both fecal and colonic mucosa samples did not change after CTS administration. However, β-diversity analysis revealed that CTS-treated mice had a fecal microbial composition that was different from the control group ($P<0.05$, both weighted and unweighted) suggesting that a short-term exposure of this peptide in the gut might change the bacterial composition profile. This is in accordance with studies demonstrating the effect of other antibacterial peptides released by Paneth cells (*i.e.* defensin) on gut microbiota [399].

Recent investigation shows that intestinal inflammatory conditions such as inflammatory bowel disease (IBD) or inflammatory bowel syndrome (IBS) are associated with altered intestinal homeostasis [359, 360]. Although microbial dysbiosis has been suggested to be a cause of intestinal pathophysiological conditions, this is still controversial, however, gut dysbiosis can take part of the entire process. In parallel, in human and animal models of IBS, it has been observed that microbial diversity is significantly altered [359, 360]. In the context of IBS, although an exact causal microbe has not yet been identified, a reduction in the microbial diversity has been documented and this temporal gut microbiota instability can result in altered host physiology, resulting in heterogeneous symptoms such as those observed in IBS patients [359, 360]. At the phylum level, IBS patients have a relative higher abundance of Firmicutes and lower abundance of Bacteroidetes [359]. In our study, we observed that CTS treatment is significantly associated with a relative reduction of Firmicutes in the feces compared with saline-treated mice. Conversely, CTS treatment was associated with a significant relative increased abundance of Bacteroidetes in the feces compared with saline-treated mice. Beside IBS, also in colitic conditions, studies have demonstrated a relative reduction in Bacteroidetes proportion [400].In a recent article, we also observed that acute dextran sulfate sodium induced colitic mice have a lower relative abundance of Bacteroidetes in their fecal samples compared to control [319]. In addition to colonic pathologies, studies demonstrated that Firmicutes are significantly more abundant relative to Bacteroidetes in obese mice compared to lean mice [366]; these results were also observed in humans [366]. In our study, CTS treatment was associated with a significant abundance of Bacteroidetes relative to Firmicutes in fecal samples, which was opposite to results from

obese animals and humans. However, these changes in Bacteroidetes and Firmicutes abundance were not observed in the colonic mucosa samples suggesting that prolonged administration of this peptide might be required to observe a possible change in the colonic wall. Overall, this study for the first time showed the *in vivo* effect of CTS on murine gut microbiota which was not predictable from the *in vitro* effect of CTS on *S. aureus, E. coli* [356, 357]. As gut microbiota is complex and composed of many bacteria which might not cultivable yet *in vitro,* their relative abundance *in vivo* can be captured through high-throughput illumina sequencing.

The CTS treatment also caused microbial alterations at lower taxonomic levels. We observed that certain bacterial taxa were positively associated with CTS treatment in both fecal and colonic mucosa samples. Among these taxa, genera *Bacteroides* and *Parabacteroides* showed a positive association with CTS treatment in the fecal samples. Both of these belong to the Bacteroidales order, which also showed a positive association with CTS treatment in the colonic mucosa samples. *Bacteroides* and *Parabacteroides* spp. represent approximately 25% of the colonic microbiota and are commensal to the host when present in the gut [401], and these anaerobic rods can transform simple and complex sugars into volatile fatty acids, which can be absorbed by the large intestine as a nutrient. *Bacteroides thetaiotaomicron* has several starch-binding genes and can produce significant amount of glycosylhydrolases, which can be crucial to prevent obesity [402]. This might explain why *Bacteroidetes* are more abundant in lean mice compared to obese mice. Beside the enormous starch-utilizing capacity, *Bacteroides* spp. are important for developing gut immunity. For example, *B. thetaiotaomicron* can stimulate Paneth cells to

produce Paneth cell protein (Ang4), which is lethal to certain pathogenic microorganisms (e.g. *Listeria monocytogenes*) [403]. In addition, *Bacteroides fragilis* produces zwitter ionic polysaccharide (ZPS), which is important for developing CD4 T cells. ZPS-activated CD4 T cells produce interleukin-10 (IL-10), which is essential to prevent abscess formation and other unchecked inflammatory responses [402, 404, 405]. Increased *Bacteroides* abundance in mice in response to CTS exposure might be beneficial to control obesity and inflammatory conditions such as IBD or IBS. These results might also explain the underlying mechanisms for improving gut inflammation that we observed previously in colitic mice exposed to CTS [317].

Finally, our metagenomic prediction analysis helped to understand the functionally of microbiota in the given environment [406]. Using this approach, we observed that certain subsystems or pathways were enriched after CTS treatment suggesting that, although, we were not able to see any effect for some markers studied after 6 days, treatment with CTS modified specific functional activities of the microbiota. In this context, CTS treatment induced functional alterations in the murine intestinal microbiota, with some metabolic pathways enriched in the mucosal microbiota of fecal and colonic mucosa samples compared to the control mice. However, since this was prediction and the specific changes observed might not directly influence the host's metabolic capacity, further studies might shed more light on this topic. In conclusion, our findings provide new insight into gut microbiota modulation by CTS. We observed an alteration in the microbial profile in response to CTS treatment, which was more prominent in the feces than in colonic mucosa-associated bacterial community. This is an observation based on a

small number of samples, and the result of this study needs to be further validated in a larger experiment associated to a paired analysis of feces. Our results suggest new avenues for the development of a potential new antimicrobial peptide, which could be used as a therapeutic agent to treat several gastrointestinal conditions such as IBD, IBS, however, further studies are warrant.

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

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MFR performed majority of the experiments like animal handling, colitis induction, clinical scoring, sacrifice, sample collection, inflammation assessment, DNA extraction and preparation for illumina sequencing. Sequencing was carried out in EK's lab and data was analyzed by MFR and PM. MFR and PM wrote the first draft of manuscript and was reviewed by EK and JEG.

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***Conflict of interest*:**

The authors have declared no conflict of interest.

4.2.i. 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. Conclusions: The development of colitis in DSS model was accompanied with reduced microbial diversity and dysbiosis of gut microbiota at lower taxonomical levels.

Keywords: Colitis, Dextran sulfate sodium, Dysbiosis, Mice, Microbiota

4.2.1. 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 [103, 407, 408]. 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) [409], defective mucosal barrier [410, 411], altered innate and adaptive immune responses and their interactions with commensal gut microbiota in the enteric environment [409, 412, 413], 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 [265, 278, 414]. 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 [415]. Most of these models require exogenous manipulation based either on chemical induction, immune cell transfer or gene targeting [265, 278, 414, 416] , and only a few models occur spontaneously without any exogenous manipulation [283, 416, 417]. 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 [418] . 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 [418, 419]or via intragastric catheter [420, 421]. The DSS induces a reproducible acute colitis [418]; 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 [267, 278, 418]. However, the model induces an acute colitis that is characterized by ulceration and infiltration, and also reflects many of the clinical features of IBD [267, 278, 422, 423] . 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 [317, 424-427] .

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 [267, 278, 422, 423]. 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 [428]. 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 [428]. On the other hand, notable shifts in gut microbiota composition (dysbiosis) have been highlighted in IBD patients at different stages of the disease [119, 429-431]. 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 [400, 432-440]. In this context, DSS treatment has been associated with changes in the composition of gut microbiota, whose dynamics shift toward an unhealthy state [400, 432-435, 437, 439]. The nature of gut microbiota was also reported to influence sensitivity to acute DSS-induced colitis independently of host genotype [38]. In addition, interdependence of the mucosa-associated bacteria and chronic inflammation has also been reported [440]. 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) [400, 433, 434, 436, 437, 440, 441], or sequencing of few clones per animal [400, 442], 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 [432, 435]. 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 [396]) 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.

4.2.2 Materials and methods

4.2.2.1. 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 [443].

4.2.2.2. 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.

4.2.2.3. 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 [336]. 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, diarrhea; 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 days 0 to 5 over the period of DSS treatment.

4.2.2.4. Macroscopic scores

Mice were euthanized on day 6, at the end of d5 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[336].

4.2.2.5. 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) anesthesia. 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 µl of Tris–HCl buffer containing protease inhibitors (Sigma, Mississauga, ON, Canada) and centrifuged for 12 min at 10,000 × 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).

4.2.2.6.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.). 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. [377]. Amplicons were verified by agarose gel electrophoresis.

4.2.2.7. Library construction and Illumina sequencing

The V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers [379] as described previously [444] 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 s, 50 °C for 60 s, and 72 °C for 90 s; 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) 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). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina) 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.uml.idm.oclc.org/sra) and are accessible through accession number SRR2830596.

4.2.2.8. Bioinformatic analyses

The PANDAseq assembler [445] 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) [446] . Chimeric reads were filtered using UCHIME [447] and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST [448] at 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier[449] and aligned with the Greengenes (v. 13.5) Core reference database [450] using PyNAST algorithms [451]. Phylogenetic tree was built with FastTree 2.1.3. [452] for further comparisons between microbial communities.

4.2.2.9. 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 [453]. 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 [454]. 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.

4.2.2.10. 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 [455], 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. R2 estimates then were used to evaluate the goodness of fit and Q2 estimate was used to evaluate the predictive value of the model. Data were 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.

4.2.2.11. 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 [396]. 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 [396]. The KEGG data were analyzed using STAMP (STatistical Analysis of Metagenomic Profiles) [397].

4.2.2.12. 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 were 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 $p < 0.05$.

4.2.3. Results

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

As shown in **Figure 4.2-1A to 4.2-1C**, 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 whereas 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 ($p < 0.0001$; **Figure 4.2-1C–4.2-1F**). These results confirmed the presence of colitis in our DSS model.

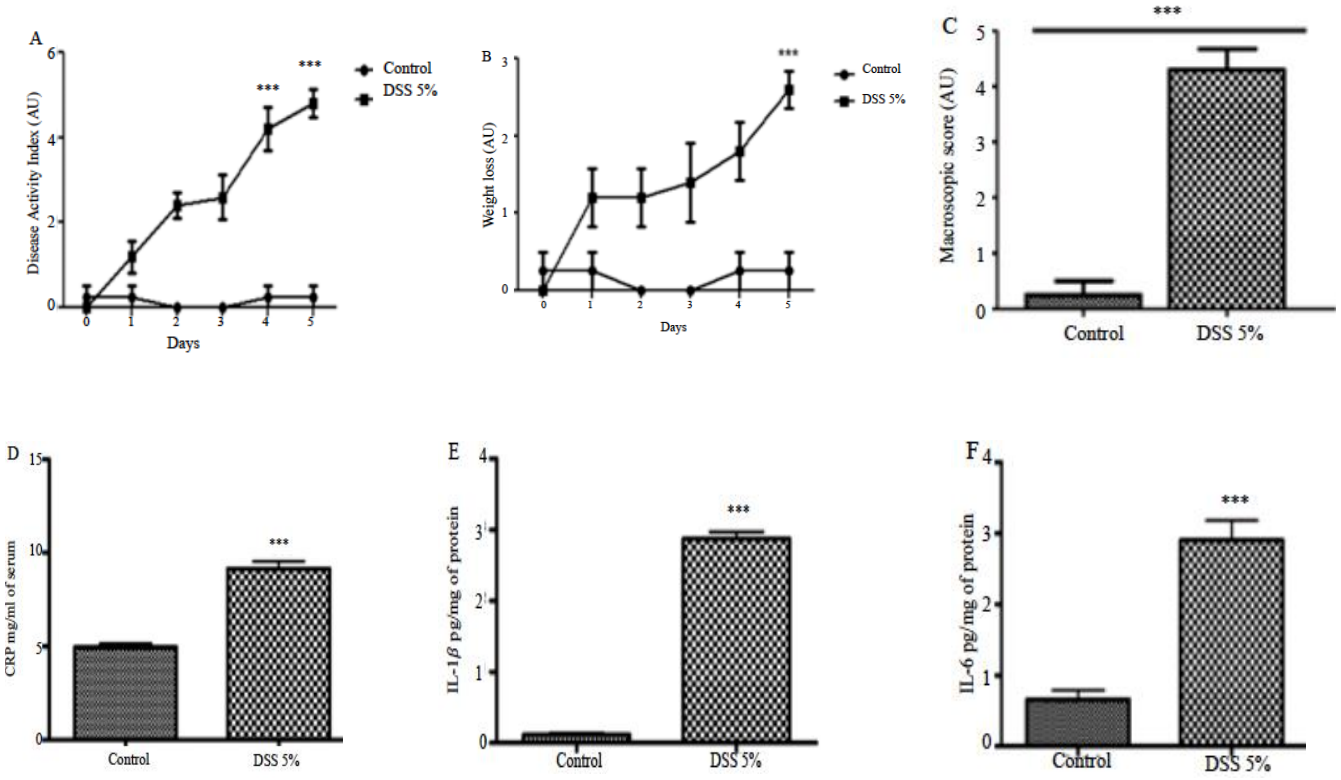


Figure 4.2-1. Dextran sulfate sodium (DSS)-induced colonic inflammation.
Five percent DSS treatment for 5 days induced a significant increase in the; (A) disease activity index, (B) weight loss score, (C) macroscopic scores, (D) C-reactive protein (CRP), (E) IL-1β, and (F) IL-6. Values are shown as the mean $\pm$ SEM. AU, arbitrary units

4.2.3.2. 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 4.2-2A**). 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 4.2-2B**). The significance levels were determined using SAS and the p values are shown on top of each bar.

4.2.3.3. 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 4.2-3A**). Although clustered separately for a large portion of communities, the colonic MAM still had an overlap of some bacterial communities, suggesting that DSS and control mice colon mucosa samples had shared bacterial communities ($P = 0.2$; **Figure 4.2-3B**).

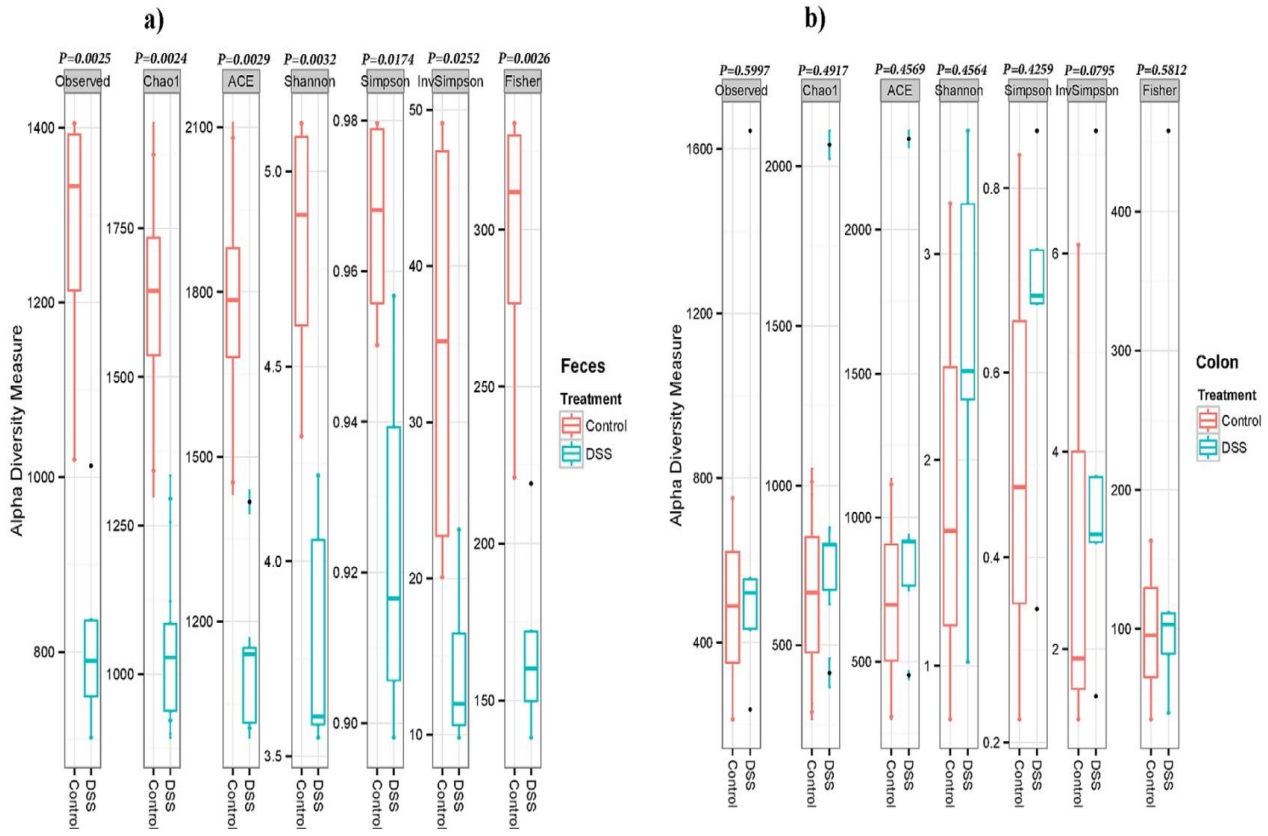


Figure 4.2-2. Alpha-diversity analysis, a measure of species richness and evenness based on different diversity indices, from dextran sulfate sodium-induced colitis and the control group studied at fecal and colon levels.

(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 had similar bacterial species richness and evenness. The p values shown on top of each bar were calculated using SAS. DSS, dextran sulphate sodium.

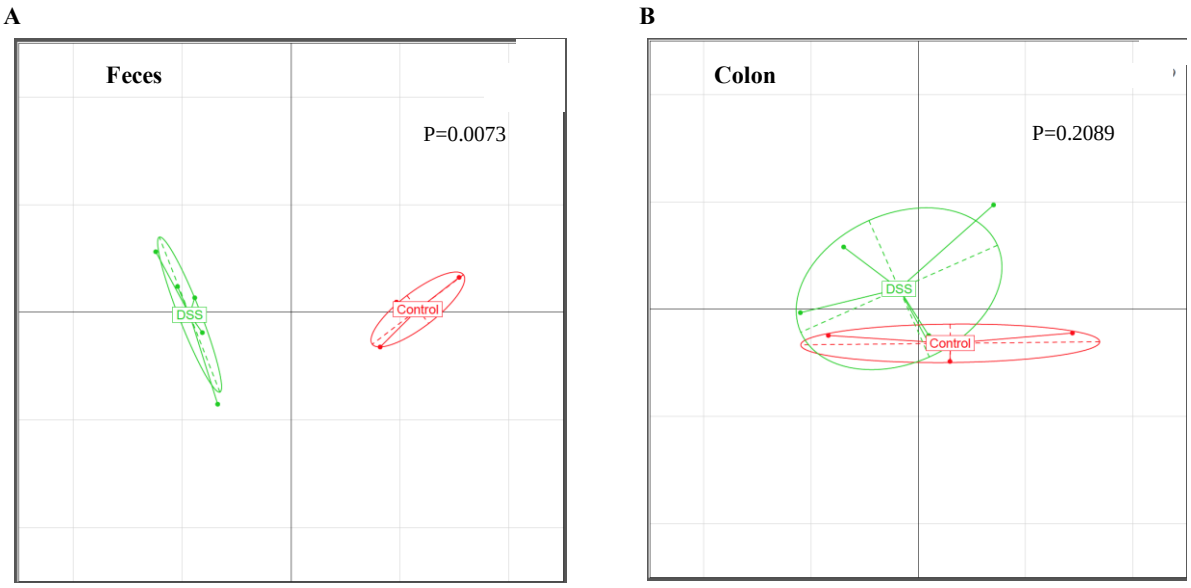


Figure 4.2-3. Principle coordinate analysis (PCoA) of weighted UniFrac distances, a measure of β-diversity of bacterial community.
(A) Fecal samples clustered separately according to treatment status of the mice, suggesting that DSS and control mice samples are composed of distinct bacterial communities; (B) colon mucosa samples have shared bacterial communities. The P values were determined using PERMANOVA. DSS, dextran sulphate sodium.

4.2.3.4. 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. Although 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 4.2-4A**.

Of the 86 taxa, 56 had abundance greater than or equal to 0.01% of community, whereas 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 4.2-5A**, 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, 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.

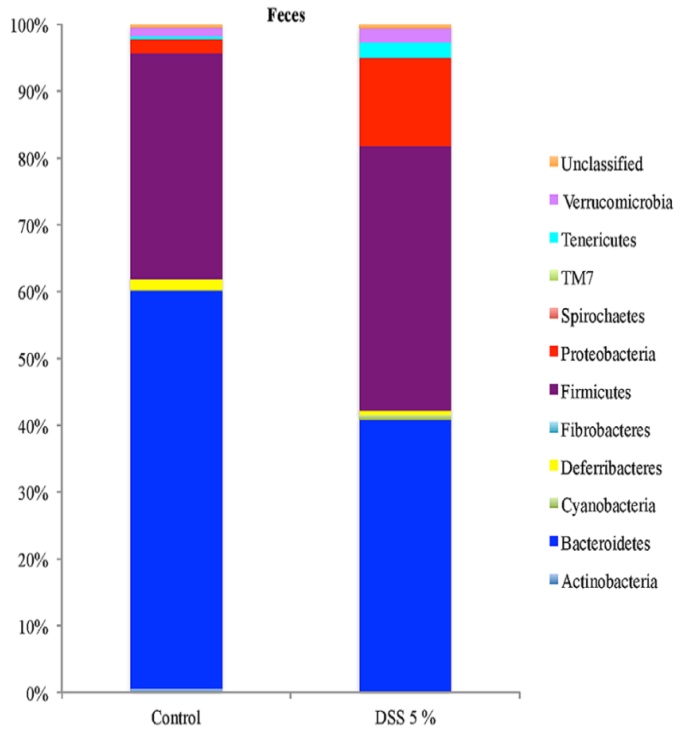
4.2.3.5. Microbiota composition at the phylum and lower taxonomic levels in colonic mucosa samples

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 community), 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 Fig. 4b.

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 Fig. 5b, 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.

A



B

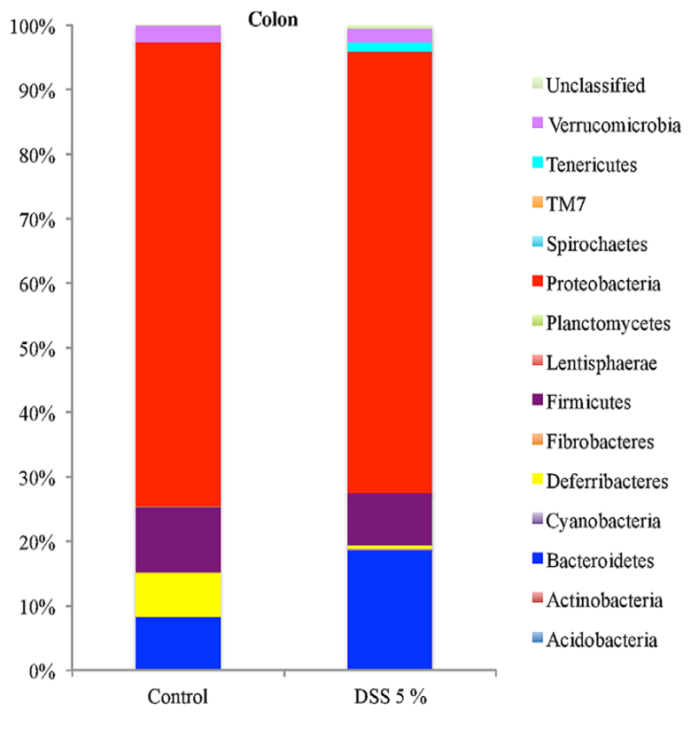


Figure 4.2-4. Percentage of relative abundances of bacterial phyla for: (A) fecal samples and (B) colon mucosa samples. The DSS significantly decreased Bacteroidetes (P=0.01) and increased Proteobacteria (P=0.004) in fecal samples but no significant difference was observed in the colonic mucosa samples. DSS, dextran sulphate sodium.

A) Feces

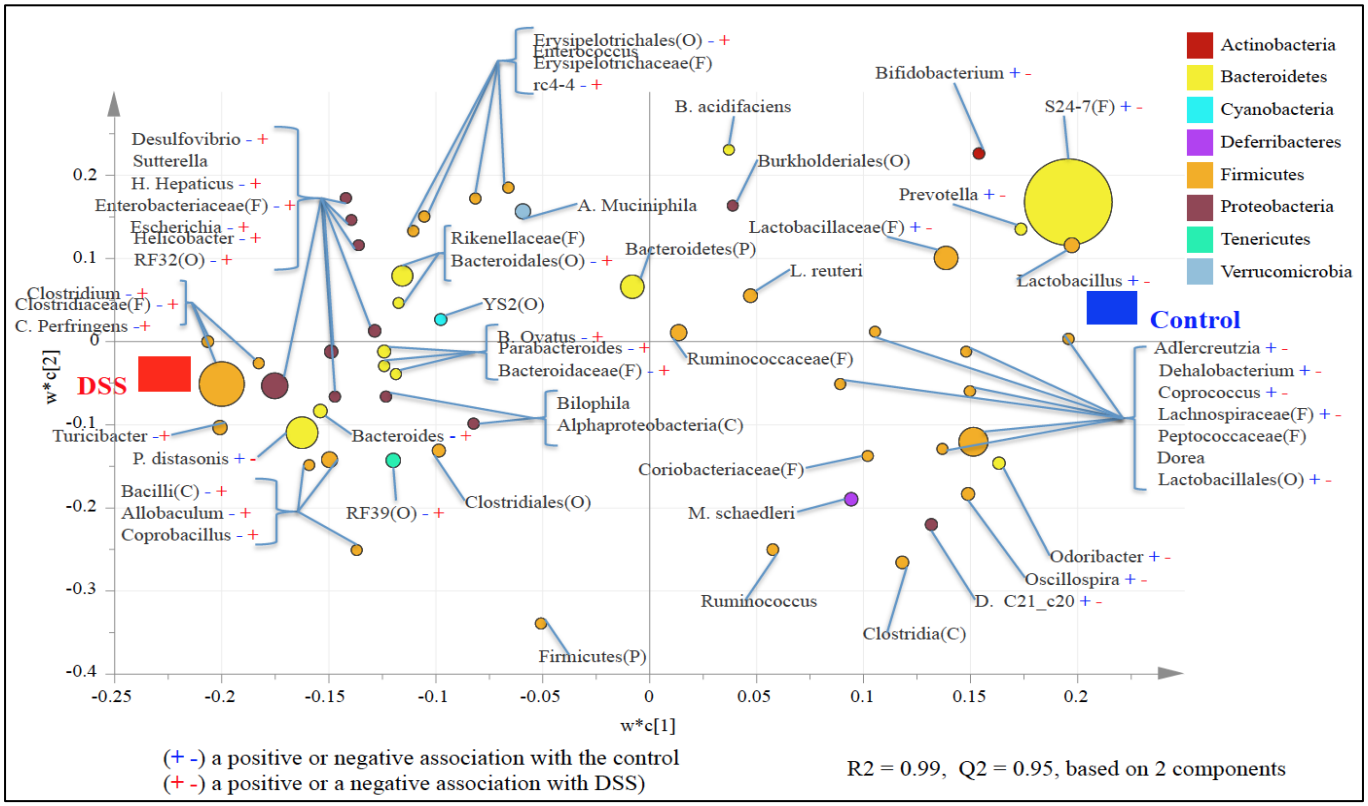


Figure 4.2-5 continued

B) Colon

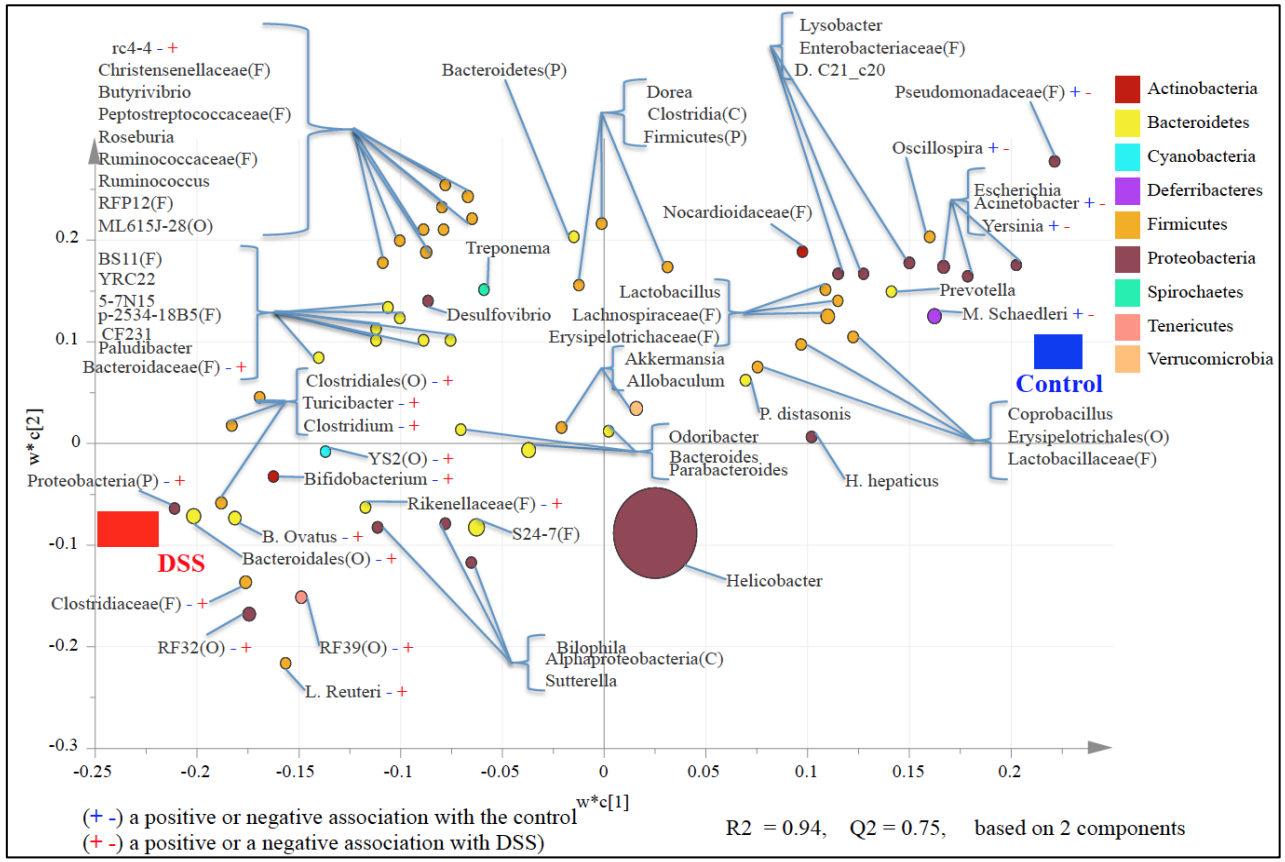


Figure 4.2-5. Partial least square discriminant analysis (PLS-DA) of bacterial communities comparing taxa that were associated with the control or DSS treatments in the mice's (A) fecal samples and (B) colon mucosal samples.
All taxa are colored based on the phyla to which they belong to. The bubble size is representative of relative abundance of each taxa within the community. While majority of taxa were classified at the genus level, some could only be affiliated to phylum (P), class (C), order (O), or family (F) levels. – or + signs and their color are indicative of significant negative or positive association between each taxon and the associated group. DSS, dextran sulphate sodium.

4.2.3.6. 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 4.2-6A**). 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 4.2-6B**).

A

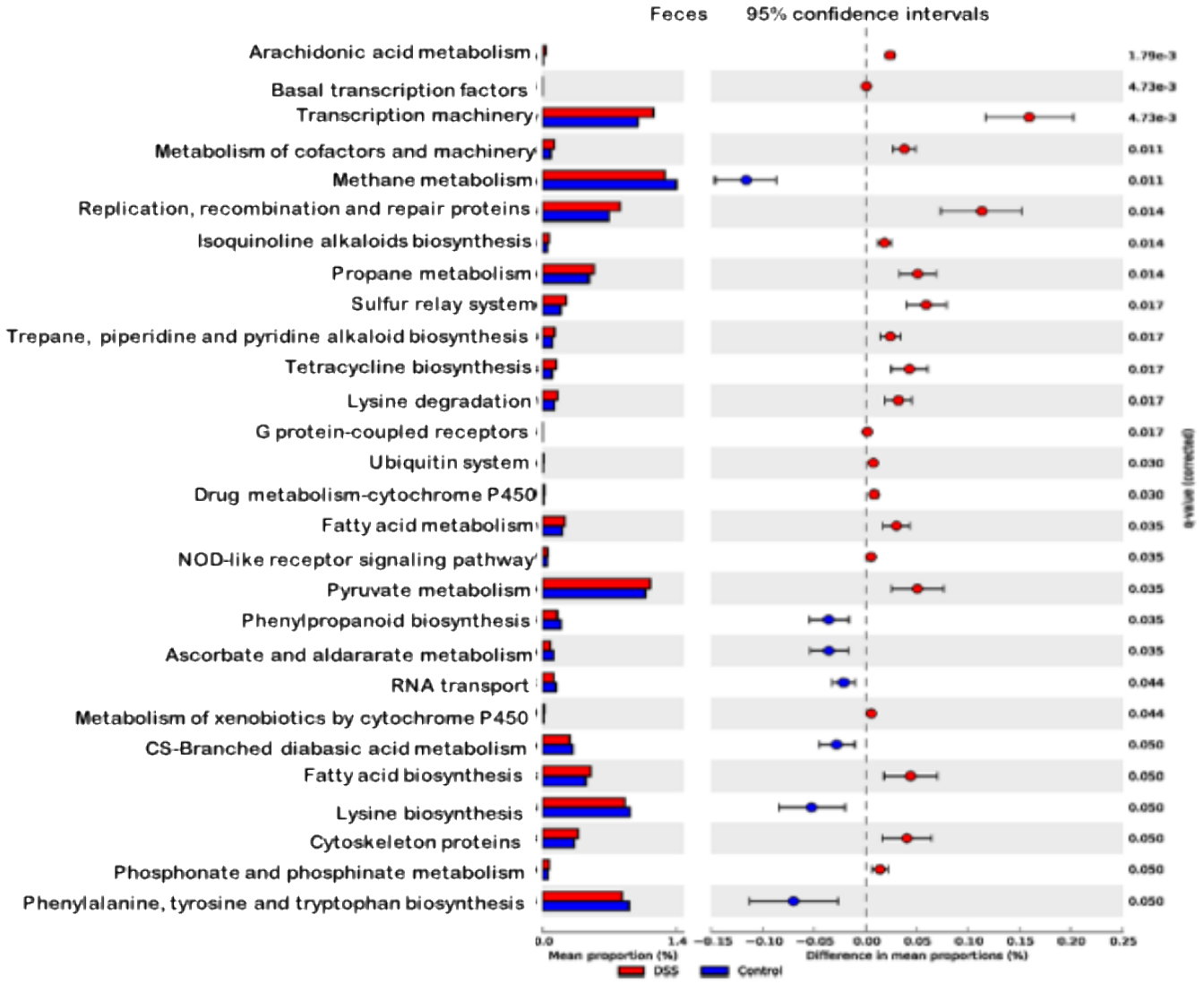


Figure 4.2-6 continued

B

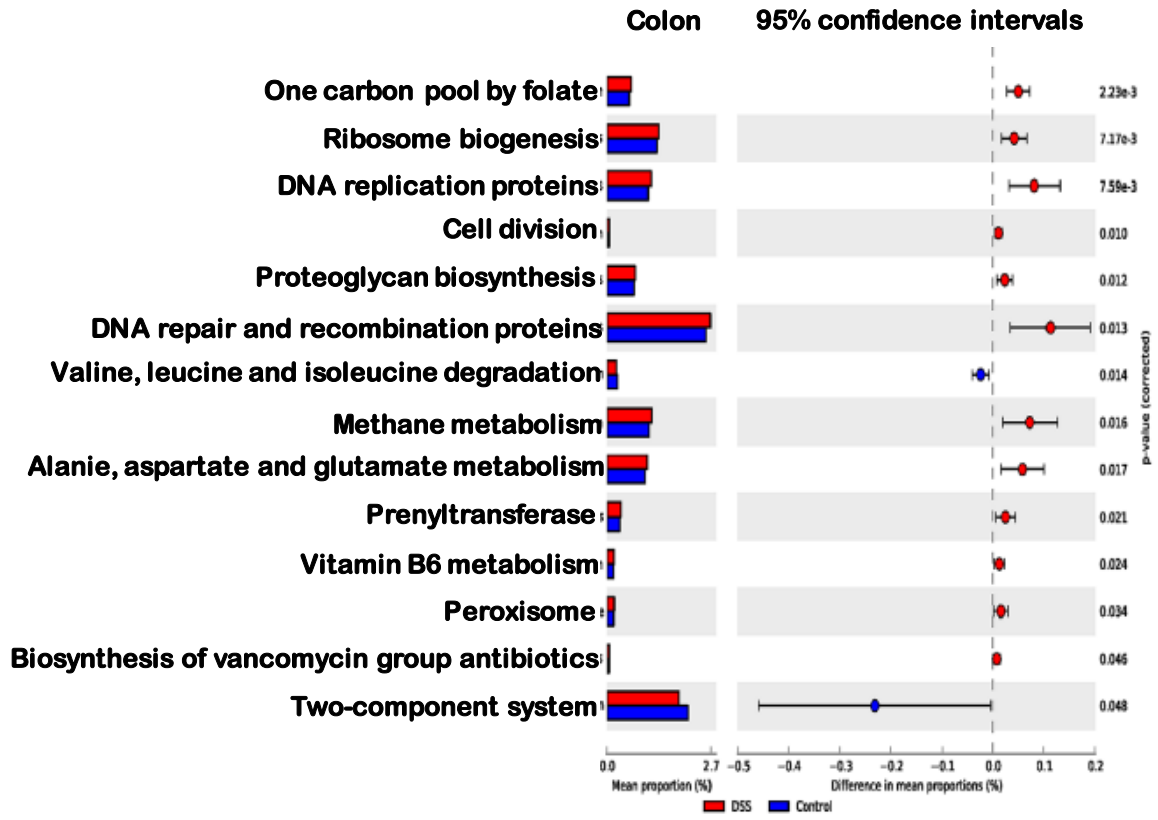


Figure 4.2-6. Functional metagenome of colonic MAM and fecal microbiome.
(A) Subsystems and pathways enriched or decreased within mice fecal samples isolated from colitic and non-colitic groups. Corrected p values were calculated using the Storey FDR correction. Subsystems or pathways overrepresented 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.

4.2.4. 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 [278] . 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 [456, 457]. Conversely, some germfree mice have been shown to have a high mortality rate when given DSS in drinking water as compared to conventional mice [456], 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 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 [317, 434]. 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 [317, 335]. 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 [317, 422].

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 [431, 434, 458-461]. 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. [432], 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 [400] and in both fecal and intestinal MAM samples of humans with IBD [70] 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 at the phylum level. 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 and community composition 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 was 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 [432] , 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. [432]also found that the overall abundances 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 [435], and were also previously shown to be capable of inducing colitis in antibiotic-pretreated

mice [462]. 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 [376, 463-467]. 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 [119, 376, 413, 431, 435, 468]. Similar findings for increased levels of Enterobacteriaceae have also been reported in murine colitis, and after antibiotic treatment or infection by enteric pathogens [442, 469, 470]. 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 [462]. 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 [400, 432, 460, 466].

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 [119]. It is possible that because DSS-induced colitis interferes directly with the intestinal epithelium and its barrier function as well as causing crypt damage [336, 411, 422, 471], this may lead to sloughing of mucosal epithelium,

thereby decreasing the number of bacteria adhering to the mucosa, and consequently increasing the abundances of bacteria in the fecal matter.

Studies applying metagenomics, metatranscriptomics or metaproteomics investigating functional capacities of gut microbiota in humans [119, 430], and in mouse model of colitis [432], 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. Although 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.

Part 05. Chapter 05

5.0 Bridge to Chapter 05

IBD patients have active, recovery and relapsing phases of gut inflammation [472]. In Chapter 03, a beneficial effect of a preventive treatment with CTS in the acute form of experimental colitis was observed. However, this does not recapitulate the natural history of the disease. Moreover, this first study demonstrated that CTS could impact the macrophage population, however, macrophages can be classified in M1-M2 type [164]. From our first study, it was not clear which macrophage type was implicated in this effect.

Moreover, in chapter 04, we observed that CTS has an impact on gut microbiota during naïve condition [318], however, the impact of CTS on gut microbiota during inflamed condition is unknown.

Taken together, we studied the effect of CTS on inflammation reactivation using a quiescence model of colitis which mimics more to the natural history of IBD. We also observed the effect of CTS on M1 and M2 macrophage population and on gut microbiota [320].

5.1. Reactivation of intestinal inflammation is suppressed by Catestatin in a murine model of colitis via M1 macrophages and not the gut microbiota.

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Contributorship:

MFR and JEG conceived and designed the study. MFR, NE, PM, LK, OE conducted the experiments and performed the analyses. MFR interpreted the data and wrote the manuscript. EK, CNB and JEG reviewed the draft manuscript.

Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Source of support:

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5.i. Abstract

While there is growing awareness of a relationship between chromogranin-A (CHGA) and susceptibility to inflammatory conditions, the role of human catestatin (hCTS; CA_{352-72}) in the natural history of established inflammatory bowel disease (IBD) is not known. Recently, using two different experimental models, we demonstrated that hCTS-treated mice develop less severe acute colitis. We have also shown the implication of the macrophages in this effect. The aims of this study were to determine (1) whether hCTS treatment could attenuate the reactivation of inflammation in adult mice with previously established chronic colitis; (2) whether this effect is mediated through macrophages or the gut microbiota. Quiescent colitis was induced in 7–8-week-old C57BL6 mice using four cycles (2%–4%) of dextran sulfate sodium (DSS). hCTS (1.5 mg/kg/d) treatment or vehicle started two days before the last induction of colitis and continuing for seven days. At sacrifice, macro- and microscopic scores were determined. Colonic pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), anti-inflammatory cytokines (IL-10, TGF-β), classically activated (M1) (*iNOS*, *Mcp1*), alternatively activated (M2) (*Ym1*, *Arg1*) macrophages markers, were studied using ELISA and/or RT-qPCR. *In vitro*, peritoneal macrophages isolated from naïve mice and treated with hCTS (10^{-5} M, 12 h) were exposed to either lipopolysaccharide (LPS,100 ng/ml, 12 h) to polarize M1 macrophages or to IL4/IL13 (20 ng/ml) to polarize M2 macrophages. M1/M2 macrophage markers along with cytokine gene expression were determined using RT-qPCR. Feces and mucosa-associated microbiota (MAM) samples were collected, and the V4 region of 16s rRNA was sequenced. Micro- and macroscopic scores, colonic IL6, IL1B, TNFA, and M1 macrophages markers were significantly decreased in the hCTS

treated group. Treatment did not have any effect colonic IL-10, TGF-β, M2 markers nor modified the bacterial richness, diversity or the major phyla in colitic fecal and MAM samples. *In vitro*, pro-inflammatory cytokines levels, as well as their gene expression, were significantly reduced in hCTS-treated M1 macrophages. hCTS treatment did not affect M2 macrophage markers. These findings suggest that hCTS treatment attenuates the severity of inflammatory relapse through the modulation of the M1 macrophages and the release of pro-inflammatory cytokines.

5.1. Introduction

Inflammatory bowel diseases (IBDs) are well known intestinal disorders that are characterized by chronic relapse and remitting stage of inflammation of gastrointestinal tract [472]. Ulcerative colitis (UC) [473]and Crohn's disease (CD) [474] are two major types of IBD and disease prevalence is rising in the Western world [13, 14, 475]. Currently, the therapeutic drugs available are not curative, and their major goals are to prolong the remission stage by suppressing inflammatory cytokines such as TNFA or various corticosteroids and thiopurines [5, 6, 476]. These drugs require life-long application, are expensive, and can cause adverse effects such as infectious disease and even malignancy because of a dysregulated immune system [312, 321]. Moreover, many patients are resistant to these drugs [297, 476, 477]. Because of these complications, clinicians have a limited choice of drugs to treat IBD. Therefore, development of a safe, cost-effective curative treatment option for IBD patients is needed.

It has been shown that a complex network of events at the molecular, cellular, and tissue levels underlie inflammation and remodeling that eventually leads to the development of IBD symptoms [323]. Mucosal and transmural inflammation is a characteristic feature of IBD that is accompanied by a massive infiltration of activated cells from both the innate and adaptive immune systems [322, 323]. In addition to this immune dysregulation, in IBD and experimental colitis [2]. gut inflammation is associated with changes in the prohormone chromogranin (CHG)-A producing enterochromaffin (EC) cells [372], but the impact of this modification on the immune activation during development of gut inflammation is unclear.

CHGA is a member of the secretory granin protein family, localized at 14q32 of the human genome, and it consists of eight exons and seven introns. It is a pro-hormone that can be cleaved at its basic residues by pro-hormone convertases (PC) 1/3 or 2, carboxypeptidase E/H22 to generate shorter bioactive peptides [243, 244]. These bioactive peptides derived from the C and N terminus are highly conserved among vertebrates, up to 88% of sequence homology between species [240-242]. CHGA-derived peptides (CGDPs) regulate several physiological activities and the immune system [250-252]. One of the most important biologically active peptides derived from the conserved C-terminal region of CHGA is the antihypertensive peptide catestatin (hCTS; $hCHGA_{352-372}$) [247-249]. hCTS is cationic in nature and is well known for its *in vitro* antimicrobial properties, it possesses immune regulation properties [262], and it can stimulate chemotaxis of human peripheral blood monocytes [252].

Recently, our group and others confirmed that serum CHGA levels are elevated in patients with UC [238, 317]. Moreover, CHGA demonstrated a positive correlation with inflammatory markers like procalcitonin, C-reactive protein, simplified acute physiological score (SAPS) [258]. Taken together, all these data provide a proof of the concept, that CHGA and CGDPs play an important role in the development of inflammatory pathologies, but the knowledge of pathophysiological mechanisms whereby CGDPs exert their action remains unclear, especially in the context of gut inflammation. Consequently, the link between CGDPs and colitis remains to be confirmed. Our lab recently showed a preventive effect of hCTS on gut inflammation using two acute experimental murine models of colitis [317]. Historically, UC patients have flares of inflammation and relapse in between, which cannot be mimicked by the use of the acute

model of colitis. Therefore, we delineated the effect of hCTS on chronic gut inflammation using the quiescence colitis model.

Macrophages are innate immune cells and are instrumental in protecting the host from the luminal content of intestinal tract [478, 479]. Depending on the stimuli, these cells can be polarized to classical (M1), and alternative (M2) activated macrophages [480]. Interferon-gamma (IFNγ) and lipopolysaccharide (LPS) are mainly responsible for the M1 polarization, and in return, M1 macrophages release or generate nitric oxide (NO) and other pro-inflammatory cytokines such as IL6, IL1B and TNFA to initiate immune activation [480, 481]. Conversely, the M2 macrophages are activated upon stimulation by interleukin (IL)4, 13 and can subsequently down-regulate the inflammatory process through the production of IL10, polyamines and prolines [482]. During UC and experimental colitis, M1 macrophages infiltrate the intestinal tissue [164], but this immune activation can be counter-balanced by the M2 macrophage population [164].

Beside immune cells, the gastrointestinal tract is heavily colonized with the microbial population. On average, 10^{14} microbes representing 1000 species are present within the human gastrointestinal tract [63]. In healthy individuals, microbial diversity in the intestine is stable over time and has a symbiotic relationship with the host, which is crucial to maintain a healthy gut immune system [369]. For example, gut microbiota can activate toll-like receptors (TLRs) in the gut epithelium, which in turn can affect the expression of antimicrobial peptides such as angiogenins [367, 368] . In addition to the innate immune system and the macrophages, gut microbiota can also control the host's adaptive immune system through T cell receptor αβ-positive intraepithelial lymphocytes, T regulatory cells, (Tregs) and T helper 17 (Th17) [69]. It has been observed that

microbial diversity is altered both in animal models of colitis and in UC patients. In our recent study, we observed that acute exposure of dextran sulfate sodium (DSS) can alter the murine gut microbiota by reducing the abundance of Bacteroides compared to Firmicutes [319]. Moreover, we showed that hCTS treatment could alter the gut microbial composition in the naïve mice [318].

The aim of our study was to demonstrate the effect of hCTS on chronic gut inflammation. Using a model of quiescence/reactivation colitis, we determined the effect of the hCTS on colitic inflammatory markers, macrophage population, and gut microbiota. We demonstrated that intrarectal administration of hCTS down-regulates the inflammatory process through down-regulation of the pro-inflammatory properties of the M1 macrophage population, but not microbiota.

5.2. Materials and Methods

5.2.1. Animals

Male C57BL/6 mice (7–9 weeks old) were purchased from Charles River (Canada) and maintained in the animal care facility at the University of Manitoba. The experimental protocol was approved by the University of Manitoba Animal Ethics Committee (15-010) and the research was conducted according to the Canadian Guidelines for Animal Research [374, 375].

5.2.2. Peptide

The hCTS ($hCHGA_{352–372}$: SSMKLSFRARAYGFRGPGPQL) was used (Biopeptide Co., Inc, San Diego, CA), and the peptide was injected intrarectally (*i.r.*) at 1.5 mg/per kg body weight per day for 7 days. Saline (0.9%) was injected into the control group. Mice were anaesthetized using Isoflurane (Abbott, Toronto, Canada). PE-90 tubing (10 cm long; ClayAdam, Parisppany, NJ), which was attached to a tuberculin syringe (BD, Mississauga, Canada), was inserted 3.5 cm into the colon. The dose was determined according to our previous published study [317].

5.2.3 Quiescence DSS colitis

To develop quiescence colitis, four cycles of DSS (molecular weight [MW], 40 kilodaltons: MPI Biomedicals, Santa Ana, CA, USA) at concentrations of 4%, 2%, 2%, and 4% (wt/vol; drinking water) were performed. Each DSS cycle lasted for 5 days and

was followed by 11 days of rest (access to normal drinking water) and treatment started two days before the reactivation.

5.2.4. Determination of colitis severity-disease activity index

The colitis severity disease activity index (DAI) is a composite score of weight loss, stool consistency and bleeding [336] was assessed on a daily basis. Scores were defined as follows: weight: 0, no loss; 1, 5–10%; 2, 10–15%; 3, 15–20%; and 4, 20% weight loss; stool: 0, normal; 2, loose stool; and 4, diarrhea; 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).

5.2.5. Sample collection

Following the final reactivation, animals were euthanized under isoflurane [483] anaesthesia and samples were collected. The macroscopic score was determined based on stool consistency, rectal prolapse and rectal and colonic bleeding [336]. A fecal sample (250 mg) and a portion of the colon were collected in individual collector tubes from each animal.

5.2.6. Microscopic score and colonic cytokines

The colonic histology (microscopic score) was assessed by hematoxylin-eosin (H&E) (Sigma, Mississauga, Canada) staining of 3-µm formalin (Sigma, Mississauga, ON, Canada)-fixed colon sections. A scoring system composed of architectural derangements, goblet cell depletion, edema/ulceration, and degree of inflammatory cell

infiltrate was used to assess the colonic damage [336]. To assess colonic cytokine levels, distal colon samples were homogenized in 700 µl of Tris·HCl buffer containing protease inhibitors (Sigma, Mississauga, Canada) and then centrifuged for 30 min at 4°C. Then, the supernatant was frozen at -80°C until assay. Commercial ELISA kits were used to determine cytokine levels (IL-1β, IL-6, TNF-α , IL-10, TGF-β) (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

5.2.7. Macrophage isolation

Naïve peritoneal macrophages were collected as described by Rabbi et al. [317]. Briefly, sterile PBS (Gibco BRL Life Technologies, Grand Island, NY) (10 ml) was injected into the caudal half of the peritoneal cavity using a 25-gauge needle (BD, Mississauga, ON, Canada) and the body was shaken for 15 sec. Resident peritoneal cells were slowly withdrawn using a 19-gauge needle (BD, Mississauga, ON, Canada), and they were purified by adhesion. Resident peritoneal cells were plated in macrophage culture medium (RPMI 1640 supplemented with 10% HI FCS containing 50 IU of penicillin, 50 µg streptomycin, and 2mM glutamine per milliliter (Gibco BRL Life Technologies, Grand Island, NY, USA)) for 60 mins at 37°C. Non-adherent cells were removed by washing five times with 500 µl of warm PBS. Overall cell viability of the adherent cell before and after treatment was greater than 97%, and more than 94% of the cells were macrophages using DiffQuick (Jorgensen Laboratory, Loveland, CO, USA) staining. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. M1 macrophages were stimulated by adding LPS (Sigma, Mississauga, Canada) to the cultures at a final concentration of 100 ng/ml. M2 macrophages were obtained by adding

IL4/IL13 combination (20 ng/ml). hCTS were added to the medium at a final concentration of 10^{-5} M one hour before the LPS or IL4/IL13. Supernatants were collected 24 h after LPS or IL4/IL13. For gene expression analysis, 1 ml of Trizol solution was added per well to collect mRNA from the cells, which were stored in −0°C until further use.

5.2.8 Gene Expression analysis

Total RNA was extracted using a kit (TRIzol® Plus RNA Purification Kit, Life Technologies, NY, USA), according to manufacturer's instructions. Quality and quantity of RNA were determined by measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples absorption ratio (A260/A280) ranged between 1.8 and 2.2 [484, 485]. To remove genomic DNA contamination, samples were treated with RQ1 RNase-Free DNase® (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Reverse transcription was performed using SuperScript VILO cDNA Synthesis Master Mix (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. qReal-time PCR (qRT-PCR) reactions were performed in a Roch lightCycler 96 Real-Time System using Power SYBR green master mix (Life Technologies) in a final volume of 20 µl reactions. All samples were tested in triplicate. Differences in the threshold cycle (ΔCt) number were determined between the target genes and the housekeeping gene Eukaryotic Elongation factor 2 (*Eef2*) [484], which was used to calculate differences in expression. The primers were designed from nucleotide sequences identified using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to

confirm the specificity of the primer design. The primer characteristics of nominated genes are listed in **Table 5-1**.

Table 5-1. RT-qPCR primers sequences.

Gene	**Forward**	**Reverse**
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnfa	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Ym1	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
Arg	TTGGGTGGATGCTCACACTG	GTACACGATGTCTTTGGCAGA
Tgfb	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG

5.2.9. Illumina sequencing

DNA was extracted from 250 mg fecal mass and colon mucosa scrapings using a ZR fecal and tissue DNA Kit (Zymo Research Corp., Orange, CA, USA). Genomic DNA was normalized to achieve a concentration of 20 ng/µL, and quality-checked by 16S rRNA gene using PCR amplification of 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') [376, 377]. Amplicons were verified by agarose gel electrophoresis. Library construction and Illumina sequencing were performed as described by Derakhshani et al. [378] targeting the V4 region of the 16S rRNA and using the MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

5.2.10. Bioinformatics analyses

Bioinformatics analyses were performed as described by Derakhshani et al. [378]. Briefly, the PANDAseq assembler [381] 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 using downstream computational pipelines in the open source software package QIIME [382]. Assembled reads were demultiplexed according to the barcode sequences and chimeric reads were filtered using UCHIME [383]. Sequences were assigned to operational taxonomic units (OTU) using the QIIME implementation of UCLUST [384] at the 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using an RDP classifier [385] and aligned with the Greengenescore reference

database [386] using PyNAST algorithms[387]. The phylogenetic tree was built with FastTree 2.1.3 [388] for additional comparisons between microbial communities.

5.2.11 Alpha (α)- and beta (β)-diversity analyses

Within-community diversity (α-diversity) was calculated using QIIME. Rarefaction curves were generated using Chao 1, an estimator of species richness [486] with 10 sampling repetitions at each sampling depth. An even depth of 25000 and 5000 sequences per sample was used for calculation of species richness in fecal and colon samples respectively. To compare microbial composition between samples, β-diversity was measured by calculating the weighted and unweighted Unifrac distances [390] using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots using PRIMER v6 software [391]. Permutational multivariate analysis of variance of Bray-Curtis distance (PERMANOVA) [392] was used to calculate *P*-values and test for significant differences in β-diversity among treatment groups. α-Diversity differences between treatment groups were determined using SAS (SAS 9.3, 2012).

5.2.12 Statistical analysis

All the results are presented as the mean ± standard error mean (SEM). The student's *t*-test and one-way ANOVA followed by the Bonferroni multiple comparison post hoc analysis was used to compare the significance between control and experimental groups. A P value of <0.05 was considered significant, with n=5 to 12 depending on the groups tested (Prism 5, GraphPad, La Jolla, CA). The SAS UNIVARIATE procedure (SAS 9.3,

2012) was used to test the normality of residuals for α biodiversity data. Non-normally-distributed data were log transformed and then used to assess the effect of sampling using the SAS MIXED procedure. The SAS MIXED procedure was used, as described above, to test for significant changes in the proportions of different phyla among the groups of interests. All the phyla were divided into two groups of abundant (above 1% of the community) and low-abundance (below 1% of the community). The differences between groups were considered significant at $P< 0.05$ while trends were observed at $P< 0.1$.

5.3. Results

5.3.1 Effect of hCTS on weight loss and macroscopic score

hCTS treatment (1.5 mg/kg/d, 7 days, *i.r.*) did not improve weight loss following reactivation of quiescent colitis (**Figure 5-1A**). DAI was also not affected by the treatment (data not shown). At sacrifice, the macroscopic score was assessed by composite scoring of fecal consistency, hyperplasia, and rectal bleeding [336], and hCTS treatment significantly improved the macroscopic score when compared with vehicle-treated colitic mice (**Figure 5-1B**).

5.3.2 Effect of hCTS on microscopic score

Colitis severity was assessed in terms of mucosal inflammation and cellular infiltration via microscopic scoring of H&E-stained formalin-fixed tissue samples [336]. As shown in **Figure 5-2A**, DSS treatment resulted in massive tissue infiltration of cells and architectural damage. Following reactivation of quiescent colitis, hCTS treatment (1.5 mg/kg/d, 7 days, *i.r.*) significantly improved tissue architecture, edema, and decreased the mixed immune cell infiltrate (mononuclear cells, neutrophils, and eosinophils) (**Figure 5-2A & 2B**).

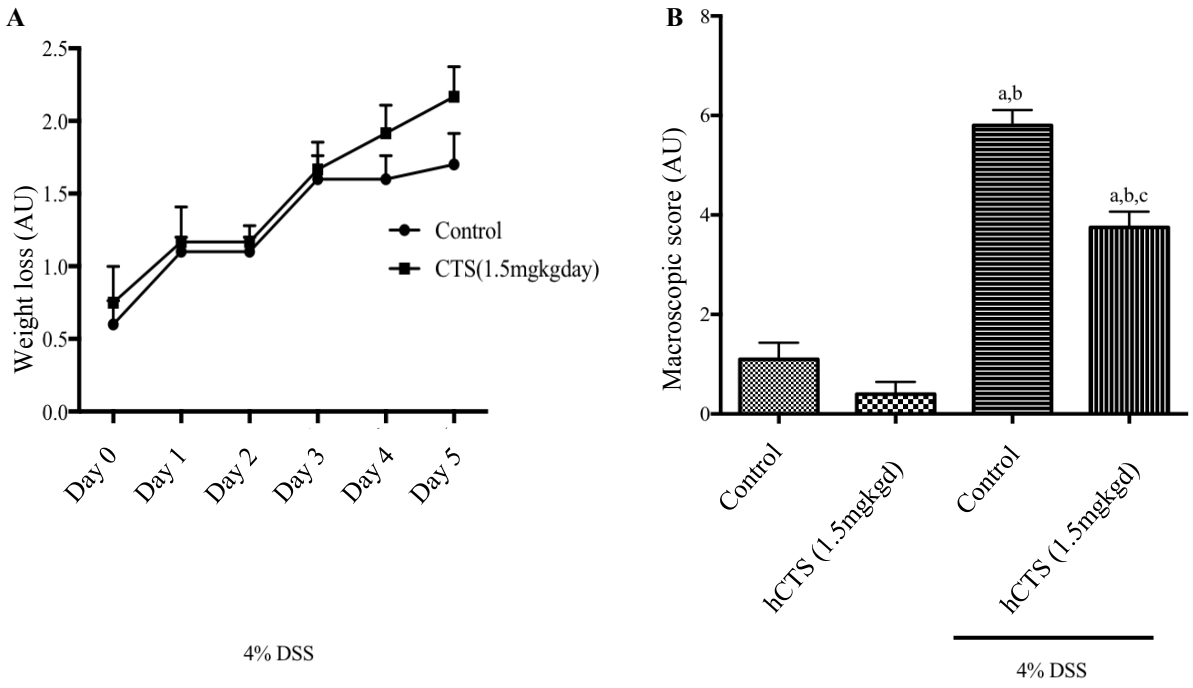

Figure 5-1. Effect of hCTS treatment (1.5 mg/kg/d, i.r. 7 days) on weight loss and macroscopic score during colitis reactivation in quiescent colitic mice.
The 7-day hCTS treatment did not improve weight loss observed in chronic quiescence colitic mice (A). However, hCTS treatment significantly improved macroscopic score in colitic mice (B). The macroscopic score was determined on the day of sacrifice considering fecal consistency, hyperplasia, and rectal bleeding. AU, arbitrary units; $n \geq 5$, $^{a}P<0.05$ compared to the Control (No DSS/No hCTS) group; $^{b}P<0.05$ compared to the hCTS (No DSS) group; $^{c}P<0.05$ compared to the DSS (No hCTS) group; Bonferroni, one-way ANOVA

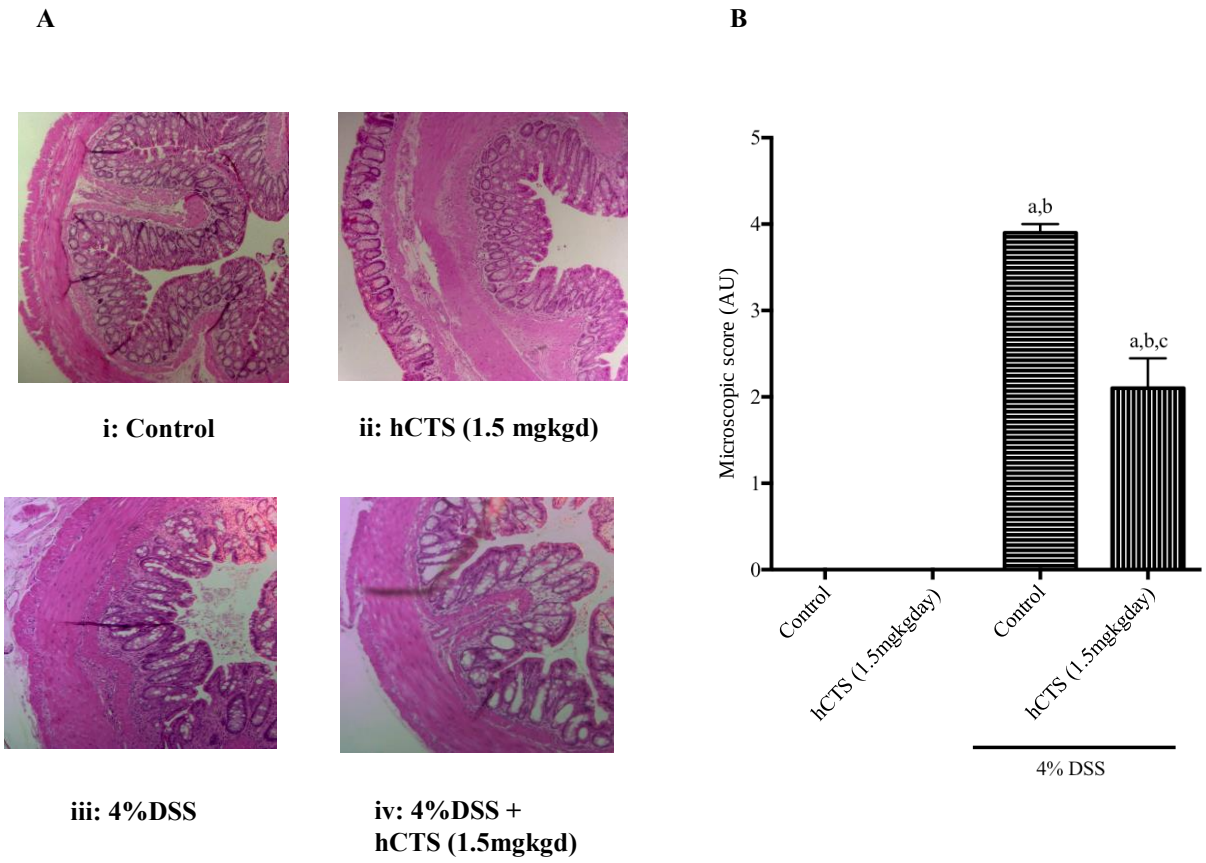

Figure 5-2. Effect of hCTS treatment (1.5 mg/kg/d, i.r. 7 ds) on (A) representative colonic architecture and (B) composite histological score.
When compared to the colonic appearance of non-colitic mice no hCTS, (A:i) and with hCTS, (A:ii), DSS treatment (A:iii) resulted in massive infiltration of immune cells and damage of intestinal architecture. This cell infiltration was inhibited together with improvement of tissue architecture in hCTS-treated DSS mice (A:iv). Thus, the composite histological (microscopic) score was significantly improved in hCTS treated colitic mice compared with vehicle treated colitic mice (B). AU, arbitrary units; $n \geq 5$; $^{a}P<0.05$ compared to the Control (No DSS/No hCTS) group; $^{b}P<0.05$ compared to the hCTS (No DSS) group; $^{c}P<0.05$ compared to the DSS (No hCTS) group; Bonferroni, one-way ANOVA

5.3.3 Effect of hCTS on colonic cytokine level

To assess the severity of colitis, levels of the major pro-inflammatory cytokines IL-6, IL-1β and TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β were determined. We confirmed that reactivation of colitis significantly increased colonic level of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α (**Figure 5-3A to 5-3C**) and decreased level of colonic anti-inflammatory cytokine TGFB (**Figure 5-3D**), but IL-10 levels were not affected (**Figure 5-3E**). hCTS treatment (1.5 mg/kg/d, 7 days, *i.r.*) significantly reduced colonic pro-inflammatory cytokines following reactivation of quiescence colitis (**Figure 5-3A to 5-3C**), but treatment did not improve colonic anti-inflammatory cytokines (**Figure 5-3D to 5-3E**).

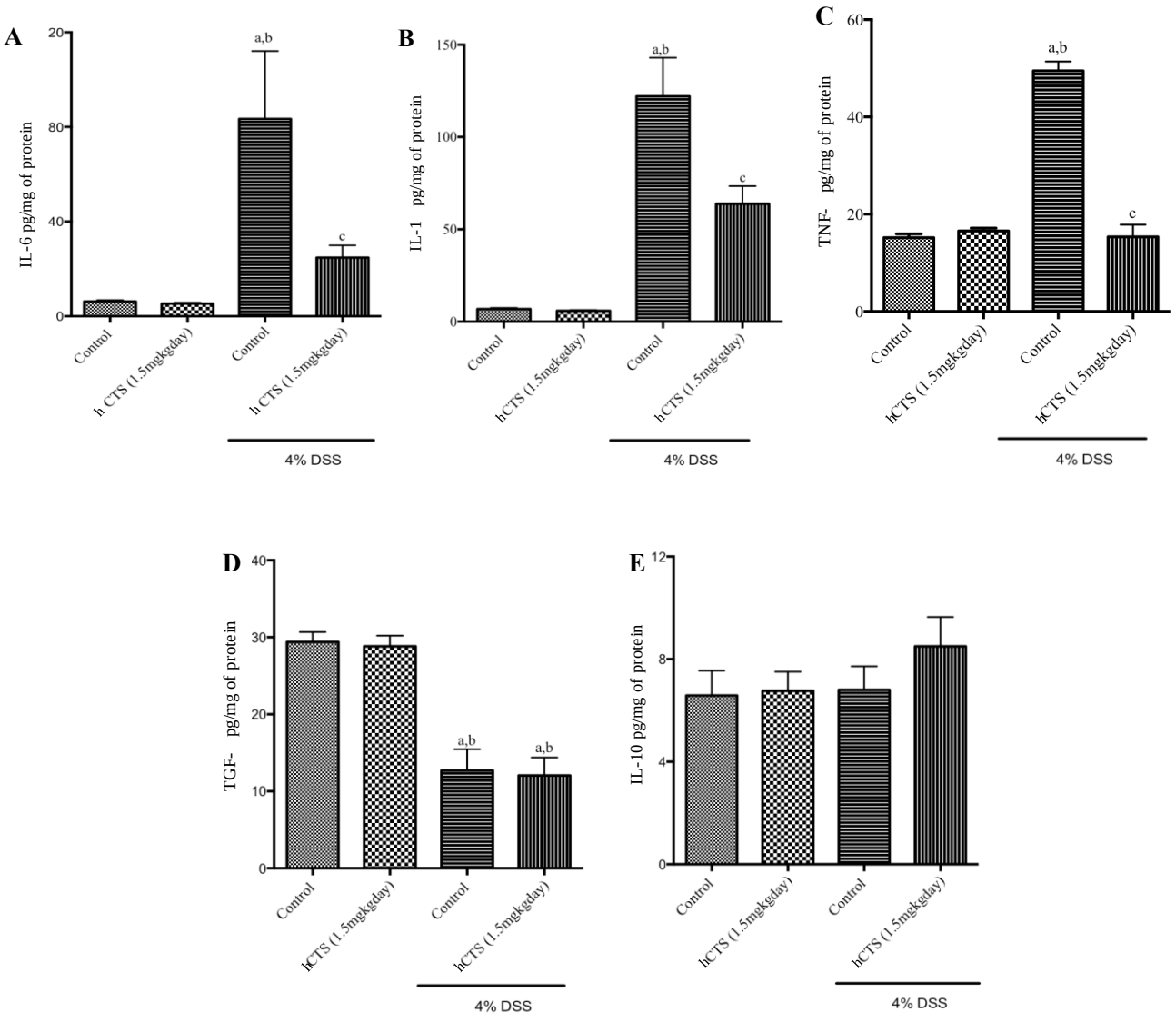

Figure 5-3. Effect of hCTS treatment (1.5 mg/kg/d, *i.r.* 7 days) on colonic cytokines. hCTS treatment resulted in significant down-regulation of colonic pro-inflammatory cytokines during colitis activation in quiescence colitic mice including IL-6 (A), IL-1β (B), and TNF-α (C). There was no observable effect of hCTS on colonic anti-inflammatory cytokines in these mice, including TGF-β (D) and IL-10 (E). $n \geq 5$; $^{a}P<0.05$ compared to the Control (No DSS/No hCTS) group; $^{b}P<0.05$ compared to the hCTS (No DSS) group; $^{c}P<0.05$ compared to the DSS (No hCTS) group; Bonferroni, one-way ANOVA

5.3.4 Effect of hCTS on colonic M1 and M2 macrophages markers/mediators

Monocytes and macrophages regulate gut inflammation and the majority of tissue infiltrating cells following reactivation of quiescence colitis are M1 macrophage and are the main source IL-6, IL-1β, and TNF-α [164]. To determine the mechanistic anti-inflammatory effect observed after hCTS treatment, mRNA expression of both M1 and M2 macrophage marker/mediators were determined. Reactivation of quiescent colitis was associated with a significant increase of M1 macrophage markers (inducible nitric oxide synthase (*iNOS*) and monocyte chemo attractant protein-1 (*Mcp1*)), which was significantly down-regulated by hCTS treatment (**Figure 5-4A & 5-4B**). Reactivation of quiescent colitis was associated with a significant increase in an M2 macrophage marker (Arginase 1 (*Arg1*)), but not Chitinase-like 3 (*Ym1*) expression (**Figure 5-4C & 5-4D**), and hCTS treatment (1.5 mg/kg/d, 7 days, *i.r.*) did not modify the markers' expression (**Figure 5-4C & 5-4D**).

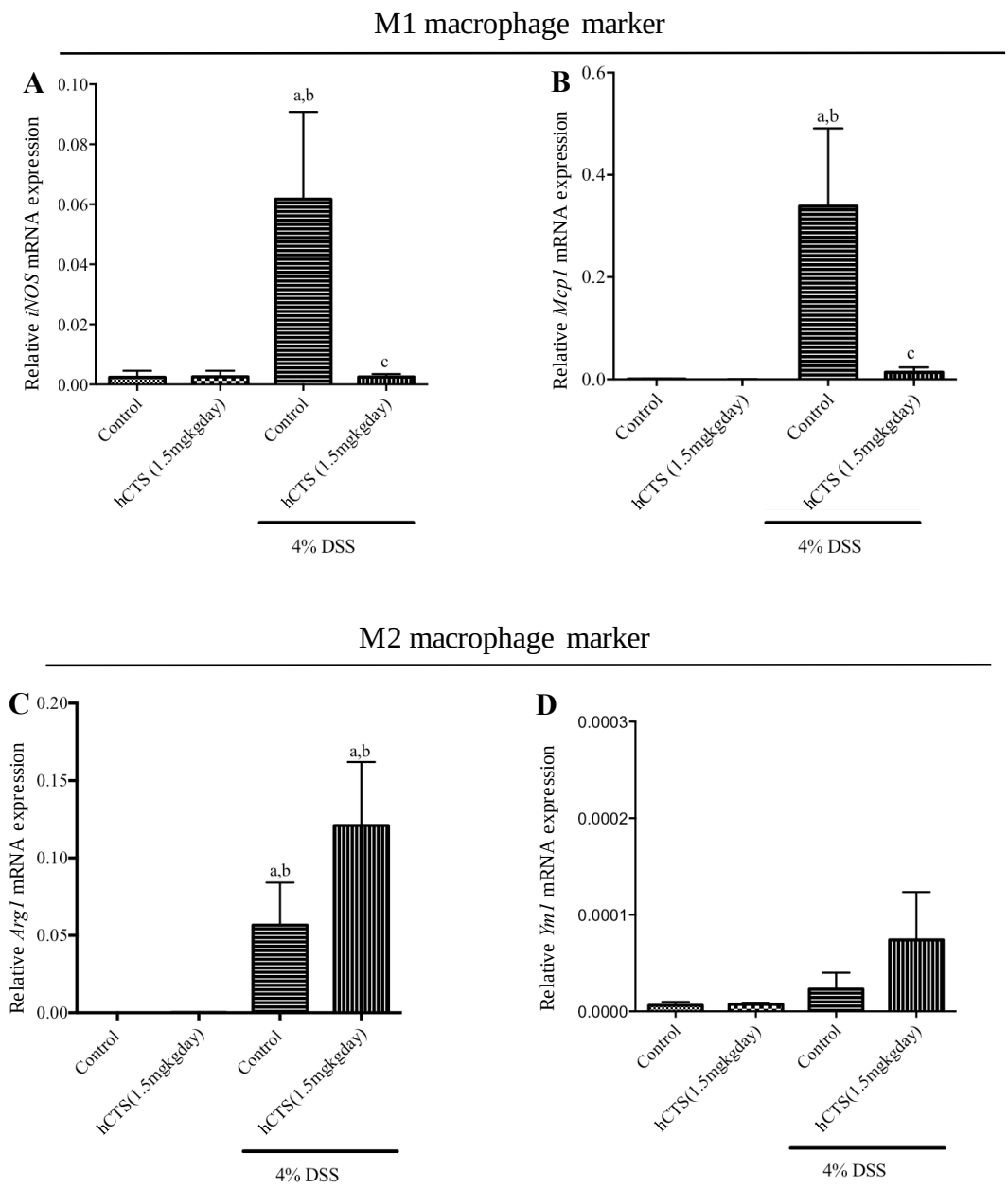

Figure 5-4. Effect of hCTS treatment (1.5 mg/kg/d, i.r. 7 days) on M1 and M2 macrophage marker expression.

hCTS treatment down-regulated the M1 macrophage markers in colitic mice, including inducible nitric oxide synthase (*iNOS*) (A) and monocyte chemoattractant protein-1 (*Mcp1*) (B). However the effect was not observed in M2 macrophage markers, including arginase 1 (*Arg1*) (C) and chitinase-like 3 (*Ym1*)) (D). $n \geq 5$; $^{a}P<0.05$ compared to the Control (No DSS/No hCTS) group; $^{b}P<0.05$ compared to the hCTS (No DSS) group; $^{c}P<0.05$ compared to the DSS (No hCTS) group; Bonferroni, one-way ANOVA

5.3.5. Effect of hCTS in vitro treatment on M1 and M2 macrophage function

Like our chronic quiescence reactivation of colitis hCTS treatment where M1 macrophage marker expression was reduced, we investigated *in vitro* the effect of this peptide on M1 macrophage. In naïve peritoneal macrophages polarized to an M1 macrophage phenotype, M1 macrophage marker (*iNOS*, *Mcp1*) and pro-inflammatory cytokine (*Il6*, *Il1b* and *Tnfa*) gene expression increased (**Figure 5-5Ato 5-5E**). Level of pro-inflammatory cytokines in the supernatant confirmed that increase (**Figure 5-5F-5-5H**). Addition of hCTS (10^{-5}M) in media 1 h before LPS simulation significantly abrogated the increase in gene expression for M1 macrophage markers and pro-inflammatory cytokines and their secretion in the supernatant (**Figure 5-5A to 5-5H**). Conversely, hCTS (10^{-5}M) treatment neither affected the expression of M2 macrophages markers *Arg1* and *Ym1* (**Figure 5-6A to 5-6B**) nor the expression of anti-inflammatory cytokines *Tgfb* and *Il10* (**Figure 5-6C to 5-6D**).

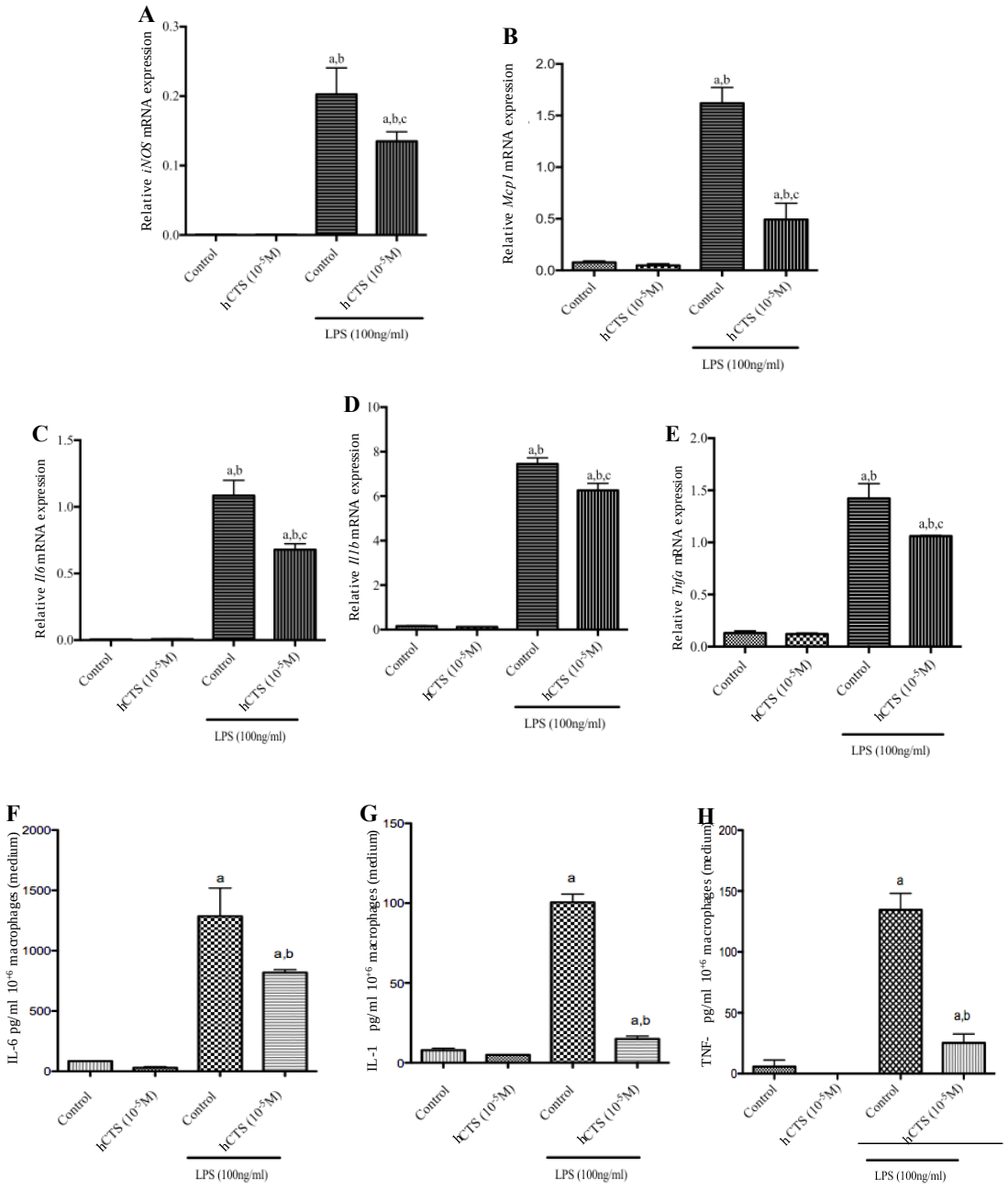

Figure 5-5. Effect of hCTS treatment (1.5 mg/kg/d, i.r. 7 days) on M1 macrophage *in vitro*.

hCTS treatment resulted in down-regulation of M1 macrophage markers and pro-inflammatory cytokine gene expression, including iNOS (A); Mcp1 (B); Il6 (C); Il1b (D); and Tnfa (E). The pro-inflammatory cytokine level in the supernatant was also decreased when CTS was present in the medium. Pro-inflammatory cytokines include IL-6 (F), IL-1β (G), and TNF-α (H). $n \geq 3$; aP<0.05 compared to the medium (no LPS)-treated group; bP<0.05 compared to the hCTS (no LPS)-treated group; cP<0.05 compared to the medium (LPS)-treated group; Bonferroni, one-way ANOVA

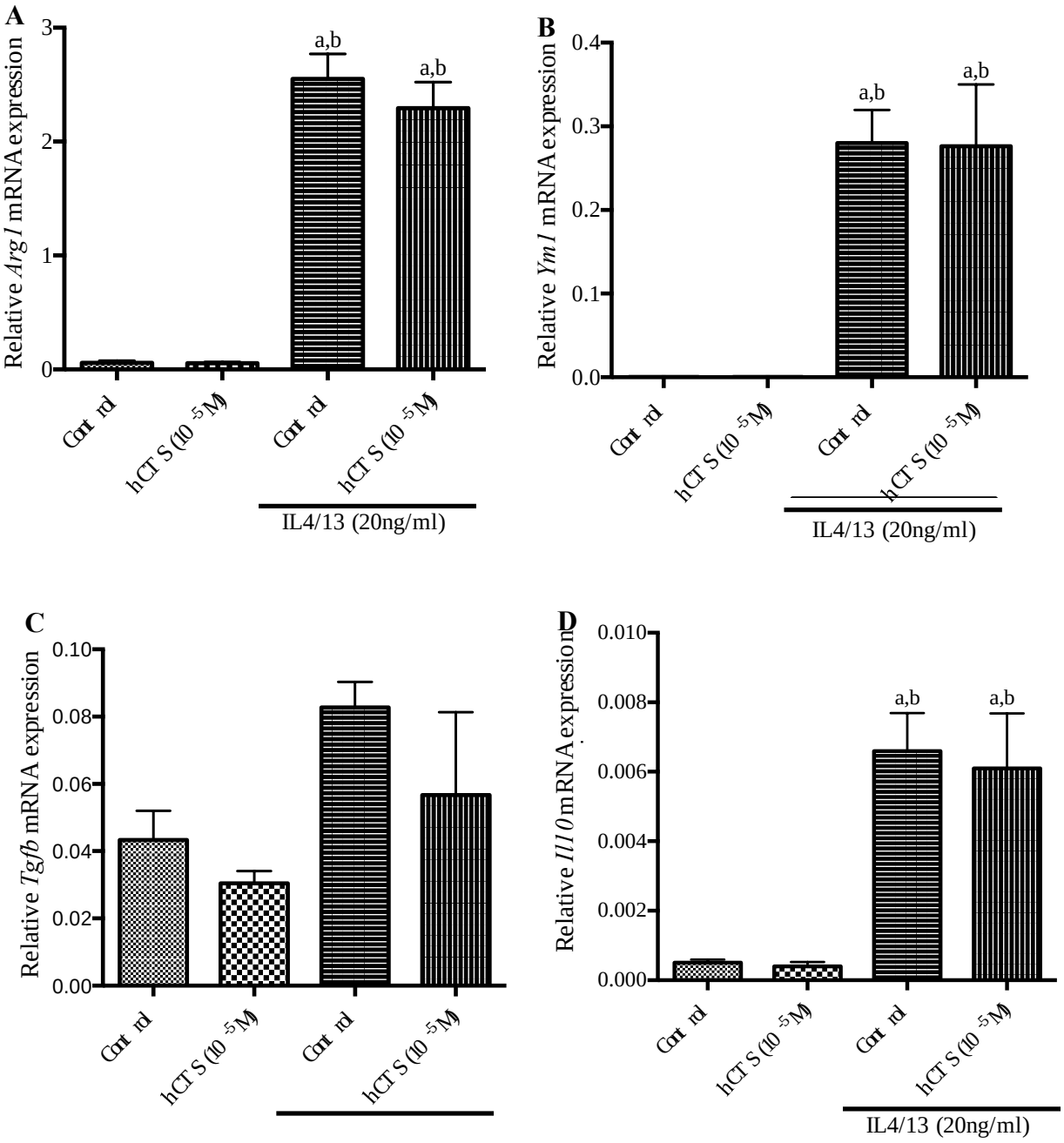

Figure 5-6. Effect of hCTS treatment (1.5 mg/kg/d, i.r. 7 days) on M2 macrophage *in vitro*.

hCTS treatment did not change gene expression of either M2 macrophage markers or the anti-inflammatory cytokines, including arginase 1 (Arg1) (A); chitinase-like 3 (Ym1) (B); Tgfb (C); and Il10 (D). n≥3, aP<0.05 compared to the medium (no IL-4/13)-treated group, bP<0.05 compared to the hCTS (no IL-4/13)-treated group; Bonferroni, one-way ANOVA

5.3.6 Effect of hCTS on gut microbiota

Gut microbial dysbiosis is evident in both UC and experimental colitis mice [319, 360]. Because hCTS is a cationic peptide and well known as an antimicrobial peptide *in vitro*, we investigated the effect of this peptide on gut microbiota. Bacterial richness and diversity from both fecal and colonic mucosa-associated samples were determined. Following reactivation of quiescent colitis, bacterial species richness in both fecal and colonic mucosa-associated samples were modified (**Figure 5-7A & 5-7B**). However, hCTS treatment (1.5 mg/kg/d, 7 days, *i.r.*) in these colitic mice was not able to modify the α-diversity when compared with vehicle-treated reactivated mice (**Figure 5-7A & 5-7B**). Using unweighted β-diversity, we observed that bacterial communities both from fecal and MAM samples collected from DSS mice clustered separately from non-colitic mice ($P < 0.05$). However, hCTS treatment in colitic mice did not change the bacterial composition in the colitic mice (**Figures 5-8A & 5-8B**). We also assessed the effect of hCTS on colitic mice in the context of abundant phyla (percentage within the community > 1%) and we observed no significant effect of hCTS treatment on the abundant phyla of colitic mice both in fecal and MAM samples (**Figure 5-9A & 5-9B**).

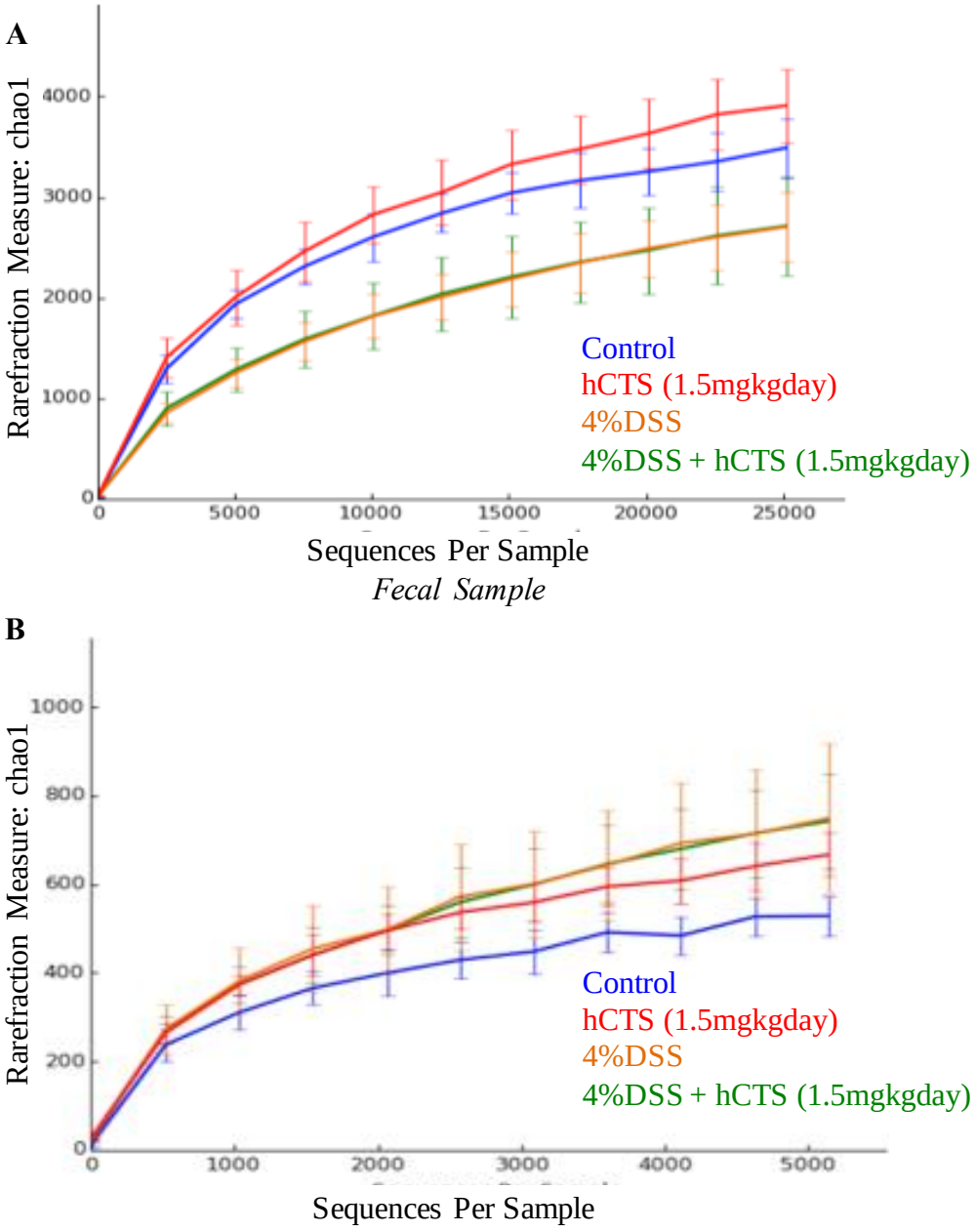

Figure 5-7. Rarefaction analysis on Chao 1, a measure of species richness based on operational taxonomic unit (OTU).
Fecal samples (A); Colonic mucosa-associated microbiota samples (B). DSS treatment significantly changed microbial diversity compared to non-colitic animals in both sample types. However, the hCTS treatment (1.5 mg/kg/d, i.r. 7 days) did not change the diversity observed in DSS mice**.**

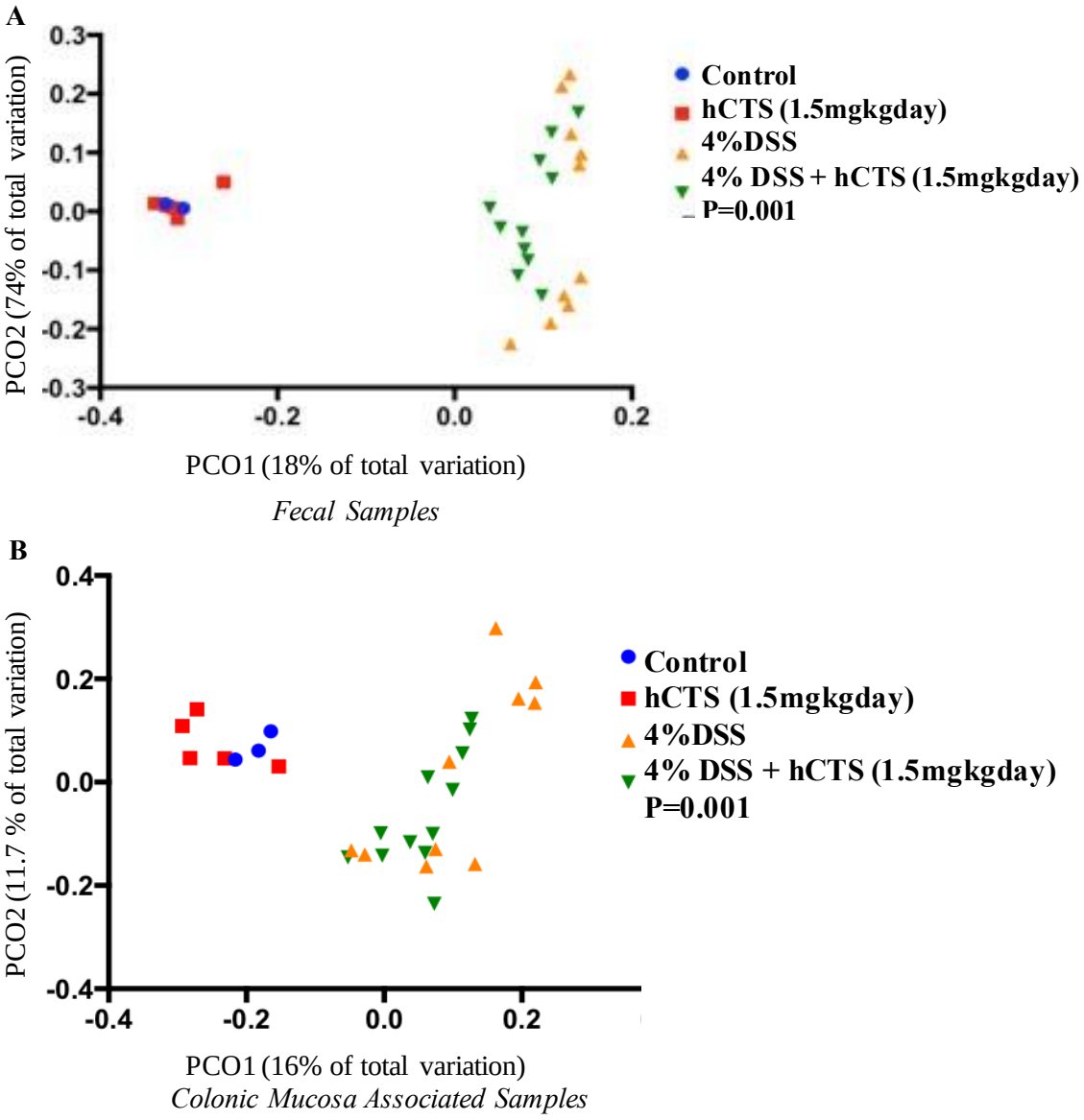

Figure 5-8. PCoA based on the unweighted UniFrac distance metric.
Fecal samples (A); Colonic mucosa-associated microbiota samples (B). Each color point represents an animal and color is shaded according to the treatment. The colitic induction clustered the microbial composition separately compared to non-colitic mice (β diversity, P values < 0.05). The hCTS treatment did not change the microbial composition of colitic mice. P values were calculated using PERMANOVA.

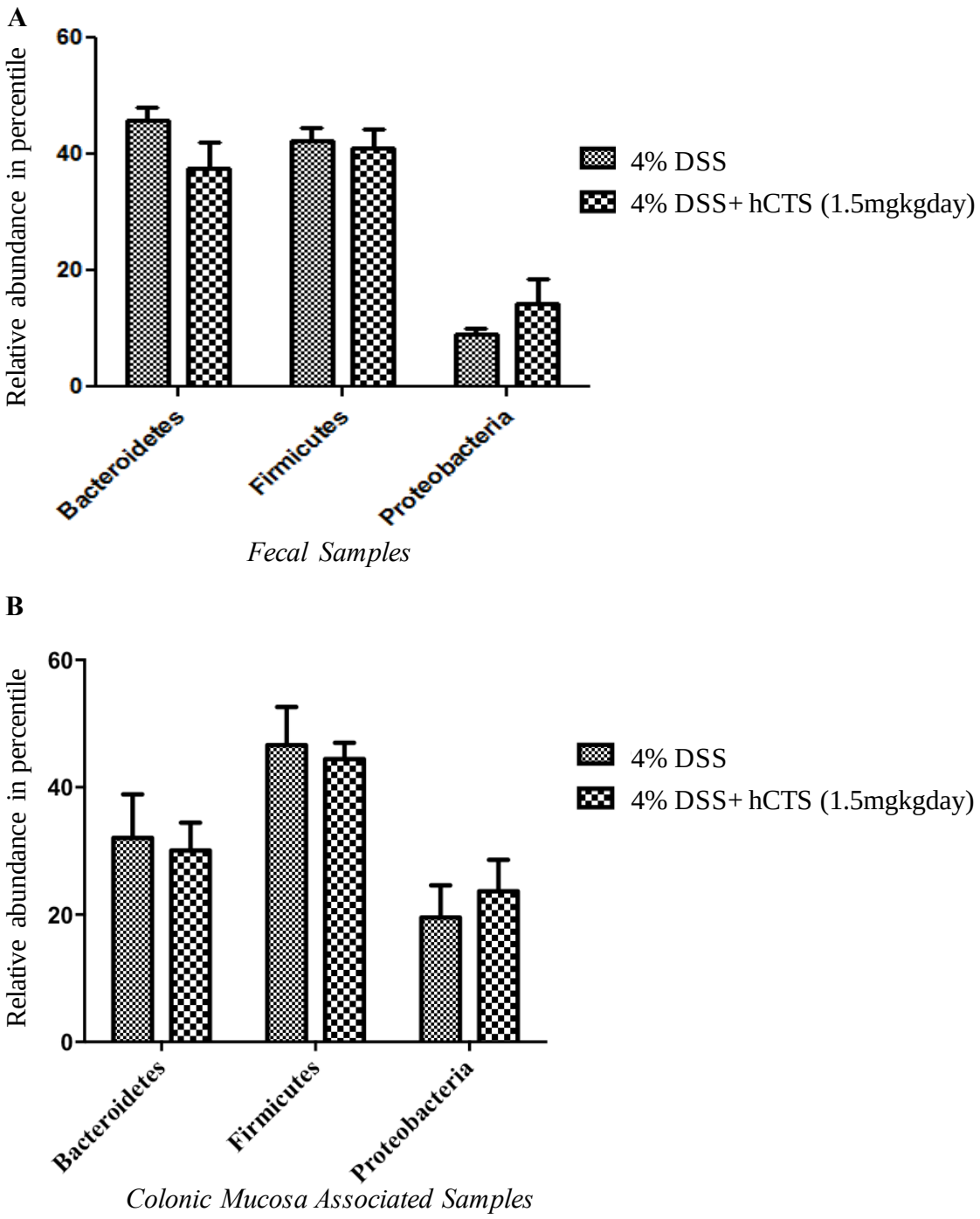

Figure 5-9. The effect of hCTS treatment (1.5 mg/kg/d, *i.r.* 7 days) on the abundant phyla (≥ 1%) present in the fecal (A) and colonic mucosa-associated samples (B).
The hCTS treatment showed no significant effect on abundant phyla during colits reactivation in quiescence colitic mice.

5.4. Discussion

The mucosa plays a major role as a first-line of defense against environmental antigen exposure in the gastrointestinal tract [398]. This mechanism of defense is aided by various antimicrobial peptides derived from the endocrine cells such as EC cells, which reside in the epithelium [157], and one of these gut antimicrobial peptides, which is hCTS [356, 357]. This study, for the first time, demonstrates the anti-inflammatory effect of hCTS (1.5 mg/kg/d, *i.r.*, 7 days) during reactivation of colitis using a quiescent model of colitis, which mimics the natural history of UC. We observed that treatment with the peptide down-regulated the macroscopic score and/or histological score and also down-regulated the M1 macrophages and the pro-inflammatory cytokine secretion. However, the peptide neither affected the M2 macrophages and the anti-inflammatory cytokine secretion in the colon nor the gut microbial dysbiosis observed in colitic mice.

During gut inflammation, macrophages infiltrate gut tissue in response to pro-inflammatory proteins [164]. These macrophages possess distinct characteristics compared to their tissue resident macrophages counterparts and are classified as M1 macrophages [164, 487]. In our study, gene expression revealed that hCTS treatment is associated with a down-regulation of *iNOS* and *Mcp1* M1 macrophages markers during reactivation of colitis. Several studies confirming the elevation of iNOS and MCP1 in IBD patients [152, 488-491] as well as experimental colitis models [492, 493] and constitutive expression of iNOS worsen UC symptoms by direct cytotoxicity, neutrophil activation [494], widening blood vessels [495], and/or elevating nitrosamines production [496]. In addition, peroxynitrite, which activates poly-ADP ribosyl synthetase (PARS),

results in cellular injury during gut inflammation via epithelial cell apoptosis, and is assumed to be increased via iNOS upregulation [497, 498]. In experimental colitis, systemic administration of specific iNOS inhibitor like amino-guanidine (AG) or N-(3-(aminomethyl)benzyl) acetamidine result in amelioration of colitic symptoms, which further validate the pro-inflammatory effect of iNOS [499, 500]. Concurrently, it is suggested that MCP1 acts as a chemoattractant by increasing the expression of integrins resulting in an increased immune cells infiltration. This statement was further validated using *Mcp1* knock-out mice (*Mcp1*$^{-/-}$), where it has been observed that dinitrobenzene sulfonic acid (DNBS)-induced colitis in *Mcp1*$^{-/-}$ mice resulted in downregulation of colonic inflammation compared to their wild type (WT) counterparts, which were associated with a lower number of classical macrophages and $CD3^{+}$ cells in their colon when compared to WT colitic mice [501]. Thus, the decrease expression of *iNOS* and *Mcp1* following hCTS administration in our chronic colitic condition might explain the abrogation of the inflammation. Conversely, M2 tissue resident macrophages are hyporesponsive to pro-inflammatory stimuli [487], and this population is crucial to control impaired activation of inflammation against commensal microbes; in this manner, the downstream signaling of TLRs and/or NF-κβ activation is abrogated [188, 487, 502]. These M2 macrophages produce IL10, which maintains the quiescent state of macrophages in the intestine. Moreover, they produce TGFB, which also down-regulates TLR signaling in blood monocytes, which is important to maintain a steady state immune environment in laminal propia (LP) [487]. Several studies confirm that defective in M2 macrophage polarization might increase the colitis severity in experimental models [503, 504]. M2 macrophages in IBD patients also have defective functioning, which is

supported by an *in vitro* study demonstrating that blood macrophages collected from CD patients and stimulated to M2 macrophages resulted in altered expression of CD40 and CD163 compared to healthy cells [505]. Arg1 is classical M2 macrophage marker [506], and studies confirm lower *Arg1* mRNA expression in UC patients [507]. The arginase enzyme (ARG1) compete with inducible nitric oxide synthase (iNOS) for L-arginine (L-ARG) as a substrate. The ARG1 metabolizes L-ARG to urea and L-Ornithine, the latter is important for cell division and collagen synthesis [508]. Conversely, iNOS metabolizes L-ARG to L-Citrulline and nitric oxide which ultimately disrupts lipid, proteins and DNA via producing peroxynitrites and/or hydrogen peroxide [508]. In our study, although hCTS treatment did not have a direct effect on *Arg1* expression but the treatment might provide a competitive advantage towards L-ARG for ARG1 enzyme via down-regulating *iNOS* expression. Concurrently, *Ym1*, another classical M2 macrophage marker [506] was not also affected by hCTS treatment. Although the functional role of Ym1 during inflammation continues to be studied, data suggests that it might help to regain immune homeostasis via modulating the toxic degradation effect of iNOS on heparin/heparin sulfate (HS) in an inflamed milieu [509]. However, because Ym1 is secretory in nature and has an intrinsic ability to form crystals, overexpression of Ym1 might be associated with fatal tissue injury. For example, in genetically modified mice like SHP-1 (protein tyrosine phosphate)- or CD-40-L-deficient mice, hyperactive macrophages overexpress Ym1, which results in eosinophilic crystal formation in the lung [510]. Thus regulation of Ym1 expression seems crucial to maintain the physiological state. Although suppression of the M2 macrophage population aggravates DSS colitis, the significance of this macrophage population in UC is controversial. Although not confirmed yet, it is

hypothesized that classical (M1) rather than anti-inflammatory macrophages (M2) are important for UC. This hypothesis is supported by granuloma formation and fibrosis that is observed more in CD rather than UC [164]. The granulomas are formed because of defective bacterial clearance by tissue resident macrophages and fibrosis that occurs because of excessive wound healing by M2 macrophage [164]. Thus, our observed hCTS effect on M1 macrophage marker and no effect on M2 macrophage marker *in vivo* might be beneficial to maintain the homeostatic environment in the gut.

To further validate our *in vivo* observation related to the anti-inflammatory effect of hCTS exerted on the M1 macrophage population, we conducted *in vitro* experiments where we collected naïve peritoneum macrophages and polarized toward either an M1 or M2 macrophage profile in the presence or absence of hCTS. In the presence of the peptide, we observed a significant decrease of M1 macrophage markers (*iNOS, Mcp1*) expression associated with a significant decrease of pro-inflammatory cytokines (*Il6, Il1b* and *Tnfa*) expression and their release in the medium. However, hCTS did not modify the expression of M2 polarized macrophage markers (*Arg1 and Ym1*) or the expression of anti-inflammatory cytokines (*Il10* and *Tgfb*). These observations support the notion that hCTS exerts its anti-inflammatory effect through the down-regulation of not only the pro-inflammatory cytokine secretion but also through the regulation of M1 macrophage markers, without modifying the M2 population. In our previous study, we demonstrated that in the context of acute colitis and *in vitro,* hCTS treatment was associated with an increased phosphorylation of signal transducer and activator of transcription 3 (STAT-3) and inhibition of STAT-3 phosphorylation abrogated the observed anti-inflammatory

effect [317]. STAT-3 phosphorylation is important to induce the anti-inflammatory response (AIR) in the host to regulate aggravated inflammation in a yet undefined mechanism [511]. The anti-inflammatory effect of STAT3 also corroborates with mice studies observing that mice having STAT3 deficient macrophages produces excessive cytokine and develop colitis [173, 180]. In addition, mice lacking STAT3 in endothelial cells also become extremely sensitive to LPS challenge [512]. Besides our lab, another group has confirmed that *in vitro* hCTS treatment stimulates STAT 3 activation in adipose tissue to derive from diet-induced obese mice [342]. Thus, we can hypothesize that the currently observed anti-inflammatory effect of hCTS seen during the reactivation of colitis occurs through the down-regulation of the M1 population and potentially through the activation of STAT-3 phosphorylation. Further confirmatory studies are required.

Gut microbiota represents the largest amount of microbes present in mammals compared to any other mammalian tissue [63]. Maintenance of healthy intestinal tissue environment largely depends on the balanced composition of commensal and pathobionts known as gut homeostasis. Alteration of the gut microbial homeostasis is known as gut dysbiosis and it is evident in UC patients [360]. Recently, we observed that acute colitis induced via DSS resulted in gut microbiota dysbiosis, which was more prominent in fecal samples compared to colonic mucosa-associated (MAM) samples [319]. In our study, we demonstrated that reactivation of quiescent colitis resulted in a distinct microbial composition when compared to control, both in fecal and MAM samples. This observation is supported by previous findings demonstrating a microbial dysbiosis in

DSS-induced colitis in dogs [461] and rats [434]. All these observations, along with our current study confirms that DSS strongly disrupts the gut microbial homeostasis both in acute and in the chronic colitic condition. However, whether the gut dysbiosis is causal or an outcome of gut inflammation in IBD and in colitic mice is remains controversial. hCTS is a well-known anti-microbial peptide [250, 356, 357], and our recent study demonstrated that administration of a 5-day regime of hCTS to naïve mice altered the gut microbial composition when compared to control, and it was associated with a prominent effect in feces rather than in MAM samples [318]. In these naïve mice fecal samples, hCTS treatment increased the relative abundance of Bacteroidetes compared to Firmicutes, which was the opposite of results observed in acute DSS colitic mice fecal samples [318, 319]. Because our previous study did not tested the effect of hCTS on gut microbiota during the development of acute colitis, in our current study, we deciphered the effect of hCTS treatment on gut microbiota composition in the context of reactivation of quiescent colitis. Surprisingly, a 7-day regime of hCTS administration did not change the microbial composition compared to vehicle-treated colitic mice. This is likely because of the long-term dysbiosis induced by the three cycles of DSS on gut microbiota, which dominates the effect of hCTS. Prophylactic hCTS treatment in the acute colitic condition or long-term hCTS treatment in the chronic colitic condition and its effect on gut microbiota need to be determined.

Finally, it is well established that environmental antigen exposure in IBD patients changes the regulatory mechanisms leading to pathological features such as gut inflammation [127, 513]. It is hypothesized that inflammation results in neuroendocrine

system activation and EC cell hyperplasia, which in turn elevates serum CHGA [238, 256]. Although the possible impact of elevated CHGA in gut inflammation remains unknown, our group for the first time, showed that hCTS derived from the distal portion of CHGA is increased both in IBD and experimental colitis mouse models [317]. Thus, we confirmed a prophylactic anti-inflammatory effect of hCTS in acute ulceration [317]. A major drawback of this previous observation was the acute murine model of colitis used. Because IBD is known to be a chronic condition associated with several flares, our previous acute experimental colitis model failed to mimic it. In the current study, we overcame this challenge by developing a quiescence colitis model. Using this model, we further validated the prophylactic anti-inflammatory effect of hCTS observed in acute colitic mice and transposed it to a model mimicking the natural history of the disease. Because therapeutic treatments are required for IBD patients, this peptide could be a new therapeutic target. However, further confirmatory studies are required.

Part 06. Chapter 06

6.1 General Discussion

As discussed in the introductions of this thesis, the incidence and prevalence of IBD are alarmingly increasing around the globe alongside with modern industrialization [9, 11, 12]. Due to an array of pathogenic factors associated with IBD, the etiology of this devastating disease is not yet well-known. Alongside with this, the scarcity of treatment for IBD and the side effects and resistance towards the available drugs [284] warrant the scientific world in performing research to investigate the underlying factors associated with disease initiation, progressions and to identify new targets in order to determine their efficacy as a treatment option. This research strategy might help to develop drugs which might be a stand-alone and/or might act in synergy with current available suppressive drugs in the market. Understanding the immense need of new therapeutic option for IBD, this PhD project focused on understanding the impact of hCTS, a peptide derived from pro-hormone CHGA in gut inflammation using murine model of colitis.

Circulating CHGA is a well-known marker for neuroendocrine tumours as well as gastrointestinal (GI) carcinoids [514-516]. Due to involvement of neuroendocrine alteration in the context of chronic inflammatory disorders, altered expression of CHGA has been observed in various diseases like rheumatoid arthritis, systemic lupus erythematosus, chronic heart failure, and chronic obstructive pulmonary disease [257, 517-519]. In line with this, altered plasma level CHGA has been observed in IBD patients, with a more pronounced level during the active stage of the disease [238]. The pro-inflammatory cytokine TNF-α is elevated during inflammatory conditions including

IBD and the deleterious effect of this cytokine in IBD outcome is well documented [201, 520]. Surprisingly, increased levels of plasma CHGA in IBD are associated with an increased level of TNF-α [238]. The availability of CHGA in the widely available enterochrommaffin (EC) cells of the intestinal epithelium and its co-expression with other hormones supports the notion that chronic inflammatory condition should activate the neuroendocrine system. Interplay of neuroendocrine peptides with inflammatory cytokines has been reviewed recently, for example, vasostatin derived from proximal part of pro-hormone CHGA can limit plasma leakage by protecting vessels from cytoskeleton rearrangement from TNF-α in inflammatory condition [263, 521]. However, the effect of the distal part of CHGA during inflammatory condition in unknown, therefore, this work targeted the hCTS derived from the distal part of CHGA and revealed its effect during gut inflammation through four major aims.

In our first aim, we confirmed the pervious observation that CHGA is increased during colitic condition. For that purpose, we collaborated with the IBD research center at the Health Science Center, University of Manitoba and collected serum samples from IBD patients. Using ELISA technique, we observed that not only the serum of CHGA but also CTS were increased in IBD patients [317]. This observation is in accordance with previously observed phenomena. Next, we targeted to verify this observation in experimental animal model.

Among the various colitic models, the chemically induced colitic models are the mostly used to study IBD [274]. We selected the DSS murine colitic model described by

a Japanese scientist and the most characterized UC model of colitis [266]. DSS is delivered though drinking water and can easily develop a reproducible colitic condition characterized by loss of weight, infiltrated immune cells, destruction of intestinal architecture [522]. By manipulating the course and the duration time of DSS treatment, both acute and chronic colitic condition can be induced [522]. In our first manuscript, we developed an acute DSS colitic condition by delivering 5% DSS in the drinking water of 7-8 weeks old C57BL/6 mice and then harvested both the serum and tissue samples for the downstream application. In this study, we for the first time demonstrated that the elevation of CHGA and hCTS is not only observed in the serum but also in the distal colon. This observation supports the notion that the EC cell hyperplasia observed during gut inflammation [235, 236] might be responsible for the elevation of serum and tissue CHGA and hCTS. This observation also strengthens our quest to verify the effect of hCTS in gut inflammation through our second aim.

In our first manuscript, we observed prophylactic effect of human (h)CTS (1.5mg/kg/d) significantly attenuate gut inflammation in DSS colitic mice [317]. The attenuation was observed in all inflammatory parameters like macro and microscopic score, pro-inflammatory cytokines, MPO and CRP level [317]. MPO sums up for 5% of total protein content of neutrophils and oxidized chloride to hypohalous acid [523]. The resulted HOCl has strong antimicrobial properties and is crucial to carry out neutrophils bactericidal activity [523]. Unfortunately, during colitis uncontrolled neutrophil activity results in tissue collateral damage and elevated colonic MPO level is reported [524, 525], thus, colonic MPO level is used as a marker to identify the intense of inflammation [523].

Besides this, during gut inflammation an array of immune cells especially macrophages infiltrate towards the intestinal epithelia [526]. These macrophage population is a major producer of pro-inflammatory cytokines like IL-6, IL-1β, TNF-α [526]. The increased accumulation of pro-inflammatory cytokines in the inflamed tissue deleteriously affects the immune hemostasis. In our study, we observed the hCTS treatment resulted in down - regulation of pro-inflammatory cytokines alongside with colonic MPO level [317]. This observation indicates the ability of this peptide to down-regulate the pro-inflammatory mediators via manipulating the gut immune cells.

Next to understand the mechanistic effect of this peptide on immune cells, we observed the effect of this peptide on macrophages. Tissue resident macrophages reside just below the intestinal epithelium and are bactericidal in nature but are inflammatory anergic which protect them to induce unwanted immune response. However, during IBD a large number of macrophages are recruited to the inflamed site [164]. ER-MP 20 is an antigen found on the differentiating monocytes and disappears when they transform to mature macrophages [527]. During acute DSS colitis, the number ER-MP 20^{+} cells is elevated throughout the colon. [526]. These newly recruited macrophages are inflammatory in nature and cause collateral damage towards the intestine by producing a vast array pro-inflammatory cytokines like IL-1β, IL-6, TNF-α and oxyradicals [526]. In our study, we observed that hCTS effectively down-regulated pro-inflammatory cytokines release from the macrophages collected from hCTS treated DSS colitic mice when compared with vehicle treated DSS colitic mice. *In vitro* study confirmed the observed *in vivo* anti-inflammatory effect of hCTS via down-regulating the pro-

inflammatory cytokine release from LPS stimulated macrophages [317]. Furthermore, we observed that the down-regulation of pro-inflammatory cytokines release by hCTS treatment was associated with an increase of STAT3 phosphorylation [317]. Although the role of STAT3 on IBD is not clear yet, it is well documented that the STAT3 activation in macrophages is crucial to down-regulate inflammation. For example, it has been observed that that inactivation of STAT3 in macrophages can result in gut inflammation [180, 181]. As deletion of *STAT3* gene is embryonically lethal, cell types specific STAT3 knockout mice were used to study the role of this protein. It has been observed that the lack of STAT3 from macrophages and colonic epithelium cells induce lethal colitis in mice [180, 181]. It is assumed that, STAT3 activation is required for the production of anti-inflammatory cytokine IL-10 production and the expression of suppressors of cytokine signaling (Socs) 3 [180, 181]. Taken together, these observations suggest that hCTS regulates the gut inflammation by manipulating the production of cytokines from macrophages via STAT3 protein and a yet unknown mechanism. In contrast to the beneficiary effect of STAT3 activation in macrophages, constitutive activation of STAT3 in T cells are detrimental to colitis [165-167]. This might be due to activation of anti-apoptotic genes like *bcl-2* and *bcl-xl* in pathogenic T cells [165]. Thus, it might be assumed that the observed anti-inflammatory effect of hCTS might be confined to inflammatory macrophages only and further studies are warranted to understand the effect of hCTS on other acquired immune cells.

The gut is known to be the biggest reservoir of microbes in the human body and their number are ten times greater than the human cells. The human health largely depends on

gut microbe homeostasis and various short chain antimicrobial peptides (AMPs) derived from intestinal epithelium immune cells are crucial to prevent dysbiosis observed in various disease conditions. Various *in vitro* studies confirmed the antibacterial [352], antifungal and antiviral activity [356, 357] of hCTS. Despite this, there were no documented study observing the effect of hCTS on gut microbiota. From the observed *in vitro* effect of CTS on *Staphylococcus aureus, Escherichia coli* [356, 357], it was not possible to predict the *in vivo* effect of CTS on gut microbiota. Thus, in our third aim, for the first time we used a sequencing technique to demonstrate the effect of hCTS on naïve murine gut microbiota *in vivo.* Although in our study, hCTS administration (1.5 mg/kg/d, for 6 days) did not affect the bacterial richness and diversity (α-diversity) in both fecal and colonic mucosa samples, hCTS treated mice had different microbial composition when compared with control group [318]. This observation was in accordance to the effect of other AMPs like defensins on gut microbiota [399]. Gut microbial dysbiosis is inevitable during gut inflammation, although controversy remains whether it is causal or the effect of altered gut inflammation. Bacteroidetes and Firmicutes are the two most abundant phyla in the gut. Studies showed that during both IBD and Inflammatory Bowel Syndrome (IBS), patients have a relative higher abundance of Firmicutes and lower abundance of Bacteroidetes [359, 400]. We also verified this observation in acute DSS colitic condition used in our animal facility [319]. In our study, we observed hCTS treatment resulted in significant abundance of Bacteroidetes relative to Firmicutes in fecal samples. However, the observed effect of hCTS on relative abundance of Bacteroidetes relative to Firmicutes in fecal samples was not pronounced in colonic mucosa-associated microbiota (MAM) samples [318]. This might indicate a prolonged administration of

hCTS is required to observe a possible alteration at the phylum level in colonic mucosa-associated microbiota.

It is noteworthy that, although hCTS effect was limited to fecal samples at phylum level, we observed that hCTS changed the microbiota at the lower taxonomic level at both fecal and MAM samples. Previously, it has been observed that microbes under the genera *Bacteroides* and *Parabacteroides* showed a positive association with hCTS treatment [318]. In general, microbes under these genera are commensal in nature and are important to transform simple and complex sugars into volatile fatty acids [401]. This transformation is crucial to transform complex food into absorbable nutrient from by the large intestine. Besides, microbes under these genera are also crucial to maintain gut homeostasis and to prevent inflammation. For example, Paneth cell protein (Ang4) production is stimulated by *B. thetaiotaomicron*, which is important to control the growth of certain pathogenic microorganisms (e.g. *Listeria monocytogenes*) [403]. In addition, abscess formation and other inflammatory responses [402, 404, 405] are inhibited by *Bacteroides fragilis.* This phenomenon is carried out by inducing the production zwitter ionic polysaccharide (ZPS) by *B. fragilis* which in turn activate CD4+ T cells to produce anti-inflammatory cytokine IL-10. These reports suggested that the observed hCTS effect on favouring the relative abundance of Bacteroidetes in naïve mice might be beneficial to control gut inflammation in colitis.

While evaluating the effect of hCTS on naïve gut microbiota *in vivo*, we also evaluated the effect of DSS on gut microbiota and predicted metabolic changes due to

DSS treatment [319]. Microbial dysbiosis is reported in IBD patients and in experimental colitis model both in rodents and vertebrates [431, 434, 458-461]. In our study, we also observed that acute DSS exposure (5% DSS, for 5 days) strongly shift the microbial composition to that of control both in fecal and MAM samples. This observation was in accordance with previously observed effect of DSS on murine gut microbiome [400, 432]. It is important to note that, the effect of DSS on phylum level was more pronounced in fecal samples rather than MAM samples. This might indicate that, the effect of DSS on microbial richness and diversity at different anatomical site might be different. Although the underlying mechanism is yet to be eluted, a similar observation was reported in human IBD samples, where microbial shift was observed more in stool samples compared to biopsy samples [119]. One hypothesis behind this notion is the crypt damage by DSS resulting in mucosal epithelium sloughing and thus preventing bacterial adherence [336, 411, 422, 471]. However, more experiments are required to verify this hypothesis.

While observing the effect of DSS on lower taxonomic levels, we learnt that bacterial communities at lower taxonomic level were altered at both fecal and MAM samples due to DSS treatment. We observed that members of various taxa like 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 MAM samples. This was in accordance with previously observed data showing that some member of these taxa are in abundance due to DSS treatment [432]. However, their exact role in gut inflammation and their efficacy as a target organism for intervening DSS treatment need

to be elucidated. The gut microbiota controls a vast array of metabolic functions. Due to microbial dysbiosis observed during colitis, functional alterations observed both in humans [119, 430], and mouse model of colitis [432]. We carried out metabolism prediction analysis and we observed several metabolic functions are associated with either hCTS or DSS treatment. Although only being a prediction analysis, this observation opens further opportunity to investigate hCTS and/or DSS treatment on host-microbe interaction in terms of functional capacity which might not be captured only by analysis of microbiota at higher taxonomic level.

As IBD is characterized as chronic gut inflammation, acute DSS colitis model does not necessarily mimic the natural history of the disease. That is why in our fourth aim, we verified the anti-inflammatory effect of hCTS using a chronic DSS colitis model [320]. We observed that hCTS administration (1.5 mg/kg/d, for 7 days) effectively down-regulated the reactivation of gut inflammation in a quiescence model of colitis. We also observed that this down-regulation was correlated with improvement of macro and microscopic score, attenuation of pro-inflammatory cytokine secretion and markers for M1 macrophage population. However, markers associated with M2 macrophage population and/or gut microbiota dysbiosis in colitic mice was not affected by the hCTS treatment [320].

In our fourth manuscript, we observed that the expression of *iNOS* and *Mcp1*, which are M1 macrophage markers were down-regulated during colitis reactivation upon receiving hCTS treatment [320]. It has been observed that exogenous nitric oxide (NO) is

associated with attenuation of intestinal inflammation by maintaining blood flow, preventing platelet and leucocyte infiltration and/or adhesion with the vasculature, attenuation of mast cell activity, alteration of oxidative stress and inhibition of NF—κβ translocation [528-531]. Further, NO can inhibit NADPH oxidase and superoxide release from neutrophils which in turn down-regulate the superoxide induce damage [528, 532]. Surprisingly, elevation of iNOS is detrimental to intestinal inflammation [533]. It is hypothesized that excessive NO production from iNOS results in direct cytotoxicity, neutrophil activation [494], widening blood vessels [495], and/or elevating nitrosamines production [496]. Peroxinitrite, is a highly toxic oxidant that reacts with proteins, lipids and initiate DNA strand leakage. It also increases epithelial permeability and cause cell apoptosis by activating poly-ADP ribosyl synthetase (PARS). The excessive production of NO from iNOS reacts with superoxide to form Peroxinitrite [489]. These observations suggest the pro-inflammatory effect of *iNOS,* which was further validated by down-regulation of gut inflammation in experimental colitis by systemic administration of specific iNOS inhibitors like amino-guanidine (AG) or N-(3—[aminomethyl]benzyl) acetamidine, results in amelioration of colitic symptoms [499, 500]. In addition to iNOS, monocyte chemoattractant protein or MCP1 are also found to be positively associated with gut inflammation. MCP1 is produced by a variety of immune cell types upon receiving stimulation from pro-inflammatory cytokines like IL-1 and TNF-α. Both in human and animal model of colitis, MCP1 has been found to be up regulated and to act as a chemokine for both innate and adaptive immune cell [152, 491]. EC cells in the gut are a major source of 5-hydroxytryptamine (5-HT; serotonin) and there are reports that elevation of serotonin worsen the IBD symptoms [163, 534]. It has been reported that

MCP-1 might be crucial to release 5-HT which indicates the pro-inflammatory role of this chemokine [535]. This phenomenon was further fueled by observing less inflammation in DNBS induce experimental colitis in *Mcp1*-knock out mice (*Mcp1*$^{-/-}$) compared to wild type [501]. Thus, down-regulation of *iNOS* and *Mcp-1* upon hCTS administration in our study might also be a mechanistic explanation of anti-inflammatory effect produced by hCTS [320]. In contrast to tissue infiltrating M1 macrophage population, tissue resident M2 macrophages are bactericidal in nature, inflammation anergic due to a down-regulated TLR signaling and NF—κβ deactivation [164]. They are crucial to maintain immune homeostasis probably by producing anti-inflammatory cytokines like IL-10 and TGF-β, with TGF-β being an important factor in regulating TLR signalling in blood monocytes. Defective M2 macrophage population is reported in IBD. Although these observations support the crucial role M2 macrophages in preventing gut inflammation, in our study hCTS did not affect M2 macrophage markers *Arg1* and *Ym1* expression during colitis reactivation. However, it is important to know that both ARG1 and iNOS compete for L-ARG as a substrate with L-ARG being metabolized to urea and L-Ornithine by the enzyme ARG1. It has been reported that L-Ornithine is a precursor for the synthesis of polyamines which are crucial for cell growth and differentiation [508]. L-Ornithine is also metabolized to L-proline by ornithine aminotransferase which is important for collagen production [536]. In contrary, L-ARG is metabolized to L-citrulline and nitric oxide by iNOS which ultimately disrupts lipid, proteins and DNA via producing peroxynitrites and/or hydrogen peroxide [508]. In our study, down-regulation of *iNOS* expression by hCTS might provide a competitive advantage for *Arg1* towards L-ARG. Another murine M2 macrophage marker is *Ym1,* which expression was also not

affected by CTS treatment in our study. It is important to note that Ym1 degrades the toxic effect of iNOS at heparin/heparin sulfate [510], and overexpression of Ym1 might result in tissue injury due to its intrinsic ability to form crystals. For example, SHP-1 (protein tyrosine phosphate)- or CD-40-L-deficient mice, possess hyperactive macrophages which overexpress Ym1, and leads to eosinophilic crystal formation in the lung [510]. This observation indicates that excessive expression of Ym1 might be detrimental to the intestine. The *in vivo* effect of hCTS on M1 macrophage population and not the M2 macrophage population was further confirmed by an *in vitro* study. Although M2 macrophages are crucial to maintain gut inflammation, it is assumed that classical (M1) macrophages are more important in UC rather than anti-inflammatory macrophages (M2). In UC granulomas and fibrosis are not observed as much as CD [164]. The granulomas are formed because of defective bacterial clearance by tissue resident macrophages and fibrosis that occurs because of the excessive wound healing by M2 macrophages [164]. The ability of hCTS on M1 macrophage population but not the M2 macrophages might explain the observed attenuation colitis reactivation in quiescence DSS colitis which mimics more to UC.

As in our third aim, we observed that both hCTS and DSS can alter gut microbial composition in naïve mice, in our final study we wanted to observe the effect of hCTS on gut microbiota in our chronic DSS colitis model. We observed that DSS induced a distinct microbial composition in both fecal and MAM samples when compared to control. However, the given hCTS treatment regime (1.5mg/kg/d, i.r., 7 days) was not able to change the microbial composition in colitis models. One hypothesis is that,

induction of quiescence colitis required three cycles of DSS treatment (4%, 2%, 2%, w/v, 5 days each followed by 11 days of rest between cycles) induces a strong symbiosis which nullify the effect of hCTS treatment [320]. Further study aimed to understand the long-term effect of hCTS treatment during experimental colitis needs to be elucidated.

Overall, this PhD project for the first time elucidated the anti-inflammatory effect of hCTS on gut inflammation using both acute and chronic experimental colitis model. Furthermore, we observed that this peptide strongly inhibited the pro-inflammatory cytokine secretion from macrophages expressing classical activated markers probably via STAT-3 activation. Although hCTS affected gut microbiota in naïve mice, during colitis its effect was not observed. These observations might open vast research fields to further study the efficacy of hCTS as a novel therapeutic option for IBD.

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7.1 Conclusion

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

1. The pro-hormone CHGA and its C-terminal derived peptide, CTS is increased both in acute murine model of colitis and serum of IBD patients [317]. Beside confirming previous published reports related to an increase of CHGA level in serum of IBD patients, this study confirmed the similar observation in experimental colitis both in serum and the site of inflammation.

2. Administration of CTS (1.5mg/kg/d for 5 days) is able to reduce the inflammation in acute model of murine colitis [317]. This unique observation revealed a previously unknown anti-inflammatory effect of CTS during the development of gut inflammation.

3. The decrease of inflammation probably occurs *via* the macrophage population and the STAT3 pathway and by modulating pro-inflammatory cytokine (IL-6, IL-1β, TNF-α) release [317]. However, the effect of CTS on other immune cells are still unknown. Furthermore, depending on cell types, STAT3 activation might be beneficial as well as detrimental [537]. Thus, further investigation considering the effect of CTS on other immune cell types are warranted.

4. Considering CTS as an antimicrobial peptide [356, 357], we, for the first time demonstrated that administration of CTS modified the gut microbial composition *in vivo* in naïve mice [318].

5. CTS administration in naïve mice were able to increase the Bacteroidetes ratio compared to Firmicutes in fecal samples [318]. This observation was unique as previous reports were suggestive of a reduction of Bacteroidetes in various colitic condition [400], experimental colitis [319] as well as other physiological conditions like obesity [366]. Although this data supports the effect of CTS on the overall gut microbiota, it opens up a research ground to further investigate the effect of this peptide in various disease conditions and physiological state, as reported y our prediction analysis.

6. Administration of DSS is widely used to induce colitis [266, 333]. However, the mechanism of action of this chemical compound is not well described. Using Illumina sequencing, we showed that 5% DSS administration result is gut-microbial dysbiosis. This observation was in accordance with other reports suggestive of microbial abnormality observed in IBD patients and experimental colitis in various vertebrates and rodent models [431, 432, 434, 458-461].

7. In our final study for this project, we investigated the effect of CTS peptide in reactivation of colitis using a chronic DSS model of colitis which mimics the natural history of IBD [320]. For the first time, we observed that CTS

administration was able to down-regulate the colitis relapse. This observation further strengthens the anti-inflammatory effect of CTS not only in acute gut inflammation but also in chronic inflammation reactivation.

8. In the same study, we observed that CTS administration down-regulated the M1 macrophage markers *iNOS* and *Mcp1* both *in vivo* and *in vitro.* However, CTS treatment neither modified M2 macrophage markers *Arg1* and *Ym1* nor the gut microbiota. Considering this, it is assumable that CTS exerts its anti-inflammatory effect via modulation of M1 macrophages and the release of pro-inflammatory cytokines. However, the effect of CTS on various other immune cells need to be investigated to determine the complete immunological effect of this peptide.

7.2 Significance

Since 2012, my PhD project focused to better understand the pathophysiology of IBD and to develop a novel therapeutic target. The rationale behind this was to counter the immense need of currently unavailable therapeutic drug for IBD. IBD is rapidly increasing worldwide and is becoming a major healthcare issue. Using both acute and chronic colitis model mimicking the natural course of IBD, we for the first time revealed the prophylactic [317] and therapeutic [320] anti-inflammatory effect of CTS on gut inflammation respectively. This observation opens up a novel research field to study CTS as a therapeutic target for gut inflammation. This unique observation of the existence of a modulating effect of CTS on infiltrating macrophage population via STAT3 activation

leads us to file a US patent (File#61/827,127, the United States), which also opened up industrial collaboration opportunity to accelerate translational research.

Beside the classical observation of the anti-inflammatory effect of CTS on gut inflammation, we, for the first time observed an *in vivo effect* of CTS on gut microbiota in naïve mice [318]. Various members of Bacteroidetes are commensal for the host and in our study, we observed a positive association of Bacteroidetes with CTS treatment. Besides, aiding the production of anti-inflammatory cytokines like IL-10 in order to maintain immune homeostasis [402, 404, 405], members of this bacterial phylum are crucial to transform complex sugars into absorbable form of nutrients. This beneficial starch utilizing capacity of *Bacteroides* is crucial to prevent obesity [402]. Obesity is a major healthcare problem and in 2010 caused around 3.9 million deaths [538]. Thus, this study opened up a possible opportunity to study the efficacy of CTS in obesity management and possible use of this peptide as a prebiotic. We also patented the effect of CTS on gut microbiota (62/155,572, the United States) which might in future help to upscale the use of this peptide commercially.

Finally, in line with industrialization IBD became a public health burden. Canada has the highest prevalence of IBD around the globe and the financial burden associated with this disease is nearly 2.8 billion dollars annually [475]. The quality of life for IBD patients is severely compromised. Unfortunately, therapeutic drugs against IBD is a rare scarcity. To date, mucosal healing treatments are based on biological and immunosuppressive therapies. These treatments have demonstrated adverse events

associated with lymphoma and opportunistic infections [284]. Therefore, a safer therapeutic option for IBD is a major need. In line with this, my PhD project for the first time provided insights about the functional role of CTS in gut inflammation and prepared the ground to further study the efficacy of this peptide as a novel therapeutic target for IBD.

7.3 Future Directions

In our project, we observed both CHGA and CTS level is increased in acute colic mice [317]. However, due to limitation ELISA technique used in our study, it is not clear whether any structural modification happens to the CTS peptide due to inflammation. Further, although we observed anti-inflammatory effect of the CTS on gut inflammation, the half-life of the injected peptide in the gut microenvironment is not clear enough. Thus, a further study using mass spectrometry (MS) to determine the true nature of elevated CHGA and its derived peptides during inflammation and any possible post-modification of the inject CTS in gut is warranted.

IBD patients generally suffer from colonic smooth muscle contraction which results in constipation and/or diarrhea. Calcium ion (Ca^{2+}) influx through voltage-gated L-type Ca^{2+} facilitate the gastrointestinal smooth muscle action potential [539, 540]. Both DSS and TNBS induced colitis models showed reduced Ca^{2+} currents observed through patch clump recordings of smooth muscle cells from these animal models [540-542]. A beneficial effect of $CHGA_{4-16}$ was observed on spontaneous mechanical activities (SMA) of human colonic motility in vitro, where acetic acid (AA) was used to reduce the colonic

motility [234]. Currently, to our knowledge there is no data showing the impact of CTS on gut SMA. Thus, a research project focused to study the effect of CTS on SMA is needed.

In our study, CTS administration resulted in down-regulation of MPO[317]. MPO is largely secreted from neutrophils facilitating its antimicrobial effect by producing hypochlorous (HOCL) acid from hydrogen peroxide (H_2O_2) and chloride (Cl^-) anion during respiratory burst or tyrosil radical production from tyrosine [543, 544]. Decrease MPO level in the CTS/CTL treated wild type mice suggests a reduction of neutrophil recruitment to the gut from the bone marrow or their decrease survival within the tissue. Furthermore, *in vitro*, CTS/CTL can modulate monocyte migration, suggesting that neutrophils are responsive to these peptides[252]. In line with other studies we have found an increased MPO level following DSS-induced colitis that is a typical feature of UC. A study thus needed to further understand the role of CTS on neutrophils.

In addition, CTS administration was associated with STAT3 activation. Depending on the cell type, STAT3 activation can be both beneficial or detrimental [170], thus the effect of CTS on STAT3 activation on various cell types need to be studied.

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